REGULATION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE FROM COMFREY BY SEVERAL PHOSPHOMETABOLITES

by

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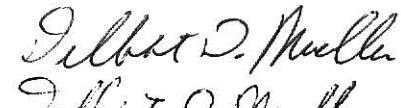

Major Professor

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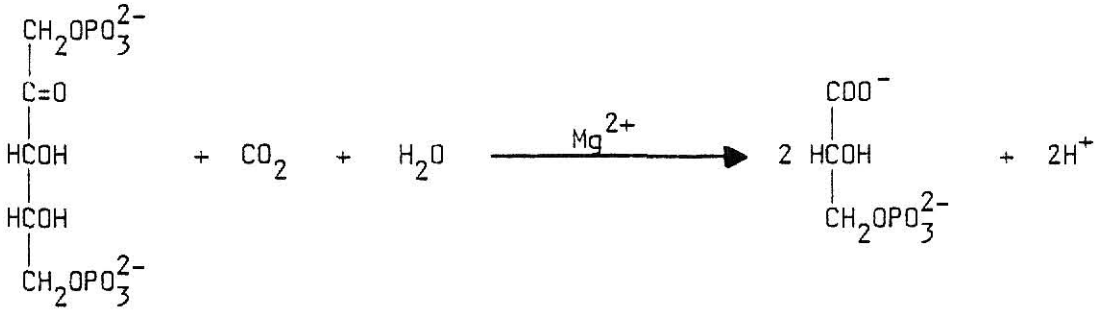
INTRODUCTION

All photosynthetic organisms and certain chemosynthentic bacteria assimilate CO_2 to form sugars by the action of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC. 4.1.1.39) (RuBPCase)[1]. Ribulose-1,5-bisphosphate carboxylase/oxygenase catalyzes the carboxylation and cleavage of ribulose-1,5-bisphosphate to yield two molecules of 3-phosphoglycerate (Quayle et al., 1954; Weisbach et al., 1954; Racker, 1955; Jacoby et al., 1956).



This enzyme also catalyzes the cleavage of RuBP in the presence of molecular oxygen to one molecule each of phosphoglycolate and 3-phosphoglycerate (Bowes et al., 1971; Andrews et al., 1973; Ryan and Tolbert, 1975).

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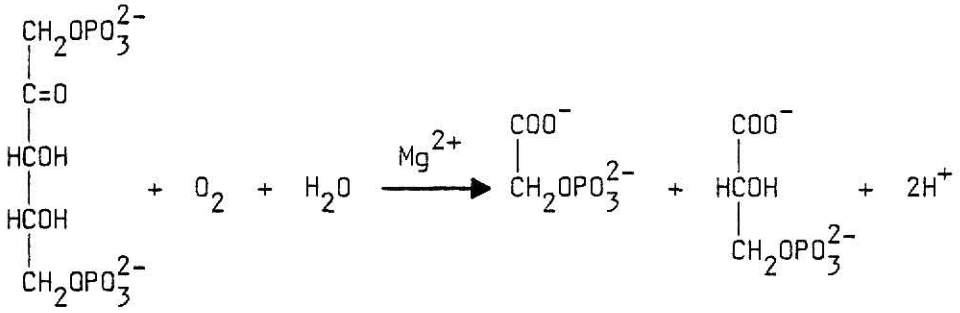
[1]Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate 6-PGA, 6-phosphogluconate; 3-PGA, 3-phosphoglycerate; FBP, fructose-1,6-bisphosphate; 2-PG, 2-phosphoglycolate; CHBP, 2-carboxyhexitol-1,6-bisphosphate; CABP, 2-carboxyarabinitol-1,5-bisphosphate; MOPS, morpholinopropane sulfonic acid; EDTA, (ethylenedinitrilo)-tetraacetic acid; POPOP, 1,4-Bis[2-(5-phenyloxazoly)] benzene; PPO, 2,5-diphenyloxazole; CPM, counts per minute; NADPH, nicotinamide adenine dinucleotide phosphate (reduced).



The two reactions appear competitive in that high levels of O_2 or CO_2 inhibit the alternate activity.

Ribulose-1,5-bisphosphate carboxylase/oxygenase often comprises more than 50% of the soluble chloroplast protein, which classifies it as the most abundant protein in nature (Kung, 1976). It is a protein of relatively high molecular weight ranging generally from 450,000 to 600,000 daltons (McFadden and Purohit, 1978) in higher organisms. It exists as a spherical aggregate of subunits. Many structures have been proposed; however, the most commonly accepted is the L_8S_8 structure which was verified by X-ray diffraction for the tobacco enzyme (Baker et al., 1975; Baker et al., 1977) and by electron microscopy (Bowien and Mayer, 1978) for a bacterial source (Alcaligines eutrophus). This structure has been established for the spinach enzyme as well in a carefully done gel electrophoresis study on ^{14}C amidated products (Gray et al., 1980). All higher plants investigated appear to have this L_8S_8 structure along with the green algae Chlamydomonas reinhardi and Chlorella ellipsoidea (Kawashima and Wildman, 1970) and also Chromatium D (Takabe and Akazawa, 1975). RuBPCases from lower organisms, however, vary more in structure, as for example, the RuBPCase of Rhodospirillum rubrum which is devoid of small subunits (Tabita and McFadden, 1974) and exists in vitro as a dimer of the large subunits.

Activation studies are fast becoming an important area of interest in RuBPCase research. Earlier investigations (Miziorko and Mildvan, 1974; Lorimer et al., 1976, 1977) indicated that activation of RuBP-Case is dependent upon pH and involves the ordered addition of CO_2 and Mg^{2+} in the following manner.

$$E - NH_2 \overset{CO_2}{\underset{\text{slow}}{\rightleftharpoons}} E - NHCO_2^- \overset{Mg^{2+}}{\underset{\text{fast}}{\rightleftharpoons}} E - NHCO_2^- - Mg^{2+}$$

CO_2 slowly and reversibly reacts with a basic lysyl amine residue on the large subunit of the enzyme followed by rapid addition of Mg^{2+} to form the active ternary complex (Pon et al., 1963; Lorimer et al., 1976; Badger and Lorimer, 1976). The CO_2 molecule involved in the formation of the carbamate compound has been found to be different from the CO_2 which is fixed in the first step of the photosynthetic process (Lorimer, 1979; Miziorko, 1979). It is known that CO_2 and Mg^{2+} are essential for RuBPCase to have any activity at all, indicating that activation in itself is essential for activity. However, in the presence of some CO_2 and Mg^{2+}, a variety of sugar phosphates have been found to stimulate the activation process (Buchanan and Schurmann, 1973; Chu and Bassham, 1975; Ryan and Tolbert, 1975, Chollet and Anderson, 1976; Lendzian, 1978; Vater and Salnikow, 1979; Whitman et al., 1979). In earlier studies (Chu and Bassham, 1975; Vater and Salnikow, 1979), these sugar phophates were believed to act at an allosteric site distinct from the catalytic site. However, more recent studies (Badger and Lorimer, 1981) indicate a strong possibility that these effectors act competitively with RuBP at the active site. Their evidence to substantiate this claim was that the binding of 6-PGA and

NADPH was completely prevented when the catalytic site was occupied by the transition - state analog, 2-carboxyarabinitol-1,5-bisphosphate (CABP) and competitively reduced in the presence of 3-PGA, the product of the carboxylation reaction. In that same study as well as in others (McCurry et al., 1981), it was found that the effectors, 6-PGA and NADPH for example, activate the enzyme by stabilizing the active ternary complex by holding the Mg^{2+} and CO_2 onto the enzyme thus decreasing the rate of deactivation. In other words, according to these investigators the effect of an activator is to reduce the "off" rate while leaving the "on" CO_2 rate largely unmodified.

Effectors such as 6-PGA, NADPH and others only exert their effects at suboptimal CO_2 concentrations (Chu and Bassham, 1973, 1975; Whitman et al., 1978; McCurry et al., 1981). Therefore, maximum activation can be established either at high levels of $NaHCO_3$ (10 - 30 mM) or at low levels of $NaHCO_3$ (~ 1 mM) in the presence of an added positive effector. Thus, in vivo, where the CO_2 levels are much closer to 1 mM (from atmospheric CO_2) than 20 mM, these effectors could be regulators of RuBPCase activity. These effectors have been found to exhibit stimulatory, inhibitory or no effect at all depending upon their concentration and order of addition to the reaction mixture (Chu and Bassham, 1973, 1974, 1975; Buchanan and Schurmann, 1973; McCurry et al., 1981). If a positive effector is added to a preincubation mixture containing enzyme, CO_2 and Mg^{2+}, there is an increase in activation at appropriate concentrations due to the stabilization of the active ternary complex. However, since the effector apparently exerts its effect by binding the active site, it must also act as an inhibitor during assay.

Recently, therefore, effector studies have utilized approximately 25-fold dilutions of the preincubation mixture for assay to minimize the inhibitory effects, but no quantitative estimates of the actual inhibition present at assay has been put forth (Hatch and Jensen, 1980). To the contrary, if these effectors are added simultaneously with RuBP, they predominantly exhibit inhibitory effects only due to their competitive action with the substrates. As a result of studies like these on the enzymes from several sources, the phosphometabolites have been classified according to their effects on RuBPCase activity. In general, 6-PGA, inorganic phosphate, FBP, carboxyribitol-P_2, 3-PGA and NADPH are said to be positive effectors, whereas ribose-5-phosphate and a potent synthetic inhibitor CHBP (at moderate to high concentrations) are generally inhibitors of RuBPCase activity (Hatch and Jensen, 1980).

The present study was undertaken for three reasons. First, and foremost, the time dependence of activation by various effectors apparently had not been investigated previously for any RuBPCase. This could be important, if nonistantaneous or variable among the effectors, in comparing results with the effector on different RuBPCases or among different effectors on the same source of enzyme. Secondly, we wanted to investigate the pH dependence of carboxylase activity and the activation process since there was some evidence (Simpson, 1980) that the RuBPCase from comfrey had a lower pH optimum than that from spinach for example. Finally, since Mg^{2+} apparently was absolutely required for activation, the degree of activation by an effector was to be investigated as a function of Mg^{2+} concentration.

There are only two instances in the literature of a carboxylase time dependence study of activation. These were done by A. S. Bhagwat (1981; 1982) on inorganic phosphate and 2-PG, the first of which appeared soon after our studies were underway. He found a maximum after about 10 min of preincubation with enzyme, CO_2, Mg^{2+} and either P_i or 2-PG. This time dependence study of activation relied upon a prior experiment where he determined the maximum preincubation concentration which would not be inhibitory when diluted for assay. A similar time dependence study of activation was also done by Martin and Tabita (1981) using FBP; however, this was done on the oxygenase activity rather than the carboxylase activity.

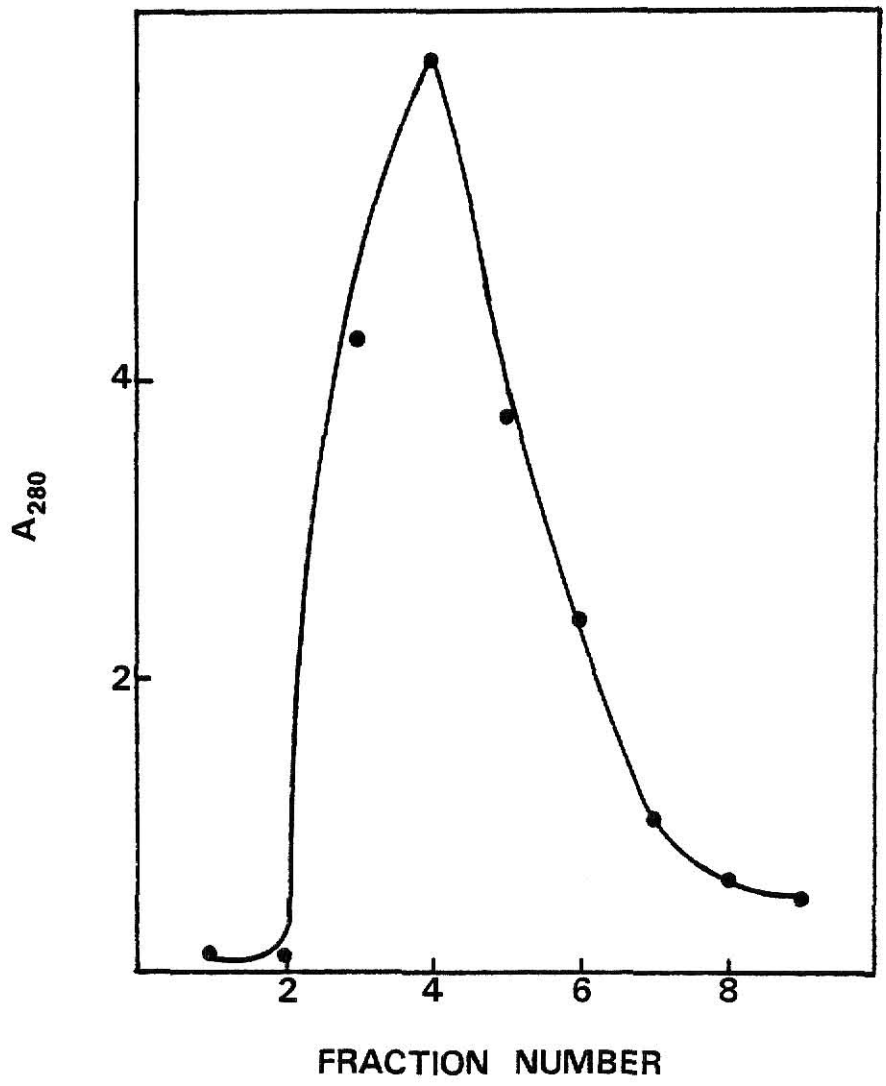
EXPERIMENTAL

The enzyme used in all experiments was isolated and prepared using the procedure developed by Simpson (1980). Briefly, his procedure involved differential centrifugation of freshly homogenized comfrey leaves to obtain the crude fraction, followed by ammonium sulfate fractionation and sucrose density gradient (0.2 - 0.8 M) centrifugation to obtain the partially purified enzyme. The pooled gradient fractions were purged with nitrogen and stored at -70°C until needed. The enzyme was further purified by application to a 3 x 1.5 cm column of DEAE-cellulose. The column was preequilibrated with a buffer suitable for the experiment, the enzyme loaded on to it and freed of sucrose with approximately 50 ml of buffer. The enzyme was eluted with the appropriate buffer made 0.25 M in NaCl. One ml fractions were collected with a Gilson micro-fractionator and the protein concentrations were measured by A_{280} on a Cary 14 Spectrophotometer using the extinction coefficient of $E^{1\%}_{1cm} = 17$ determined for the comfrey enzyme (Bolden, 1982). The tubes containing the highest concentrations of enzyme were then pooled and used in the experiment (Fig. 1). The purified enzyme was electrophoretically homogeneous. The exact procedure for each particular experiment will be described individually below.

Initial Velocity of RuBPCase Activity with and without Cl^-

In these experiments RuBPCase was eluted from the DEAE-column with TME-DTE (50 mM Tris-HCL, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM

Figure 1. A_{280} *vs* fraction number profile of RuBPCase from the DEAE-cellulose column. The enzyme was applied and washed free of sucrose with 50 ml of an appropriate buffer. RuBPCase was eluted with the same buffer made 0.25 M in NaCl.


A_{280}
4
2
2
4
6
8
FRACTION NUMBER

dithioerythritol, pH 7.5) buffer made 0.25 M in NaCl. In one experiment the enzyme was used directly from the column, whereas in the other experiment, the enzyme was dialyzed overnight against TME-DTE buffer to remove the excess chloride ions. Normal assays were run for both enzyme solutions in which 20 ug of RuBPCase were incubated with approximately 520 ul TME-DTE made 1 mM in $NaH^{14}CO_3$ for 10 minutes at 25 ± 1°C in sealed 7 ml scintillation vials to allow for isotopic equilibrium and CO_2 activation of the enzyme. Following incubation, 10 ul of .03875 M D-ribulose 1,-5 bisphosphate (tetrasodium salt, Sigma Chemical Co.), were added and the reaction quenched with 0.2 ml of glacial acetic acid at 10 s intervals for 90 s and at longer intervals up to 300 s. The samples were dried overnight in a fume hood by placing the vials in an aluminum block maintained at ~ 70°C and finally for ~ 2 hrs at ~ 85°C. Heated and unheated controls were run using the same reagents but without RuBP. After drying, 0.5 ml of deionized water and 6.5 ml of liquid scintillation cocktail (8 g PPO, 0.2 g POPOP, 2 liters toluene and 1 liter of Triton X-100) were added and the residual radioactivity was measured on a Beckman Liquid Scintillation Spectrometer using a ^{14}C isoset and a gain setting of 2.5. In all experiments except where indicated, no effort was made to exclude atmospheric CO_2, which at pH 7.5 would contribute about 1 mM HCO_3^-. All experiments were done in duplicate and all results reported as average values.

Activation and Inhibition of RuBPCase Activity by 6-Phosphogluconate and 3-Phosphoglyceric Acid

RuBPCase for these experiments was prepared as described previously including overnight dialysis against TME-DTE. Both 6-PGA and 3-PGA solutions were prepared by diluting a 0.317 M stock solution in TME-DTE with appropriate volumes of the same buffer. In addition, tests for inorganic phosphate and total phosphate were done using the Ames method on both 6-PGA and 3-PGA. An ammonium molybdate analysis with ascorbic acid as the reductant was used to determine inorganic phosphate. Total phosphate was determined by complete digestion of the 6-PGA and 3-PGA solutions by $Mg(NO_3)_2$ followed by analysis for inorganic phosphate. The results indicated that both solutions were relatively pure containing less than 3% inorganic phosphate and 106% total phosphate based on a theoretical amount. For the activation experiments, an appropriate quantity of RuBPCase solution was made 2 mM in $NaH^{14}CO_3$. A 20 ug aliquot (10 - 15 ul) of RUBPCase was added to an equal volume of a 6-PGA solution at twice the desired concentration and incubated for 50 min. Following activation appropriate amounts of TME-DTE made 1 mM in $NaH^{14}CO_3$ and containing 0.7 mM RuBP were added (final volume 553.5 ul) to initiate the reaction which was quenched after 90 s. For the inhibition experiments, RuBPCase was made 2 mM in $NaH^{14}CO_3$ as before and diluted 1:1 with TME-DTE instead of 6-PGA and allowed to incubate for 50 min. Again appropriate volumes of TME-DTE made 1 mM in $NaH^{14}CO_3$ were added followed by the simultaneous addition of 10 ul of both RuBP (39 mM) and a 6-PGA concentration corresponding to that obtained in the preceeding experiment. The reaction was run as before.

Heated and unheated controls were run in the presence of 6-PGA without RuBP. Normal assays were run with RuBP but no 6-PGA. Samples were dried and analyzed as previously reported. Similar experiments were done using 3-PGA.

Time Dependence of Activation of RuBPCase by Several Phosphometabolites

A concentrated sample (2 mg/ml) of RuBPCase from the DEAE-cellulose column was made 2 mM in $NaH^{14}CO_3$ and incubated for 50 minutes. An equal volume of 8 mM 6-PGA was added and after times ranging from 10 s to 20 min, 20 ug aliquots were added to appropriate volumes of TME-DTE made 1 mM in $NaH^{14}CO_3$ and 0.7 mM in RuBP and reacted for 90 s at 25 ± 1°C. Normal and control assays were run with and without RuBP respectively. Samples were dried and analyzed as previously described. The same experiments were done with 3-PGA, FBP, 2-PG and the synthetic inhibitor CHBP replacing the 6-PGA. An additional experiment was done using the same procedure, except an 8 ul aliquot (15 units/assay) of carbonic anhydrase was added simultaneously with the 6-PGA. This amount of carbonic anhydrase was found to equilibrate the enzyme with CO_2 in less than 30 s (Bolden, unpublished data). Once the initial shorter times were completed the vial containing carbonic anhydrase was sealed to prevent loss of CO_2 to the atmosphere. The effect of assay time on the apparent time dependence of activation was studied by repeating the above experiments, except the carbonic anhydrase one, using a 20 s assay time with 3-PGA, and FBP. With 3-PGA additional experiments were done using both a 20 s and 90 s assay where the enzyme and 3-PGA were preincubated separately in 1 mM $NaH^{14}CO_3$ instead of 2 mM and 0 mM,

respectively, before being combined.

Time Course of Catalysis at Maximum Activation

Separate pools of RuBPCase, effector and TME-DTE were incubated for 25 min in 1 mM $NaH^{14}CO_3$, after which 10 ul of RUBPCase and effector solutions were combined and activated for various additional times depending on the effector (3-PGA - 90 s, 6-PGA - 15 min, FBP - 70 s, 2-PG - 40 s), then 533.5 ul of the equilibrated TME-DTE containing 0.7 mM RuBP were added and the reaction quenched at various times up to 90 s. Normal assays activated with 1 mM $NaH^{14}CO_3$ only, were run before each effector. Controls and standard assays were also done. The standard assays (saturating conditions of substrate and cofactors) contained in a pool 60 ug of RuBPCase in 20 mM $NaHCO_3$ (0.74 mM in $NaH^{14}CO_3$, 19.26 mM in $NaHCO_3$) and an appropriate volume of TME-DTE. This mixture was incubated for 50 minutes where upon two 543.5 ul aliquots were removed and 10 ul of RuBP were added and reacted for 60 s. To the remaining 543.5 ul, 10 ul of TME-DTE were added and used as a control. All samples were treated as before.

Effect of Assay pH on RuBPCase Activity when Activated in the Presence of 6-PGA at pH 7.5

A 20 ug aliquot of purified enzyme made 2 mM in $NaH^{14}CO_3$ was incubated with an equal volume of either 1.6 mM, 16 mM or 32 mM 6-PGA in TME-DTE for 50 minutes. Appropriate volumes of T(MOPS) ME-DTE (25 mM Tris-HCL, 25 mM MOPS, 10 mM Mg^{2+}, 1 mM EDTA, 2 mM dithioerythritol) at a desired pH (pH was adjusted with 1 N HCL and 2.5 N NaOH where necessary),

containing 1 mM $NaH^{14}CO_3$ and 0.7 mM RuBP were added to give a total volume of 553.5 ul. The reaction was allowed to run for 90 s. The pH values of the solutions ranged from 6.8 to 8.5. Controls were run at each pH using the same reagents, but without RuBP, as well as normal assays at each pH without 6-PGA. Residual ^{14}C-activity was determined as previously described.

Effect of Activation pH on RuBPCase Activity in the Presence of 6-PGA when Assayed at pH 7.5

RuBPCase was eluted from a DEAE-cellulose column with T(MOPS)ME-DTE made 0.25 M in NaCl and adjusted to the desired pH. A 20 ug aliquot of RuBPCase was then incubated as before with an equal volume of 1.6 mM, 16 mM or 32 mM 6-PGA in T(MOPS)ME-DTE; appropriate volumes of TME-DTE (pH 7.5), made 1 mM in $NaH^{14}CO_3$ and 0.7 mM in RuBP were added to give a final volume of 553.5 ul and reacted for 90 s. Controls were run as before at pH 7.5 only. Normal assays containing no 6-PGA were run at each pH.

Effect of Assay pH and Mg^{2+} Concentrations on RuBPCase Activity when Activated with 6-PGA at pH 7.5 and 10 mM Mg^{2+}

RuBPCase was eluted from a DEAE-cellulose column with TME-DTE (pH 7.5, 10 mM Mg^{2+}). Enzyme made 2 mM in $NaH^{14}CO_3$ was incubated with an equal volume of 1.6 or 16 mM 6-PGA for 50 minutes. Following activation appropriate volumes of T(MOPS)ME-DTE at various pH's and Mg^{2+} concentrations containing 1 mM $NaH^{14}CO_3$ and 0.7 mM RuBP were added to give a final volume of 553.5 ul and reacted for 90 s. Controls were

run using the same procedure along with standard assays containing saturating conditions of substrate and cofactors.

Effect of pH and Mg^{2+} Concentrations on Activation of RuBPCase by 6-PGA when Assayed at pH 7.5

RuBPCase was eluted from a DEAE-cellulose column at various pH's and 1 mM Mg^{2+}. Microliter amounts of 2.8 M $MgCl_2$ were added to bring the Mg^{2+} concentration up to 2, 4, 10 and 20 mM after which the solution was diluted 1:1 with 16 mM 6-PGA in TE-DTE buffer (50 mM TRIS-HCL, 1 mM EDTA, and 2 mM dithioerythritol) giving 0.5, 1, 2, 5 and 10 mM Mg^{2+} at activation in 1 mM $NaH^{14}CO_3$. Following 50 minutes of incubation appropriate volumes of TME-DTE (pH 7.5, 10 mM Mg^{2+}), 1 mM $NaH^{14}CO_3$ and 0.7 mM RuBP were added and reacted for 90 s. Normal assays with no 6-PGA were run at each pH and Mg^{2+} concentration along with standard assays (20 mM $NaHCO_3$, 10 mM Mg^{2+}).

Stabilization of the CO_2 - Activated Complex by 6-PGA in Low Mg^{2+}

RuBPCase was eluted from a DEAE-cellulose column with TME-DTE made 0.25 M in NaCl. Aliquots of the pooled RuBPCase fractions adjusted to 2 mg/ml of protein were made 2 mM in $NaH^{14}CO_3$ and incubated for 25 minutes with an equal volume of TME-DTE buffer or 3.2 mM 6-PGA in TME-DTE buffer. A 20 ug aliquot (20 ul) was added to 523.5 ul of TM-DTE buffer (50 mM TRIS-HCL, 1 mM Mg^{2+}, 2 mM dithioerythritol), made 1 mM in $NaH^{14}CO_3$ for the control. Where appropriate 3 ul of 0.317 M 6-PGA were added to other aliquots. These mixtures were then incubated again for various times whereupon 10 ul of RuBP in TM-DTE buffer

were added to initiate the reaction. The reaction was quenched after 20 s. Standard assays were run as before for the fully activated controls. EDTA was present throughout the experiment at a concentration of 0.5 mM. The amount of Mg^{2+} bound by EDTA was determined at pH 7.5 from the known ionization constants for EDTA and the binding constants of Mg^{2+} to each of the ionic species. It was calculated that EDTA bound 1 - 2% of the 1 mM Mg^{2+} present during assay.

Km for CO_2 in the Absence and Presence of 6-PGA

RuBPCase was eluted from a DEAE-cellulose column with TME-DTE (pH 8.0) buffer which was purged with N_2 gas for 30 min prior to use. RuBPCase was incubated for 45 minutes in 20 mM $NaH^{14}CO_3$, after which a 20 ug aliquot (11.5 ul) was added to a 542 ul portion of N_2 purged TME-DTE, which was made 0.7 mM in RuBP and in which the $NaH^{14}CO_3$ had been adjusted to give final CO_2 concentrations ranging from 26.9 uM to 116.8 uM based on the overall $H_2O+CO_2 \rightleftarrows HCO_3^- + H^+$ equilibrium constant (7.4×10^{-7} M) of Gibbons and Edsall (1963). The reactions were quenched after 6 s to 22 s reaction time at ~ 7 s intervals. For the experiments with 6-PGA, the same procedure was followed except for the addition of 6 ul of 16 mM 6-PGA to the incubation mixture. This changed the 20 ug aliquot to 12. 5 ul and the final CO_2 concentrations now ranged from 28.5 uM to 118.4 uM. Controls and standard assays were again run. All samples were dried and analyzed as previously described.

RESULTS

Initial Velocity of RuBPCase Carboxylase Activity with and without Cl^-

Time dependent studies on RuBPCase carboxylase activity using the normal assay and enzyme which had, and had not, been dialyzed to remove the 0.25 M NaCl needed to elute the RuBPCase from the DEAE-column indicated a linear increase in velocity over at least 90 s in both cases (Fig. 2). The close agreement between the two indicated little effect from having the enzyme in 0.25 M added NaCl. Thus, undialyzed enzyme was used for most future studies and 90 s assays were used where possible to maximize the incorporation of radio label. After 90 s, a somewhat slower rate was seen which can be maintained for at least five minutes in the normal assay medium. This no doubt represents the steady state rate reported by other investigators (McCurry et al., 1981).

Activation by Effectors at Different Levels of Bicarbonate

6-Phosphogluconate and other phosphometabolites are effectors of RuBPCase activity only at low concentrations of $NaHCO_3$ (Table 1). Percent enhancement was calculated from

$$\frac{CPM_B - CPM_A}{CPM_A} \times 100,$$

where CPM_B indicates the enzyme was preincubated with 6-PGA and diluted at assay, whereas for CPM_A, 6-PGA was added at the same final diluted concentration simultaneously with RuBP. Increased RuBPCase activity can be seen either at saturating concentrations of $NaHCO_3$ (20 mM) or at

Figure 2. Initial velocity of RuBPCase activity in the presence, (●) and absence, (■) of 200 mM in added NaCl. Dialyzed (57 mM Cl^-) and undialyzed (257 mM Cl^-) 20 ug (20 ul) aliquots of RuBPCase eluted from the DEAE-cellulose column were incubated with approximately 520 ul of TME-DTE (57 mM Cl^-) pH 7.5, made 1 mM in $NaH^{14}CO_3$ for 10 min in sealed vials. A 10 ul portion of RuBP (39 mM) was then added to initiate the reaction. The reactions were quenched at 10 s intervals for the first 90 s and at longer intervals up to 300 s. Average heated control (background) CPM were subtracted from the average of duplicate determinations of each point. The data in the absence of Cl^- was normalized to that in the presence of Cl^-.

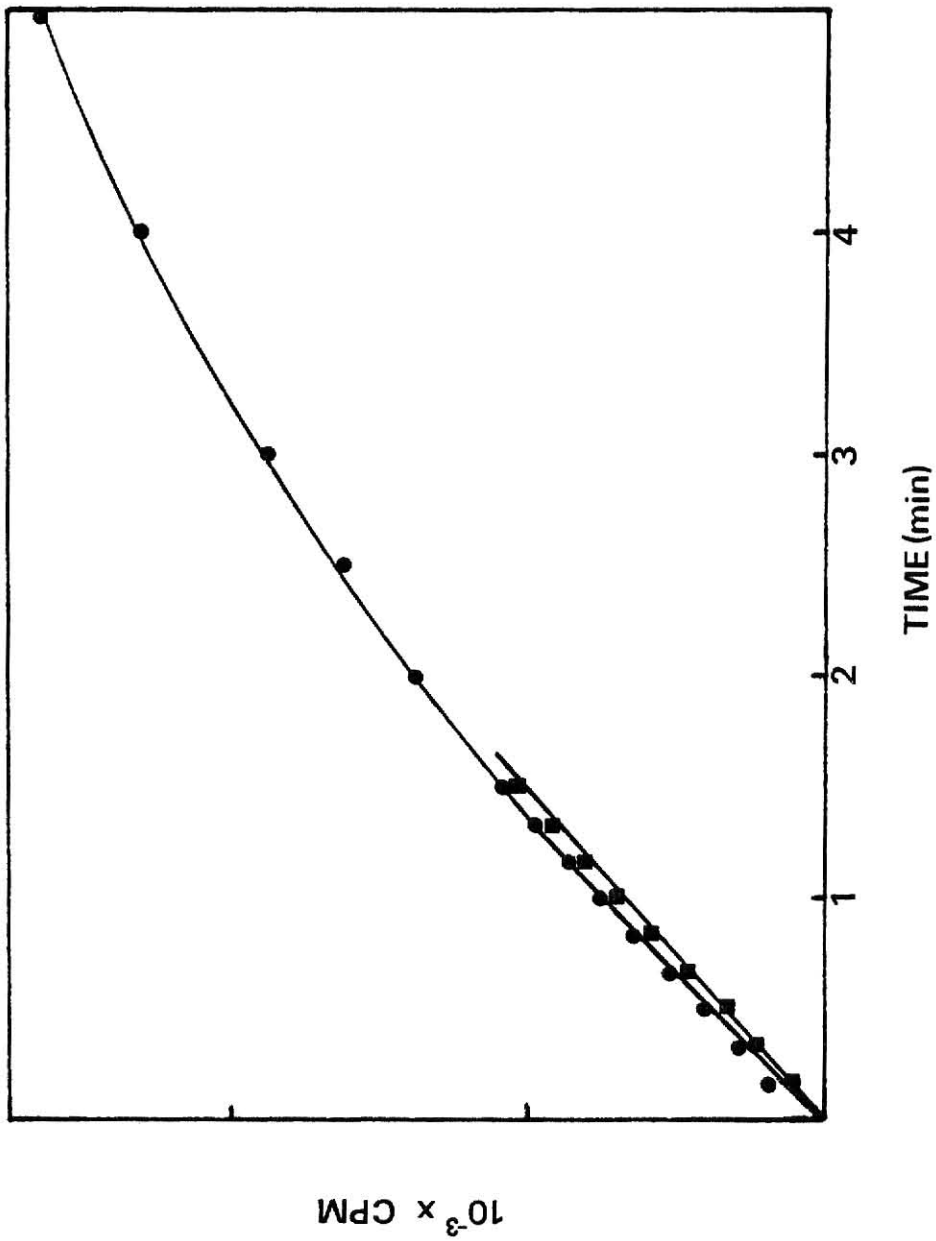

10^{-3} x CPM
TIME (min)
1
2
3
4

Table I

Percent Enhancement of RuBPCase Activity by 16.0 mM 6-PGA at preincubation [1000/1 (6-PGA)/(active sites)] as a function of bicarbonate concentrations

mM Bicarbonate	Percent Enhancement
1	139
10	42

low concentrations of $NaHCO_3$ (1 mM) in the presence of certain added positive effectors. The positive effector acts to hold CO_2 and Mg^{2+} on the activating site (Badger and Lorimer, 1981), which results in increased enzymatic activity (Tabita and McFadden, 1972; Buchanan and Schurmann, 1973; Chu and Bassham, 1973, 1975; Lorimer, et al., 1976).

Time Dependence of RuBPCase Activation by Several Phosphometabolites

For these studies concentrated enzyme (2 mg/ml) was incubated for at least 25 min at 25 ± 1° in the normal 50 mM Tris buffer which was made 2 mM in added $NaH^{14}CO_3$ and 10 mM in Mg^{2+}. An equal volume of a particular phosphometabolite was added and the 20 ug aliquots removed at various times were used to initiate the carboxylase reaction in the same buffer made 1 mM in $NaH^{14}CO_3$ and 0.7 mM in RuBP. The time dependence of activation by 6-PGA showed a maximum after ~ 20 min of preincubation at 25 ± 1° with 4 mM effector (Fig. 3). However, some activation can be seen at the earliest times after addition of 6-PGA, therefore some activation no doubt occurred during the 90 s assay. When carbonic anhydrase was added simultaneously with 6-PGA, there was no change in the shape of the time dependence curve. There was, however, increasing divergence between the sets of data up to about 10 min at which time a 30% increase had occurred when carbonic anhydrase was present (Fig. 4). At longer times the two curves appeared to converge again.

Time dependent experiments done with 3-PGA, FBP, and 2-PG showed very different profiles (Figs. 5, 6, 7 respectively). In each case

Figure 3. Time dependence of activation of RuBPCase by 6-PGA. A concentrated sample of RuBPCase (2 mg/ml) made 2 mM in $NaH^{14}CO_3$ was incubated in a sealed vial for 50 min at 25 ± 1°. An equal volume of 8 mM 6-PGA was added and at times ranging from 10 s to 20 min 20 ug aliquots were withdrawn and added to appropriate volumes of TME-DTE made 1 mM in $NaH^{14}CO_3$ and 0.7 mM in RuBP to initiate the reaction. The reactions were quenched after 90 s. The arrow indicates the normal assay (NO 6-PGA). The points are averages of duplicate determinations.

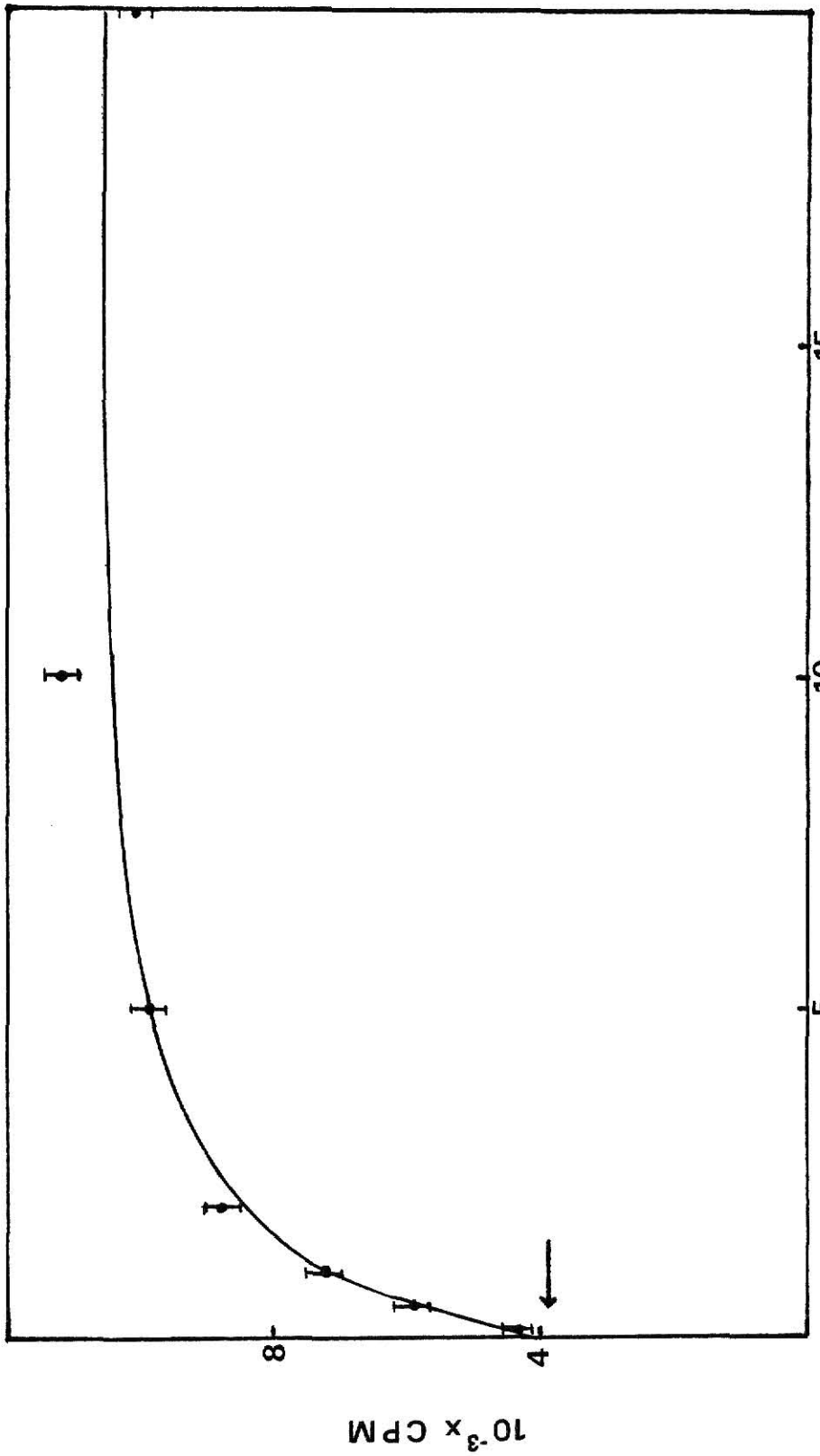

10^{-3} x CPM
4
8
5
10
15
PREINCUBATION TIME(min)

Figure 4. Time dependence of activation of RuBPCase by 6-PGA in the presence, (■) and absence, (●) of carbonic anhydrase (15 units/assay). The same procedure was followed as for Fig. 3, except the enzyme and 2 mM $NaH^{14}CO_3$ was incubated for 25 min instead of 50 min and where appropriate 5 ul of carbonic anydrase were added simultaneously with the 8 mM 6-PGA. The arrow indicates the normal assay.

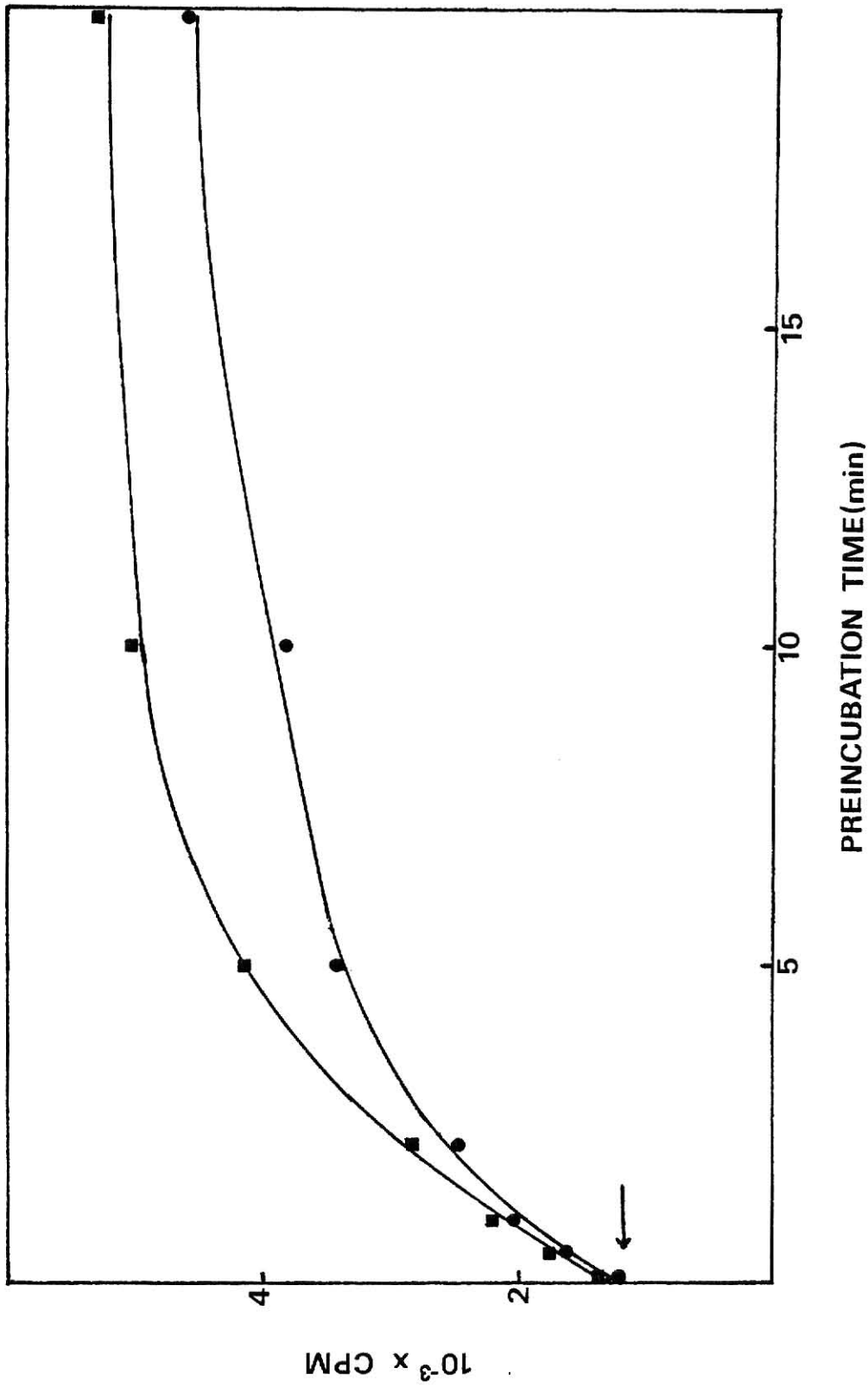

10^{-3} x CPM
4
2
5
10
15
PREINCUBATION TIME(min)

Figure 5. Time dependence of activation of RuBPCase by 3-PGA: 0.8, (■); 8.0, (▲); and 80 mM, (●) at preincubation. These experiments were done similar to those in Fig. 3, except for the replacement of 6-PGA by 3-PGA. The arrow indicates the normal assay. Note the change in the abscissa scale following the break.

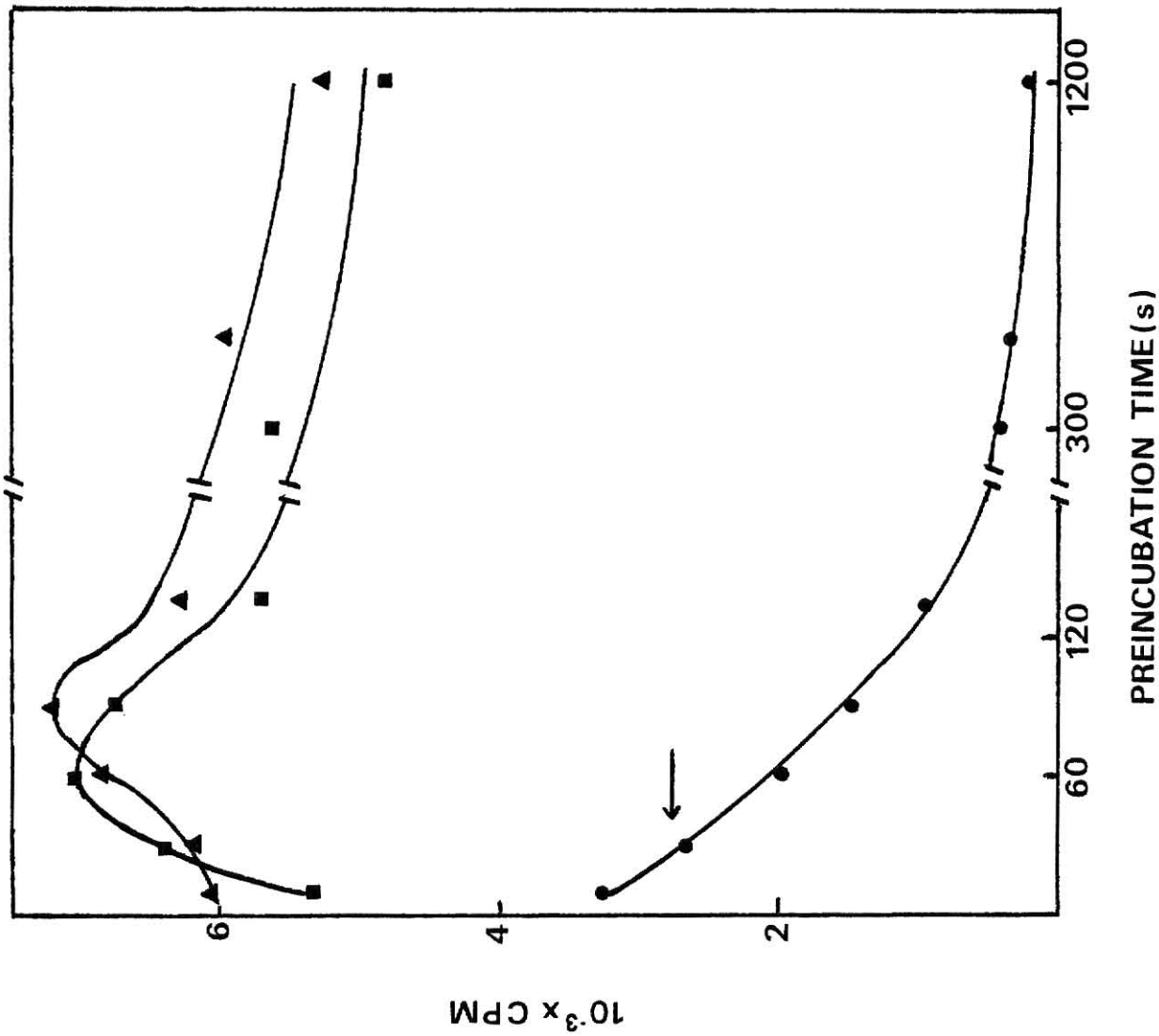

10.3x CPM
6
4
2
60
120
300
1200
PREINCUBATION TIME(s)

Figure 6. Time dependence of activation of RuBPCase by FBP: 0.16, (▲); 0.8 (■); and 8 mM, (●) at preincubation. These experiments were done similar to those in Fig. 3, except for a 25 min incubation of the enzyme with $NaH^{14}CO_3$ instead of 50 min and replacement of 6-PGA by FBP. Note the change in the abscissa scale following the break. The arrow indicates the normal assay.

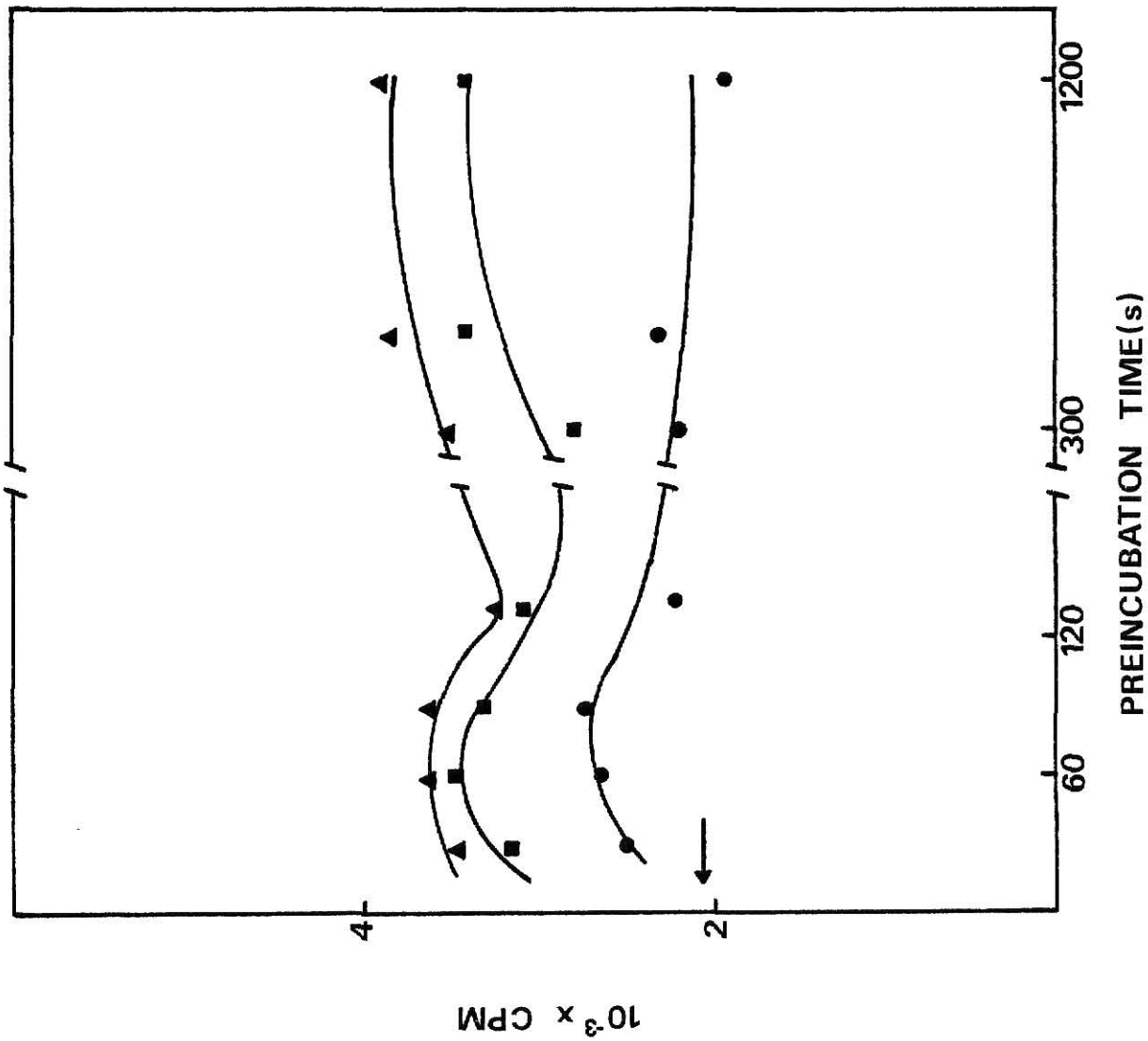
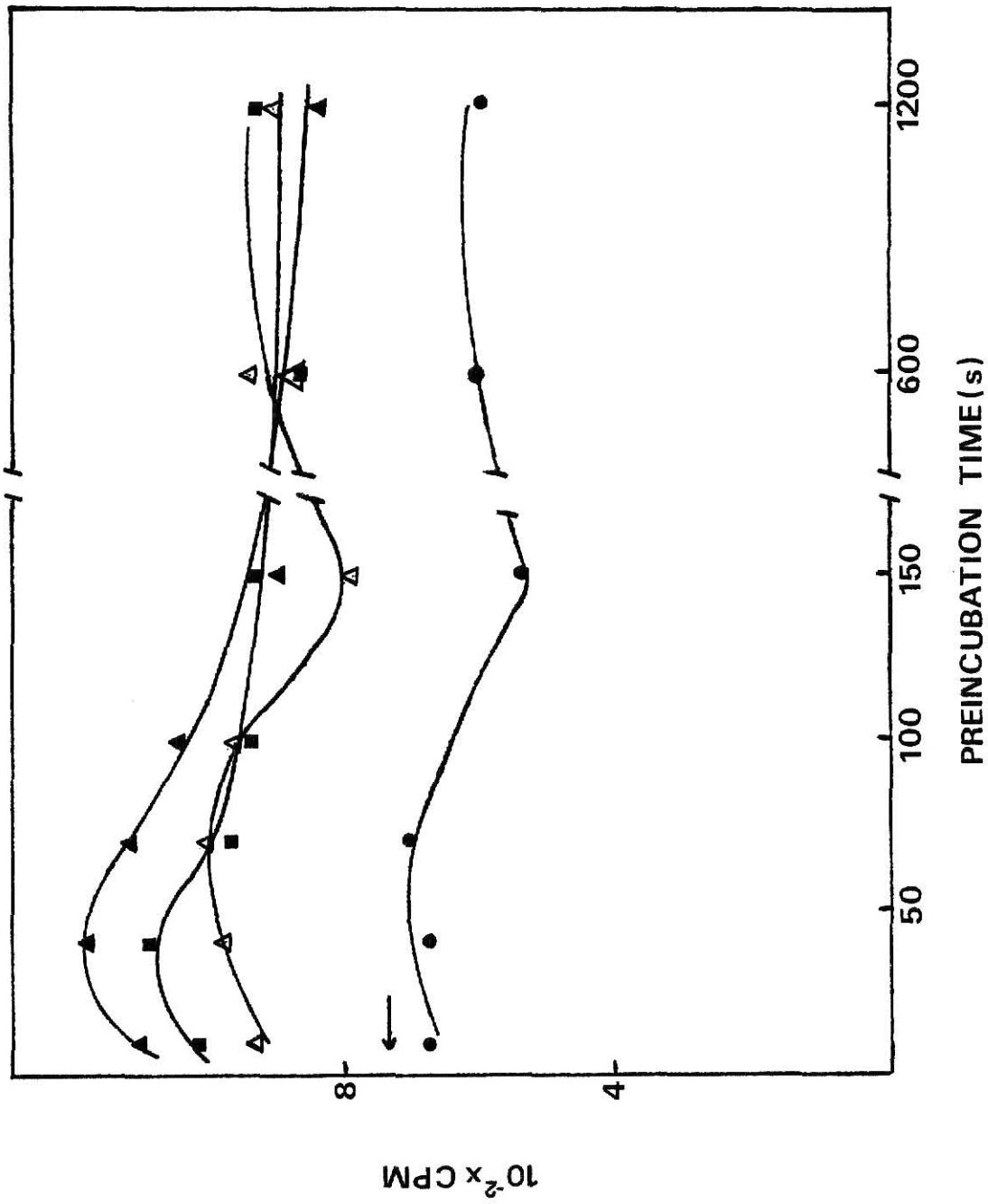

4
2
10^{-3} x CPM
60
120
300
1200
PREINCUBATION TIME(s)

Figure 7. Time dependence of activation of RuBPCase by 2-PG: 0.16, (▲); 0.8, (■); 4.0, (Δ); and 16.0 mM, (●) at preincubation. These experiments were done similar to those in Fig. 6. The 4.0 mM 2-PG data (Δ) was taken from a different experiment and normalized to the other data from comparison of the normal assay values. The arrow indicates the normal assay value to which these data were normalized.


8
4
10^{-2} x CPM
50
100
150
600
1200
PREINCUBATION TIME (s)

there was an initial rise between 40 - 90 s with a subsequent decline after 90 s of activation when using concentrations of 0.8 and 8.0 mM for 3-PGA; 0.16, 0.8 and 8 mM for FBP and 0.16, 0.8, 4 and 16 mM for 2-PG. At very high concentrations of 3-PGA (80 mM, the enzymatic activity dropped off continuously from the earliest times until it reached a nearly fully inhibited steady state after 20 min. Although the three effectors acted qualitatively similar up to about 2 min, afterwards they behaved differently. With 3-PGA there was a continuing decline out to the longest times measured for one set of data (Fig. 5) whereas for the other two sets of data and for 2-PG at low concentrations, the activity appeared to reach a steady state. To the contrary 2-PG at high concentrations and FBP low concentrations increased again at longer times (Figs. 7 and 6 respectively). The same experiments run on CHBP, a potent synthetic inhibitor of RuBP-Case activity, indicated yet another type of time dependence profile. There seemed to be a slow continous decline in activity from the shortest time of 10 s to the longest time of 20 min (Fig. 8).

These data, however, are complicated by a small but steady decline in enzymatic activity during the course of the experiment as illustrated in Table II. This decrease was linear with time and the data were corrected by estimating as closely as possible the time after the first normal assay each reaction was initiated. Application of these corrections to the 3-PGA data (Figs. 9 and 10) maintained the qualtitative features noted earlier. At longer preincubation times, the appearant steady decline noted in Fig. 5 was not completely eliminated but it declined less sharply (Fig. 9). In future experiments the decrease in inherent enzymatic activity was

Figure 8. Time dependence of activation of RuBPCase by CHBP: 0.032, (■); 0.16, (▲); and 1.6 uM, (●) at preincubation. These experiments were done similar to those in Fig. 6. The 1.6 uM CHBP data (●) was taken from a different experiment and normalized to the remaining data from comparison of the normal assay values. The arrow indicates the normal assay value to which this set of data had been normalized.

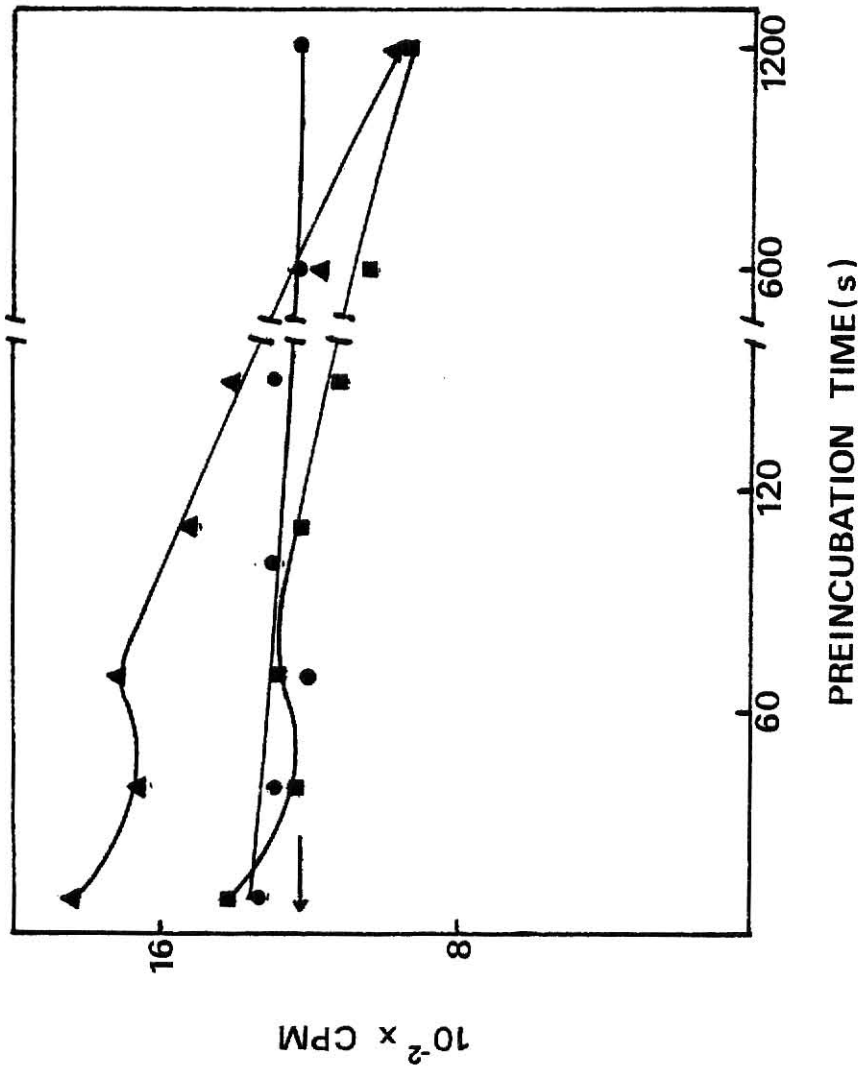

16
8
10^{-2} x CPM
60
120
600
1200
PREINCUBATION TIME(s)

Table II

Inherent Enzymatic Activity Loss during a typical Activation Study (~3h)

Normal Assay	Activity (CPM)
Beginning	530
Middle	476
End	401

Figure 9. Time dependence of activation of RuBPCase by 3-PGA. This is the same 3-PGA data [0.8, (■); and 8.0 mM, (▲)] as in Fig. 5, corrected for the inherent enzymatic activity loss (~ 20%) over the course of the experiment. The arrow indicates the normal assay value from the beginning of the experiment to which all data were adjusted.

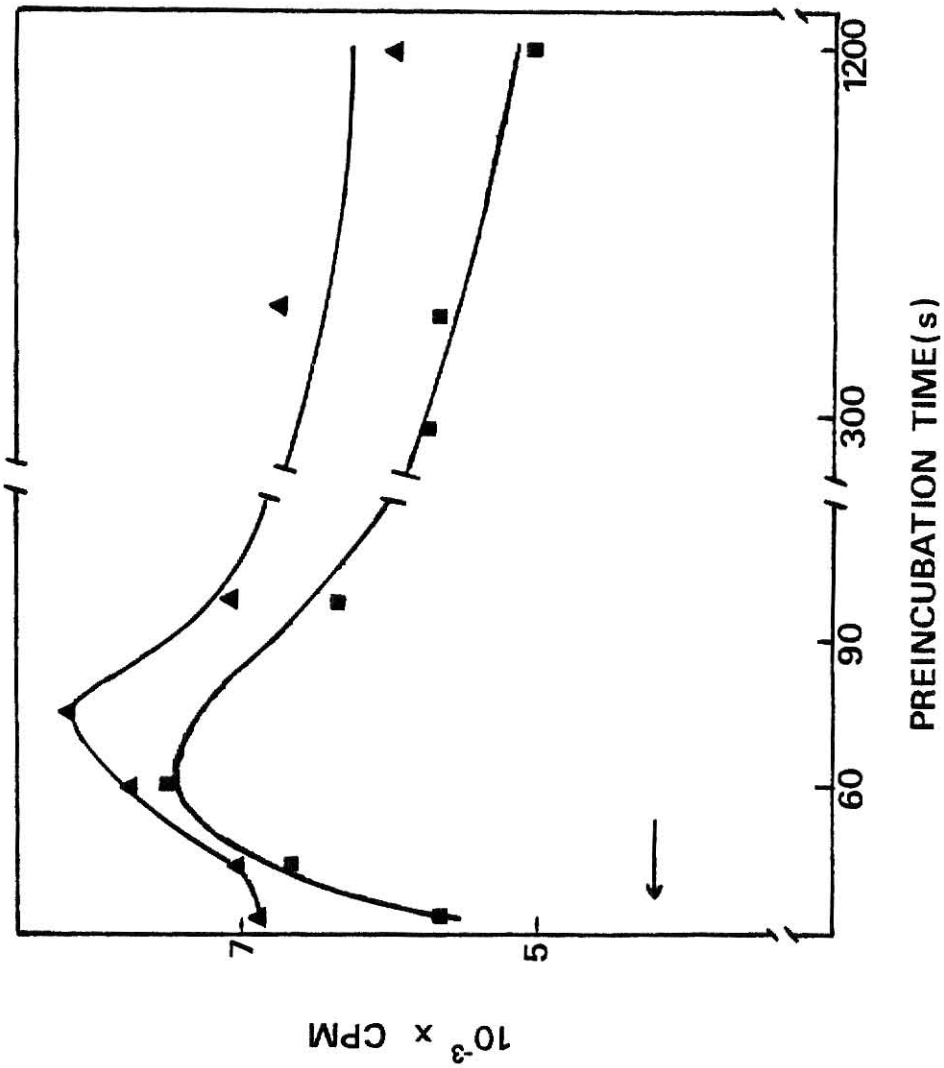
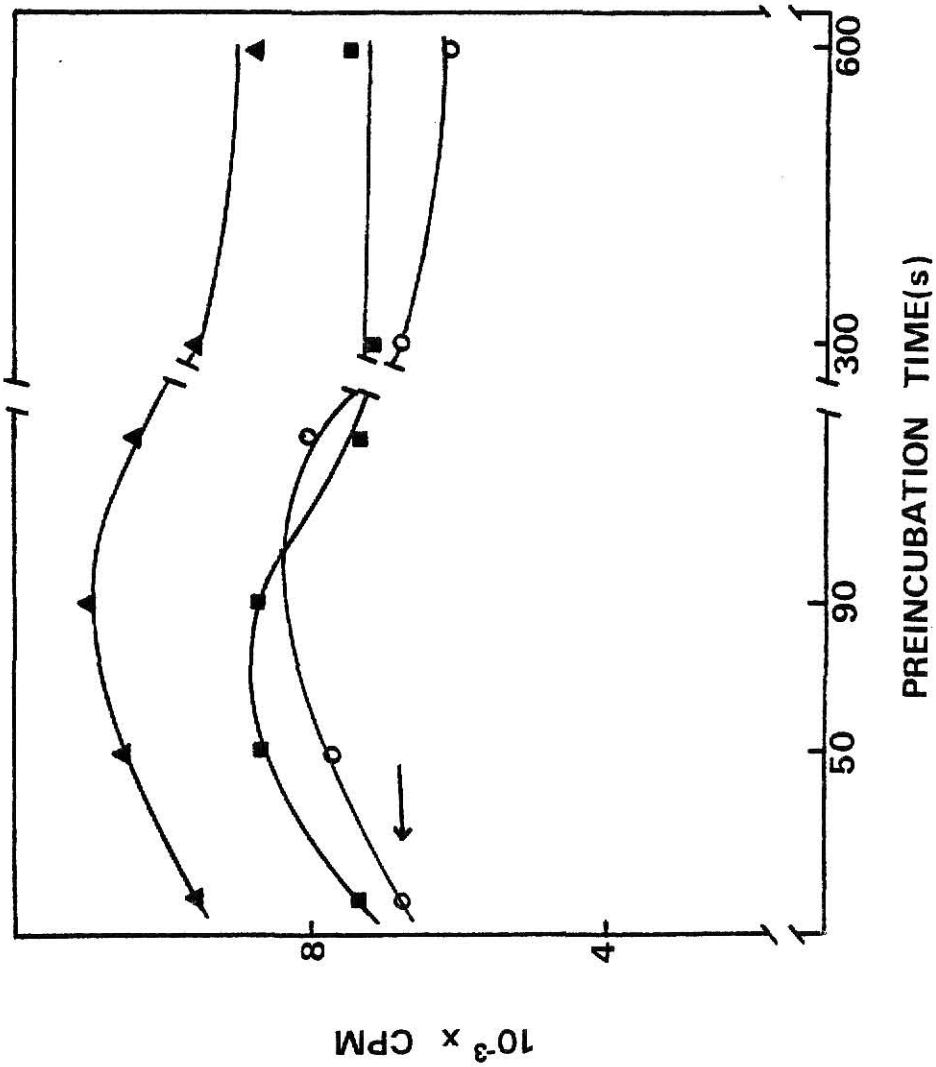

7
5
60
90
300
1200
PREINCUBATION TIME(s)
10-3 x CPM

Figure 10. Time dependence of activation of RuBPCase by 3-PGA: 0.8, (■); 8.0, (▲); and 16 mM, (O). These experiments were done similar to those in Fig. 6, except both the enzyme and 3-PGA were incubated separately in 1 mM $NaH^{14}CO_3$ for 25 min before mixing. These data, including the normal assay (arrow) were corrected for the inherent enzymatic activity loss (~ 20%) over the course of the experiment.


10⁻³ x CPM
8
4
50
90
300
600
PREINCUBATION TIME(s)

corrected in another way. For the 3-PGA data shown in Fig. 11, normal assays were run at the beginning and end of each set of 3-PGA concentrations and the reported activities were measured relative to these values. The same approach was used for the 2-PG and CHBP data.

To test for the possibility of $^{14}CO_2$ - enzyme equilibration effects on these profiles another set of experiments were performed in which the enzyme and 3-PGA were separately equilibrated for 25 min with 1 mM $NaH^{14}CO_3$ and then mixed at time zero. No substantial differences in the shapes of these profiles were noted whether assayed for 20 or 90 s after initiating the reaction with the concentrated 3-PGA-enzyme mixture (compare figures 11 and 12). However, the extents of activation by the 0.8 (50/1) and 8 mM (500/1) 3-PGA were reversed in the 20 s assay (Fig. 11) compared to that obtained with the 90 s assay (Fig. 12), this was true whether or not the data were corrected for activity changes during the course of the experiment.

A time course of catalysis at maximum activation experiment was done on each effector studied. Except for 6-PGA (Fig. 13), the other effectors, as illustrated for 3-PGA (Fig. 14), remained linear over the 90 s assay time.

Activation of RuBPCase by Several Phosphometabolites

6-Phosphogluconate and other phosphometabolites are effectors of RuBPCase activity only at low concentrations of $NaHCO_3$ (Table I). At 1 mM $NaHCO_3$ and 10 mM $MgCl_2$, pH 7.5, activity increased with increasing 6-PGA concentration attaining a maximum at 1.6 mM (100/1 molar ratio of 6-PGA to active sites) at activation which corresponds to about a

Figure 11. Time dependence of activation of RuBPCase by 3-PGA: 0.8, (■); and 8.0 mM, (▲). These experiments were done similar to those in Fig. 10, except a 20 s assay was used in place of a 90 s assay.

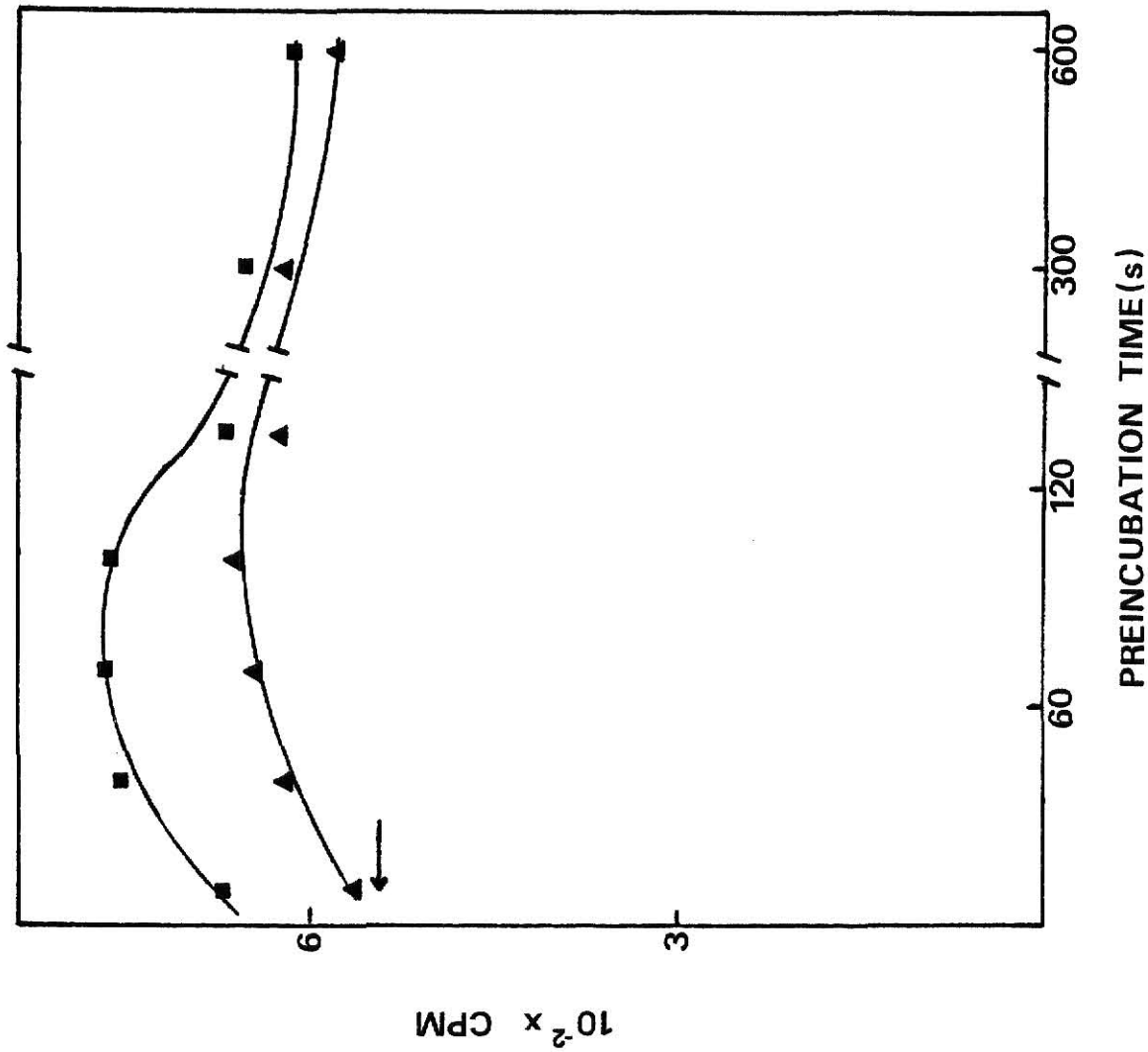

PREINCUBATION TIME(s)
60
120
300
600
3
6
10⁻² x CPM

Figure 12. Time dependence of activation of RuBPCase by 3-PGA; 0.8, (■); 8.0 (▲); and 16 mM, (O). This graph illustrates the original uncorrected data corresponding to the corrected data of Fig. 10.

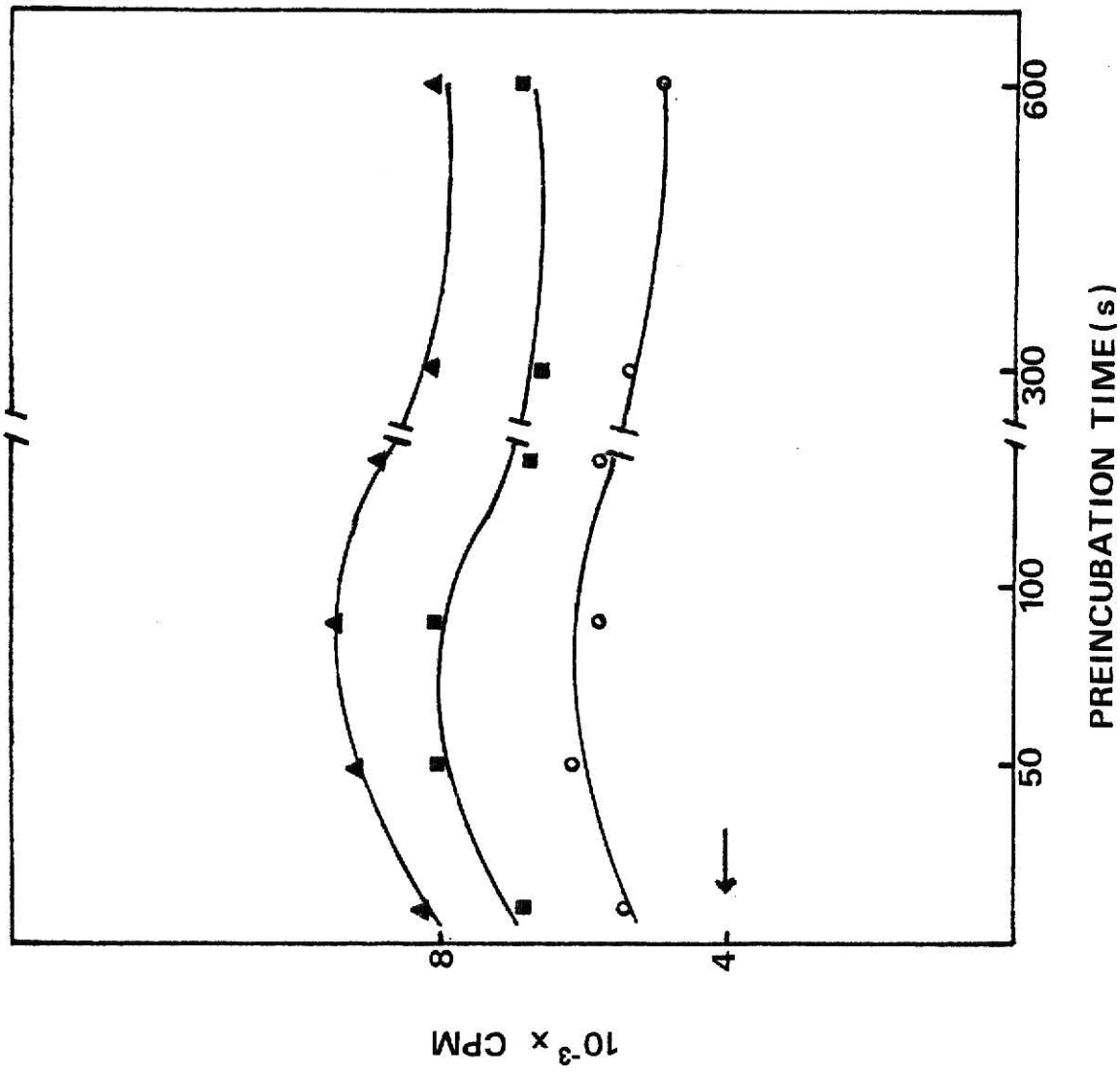

8
4
10^{-3} x CPM
50
100
300
600
PREINCUBATION TIME(s)

Figure 13. Time course of catalysis following maximum activation by 6-PGA; 0.16, (Δ); 1.6, (□); and 16 mM, (○); 0 mM (●), normal assay at preincubation. The enzyme (2 mg/ml), 6-PGA and TME-DTE solutions were all made 1 mM in $NaH^{14}CO_3$ and incubated separately for 25 min, then 10 ul of both enzyme and 6-PGA were mixed and preincubated for 15 min to allow for maximum activation; then 533.5 ul of TME-DTE made 1 mM in $NaH^{14}CO_3$ and 0.7 mM in RuBP were added and reacted for the times indicated. The normal assays were done using the same procedure except for replacing the 6-PGA by TME-DTE. Controls were run similar to the normal assays but without RuBP.

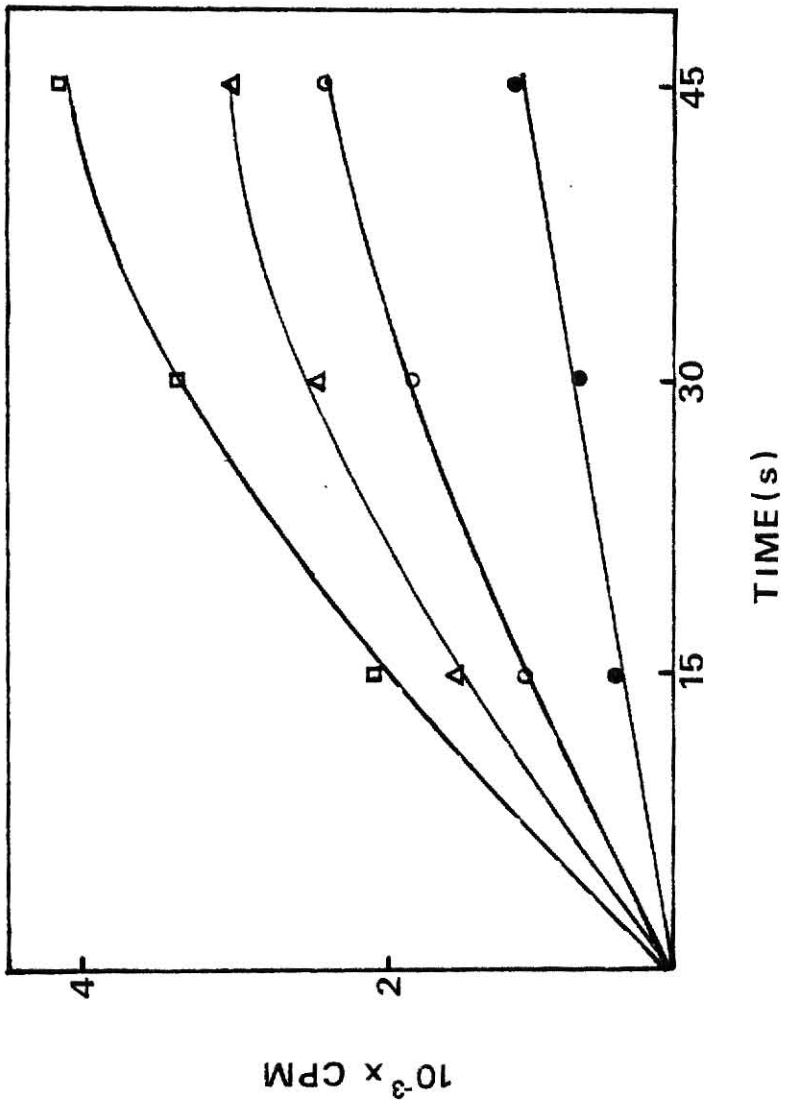
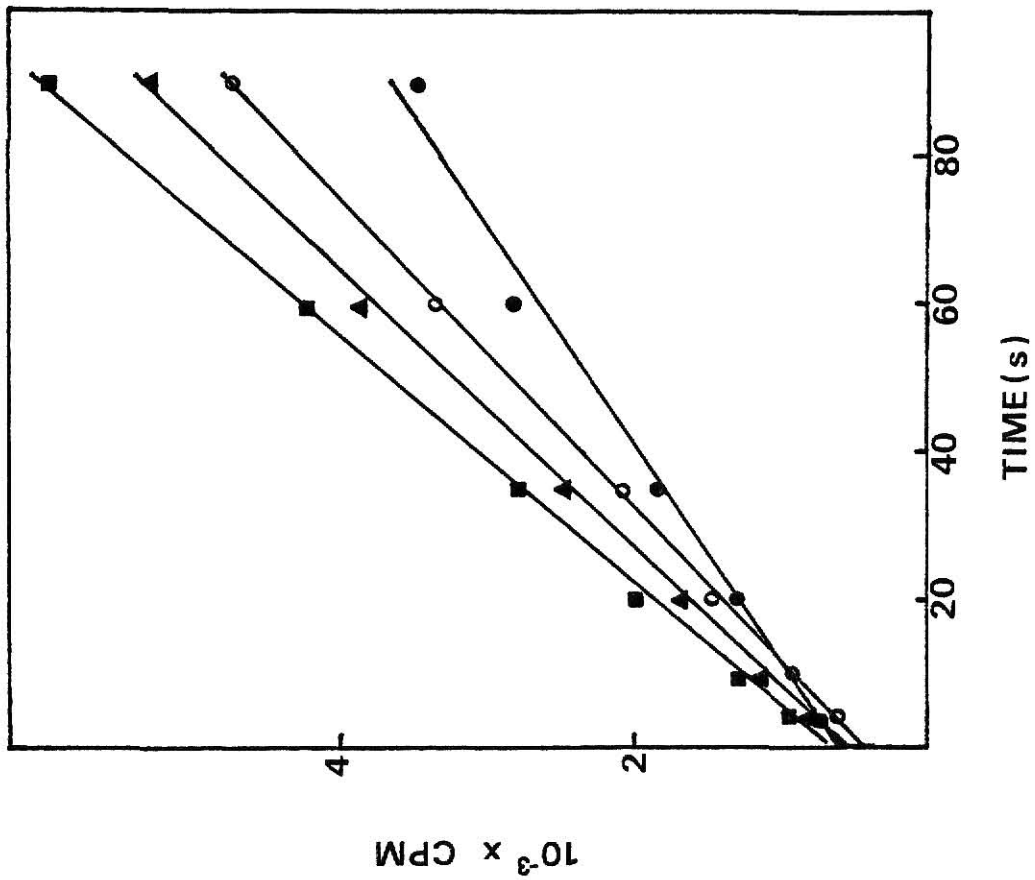

4
2
10^{-3} x CPM
15
30
45
TIME(s)

Figure 14. Time course of catalysis following maximum activation by 3-PGA: 0.8, (■); 8.0, (▲); and 16 mM, (O); 0 mM (●), normal assay at preincubation. These experiments were done similar to those in Fig. 13, except the enzyme and 3-PGA mixture was preincubated for 90 s instead of 15 min as with 6-PGA. These data were not corrected for background CPM.


TIME (s)
20
40
60
80
2
4
10⁻³ x CPM

5-fold increase in activity (Fig. 15). Furhter increases in 6-PGA had an inhibitory effect on activity as shown by the decline in activity beyond 1.6 mM 6-PGA.

The traditional enhancement plot (Chu and Bassham; 1973) when applied to similar data with the comfrey enzyme was biphasic (Fig. 16). The second maximum probably results from the lowering of the pH optimum for activation at higher 6-PGA concentrations (vida infra). Therefore, as the 6-PGA concentration was raised the pH optimum for activation approached the pH of the assay (7.5) giving additional activation during the 90 s assay and resulting in a second maximum. Enhancement was calculated by $\frac{(CPM_B - CPM_A)}{CPM_A}$ where CPM_B indicates the activity when 6-PGA was preincubated with concentrated enzyme and CPM_A, the activity when the final concentration of 6-PGA was added simultaneously with RuBP. This method gave an enhancement of 5.5 fold at 4.0 mM 6-PGA at activation which corresponded to a molar ratio of 250/1 6-PGA to active sites (Fig. 16). An identical experiment on the same enzyme preparation after three more weeks of storage gave the same biphasic appearance with a slightly smaller enhancement (Fig. 16). Similar experiments done with 3-PGA showed the same biphasic appearance to the enhancement plot as seen with 6-PGA (Fig. 17); however, maximum activity resulted from about 8 mM (500/1) 3-PGA at activation. This gave an enhancement of activity of ~ 30% (Fig. 17). For comparisons, apparent maximum activations for 6-PGA, 3-PGA and several other phosphometabolites, taken from the time dependence data (Figs. 3, 5, 6, 7, 8) are summarized in Table III. Clearly degrees of activation by the enhancement and time dependence method do not agree well quantitatively but do show the same general trends for 6-PGA and 3-PGA.

Figure 15. Effect of 6-PGA on the activity of RuBPCase. A 20 ug sample of enzyme was incubated for 50 min with an equal volume of 6-PGA at the molar ratios shown and diluted 28-fold for assay by the normal procedure. The abscissa was made nonlinear to display the data conveniently. The following concentrations (mM) correspond to the molar ratios indicated: 10/1, 0.16; 100/1, 1.6; 500/1, 8.0 and 5000/1, 80. The origin represents a 1/1 molar ratio of 6-PGA to active sites.

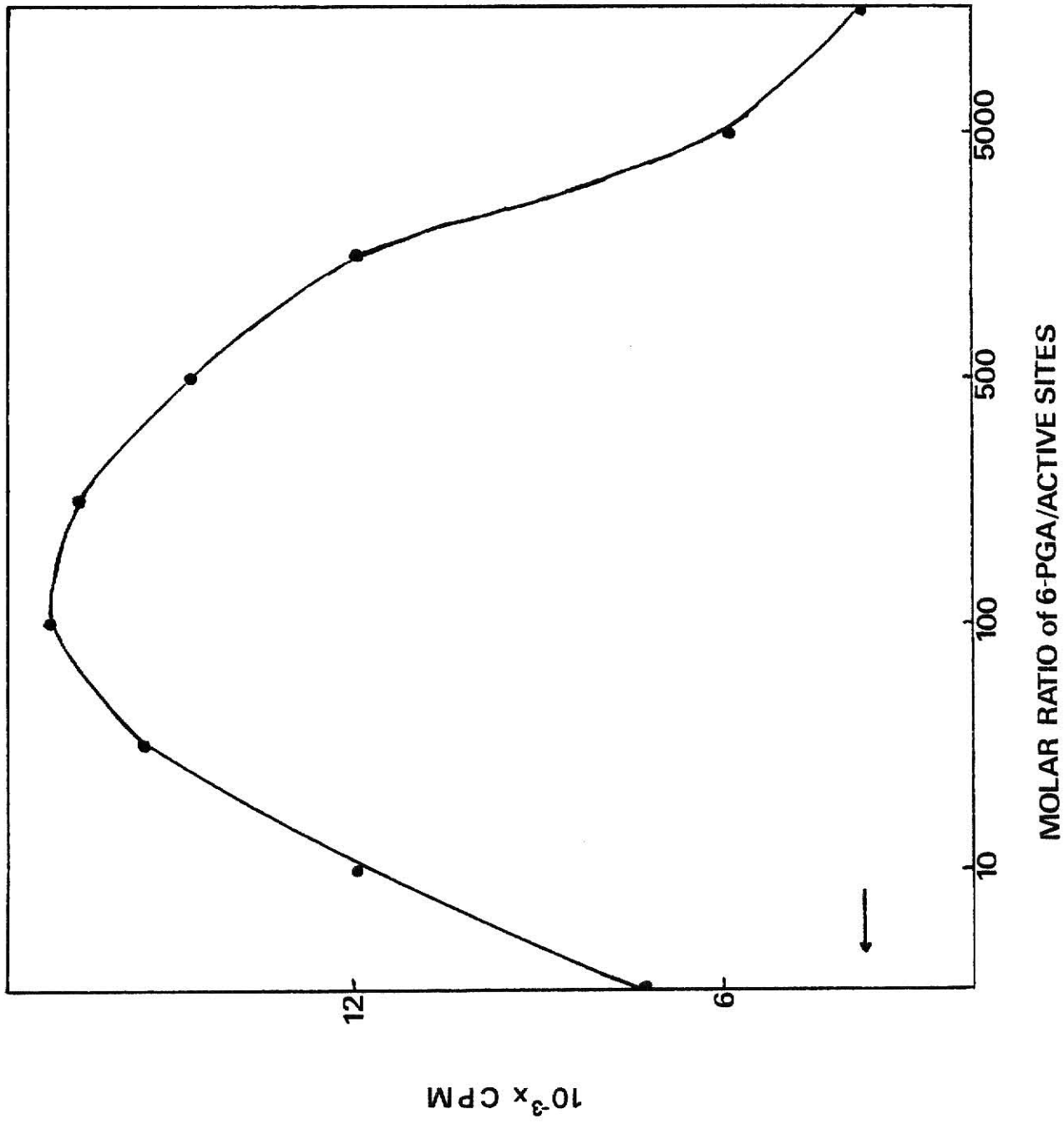

10^{-3} x CPM
12
9
6
10
100
500
5000
MOLAR RATIO of 6-PGA/ACTIVE SITES

Figure 16. Enhancement plot of the data shown in Fig. 15, (■) after including the inhibition data. Enhancement is defined as $(CPM_B - CPM_A)/CPM_A$ where CPM_B is the activity obtained when the enzyme is preincubated with 6-PGA and CPM_A is the activity obtained when the same 6-PGA concentration was added simultaneously with the RuBP. A log scale is used arbitrarily to display all the data on the plot. (●), indicates data from a similar experiment done on the same enzyme preparation after 3 additional weeks of storage.

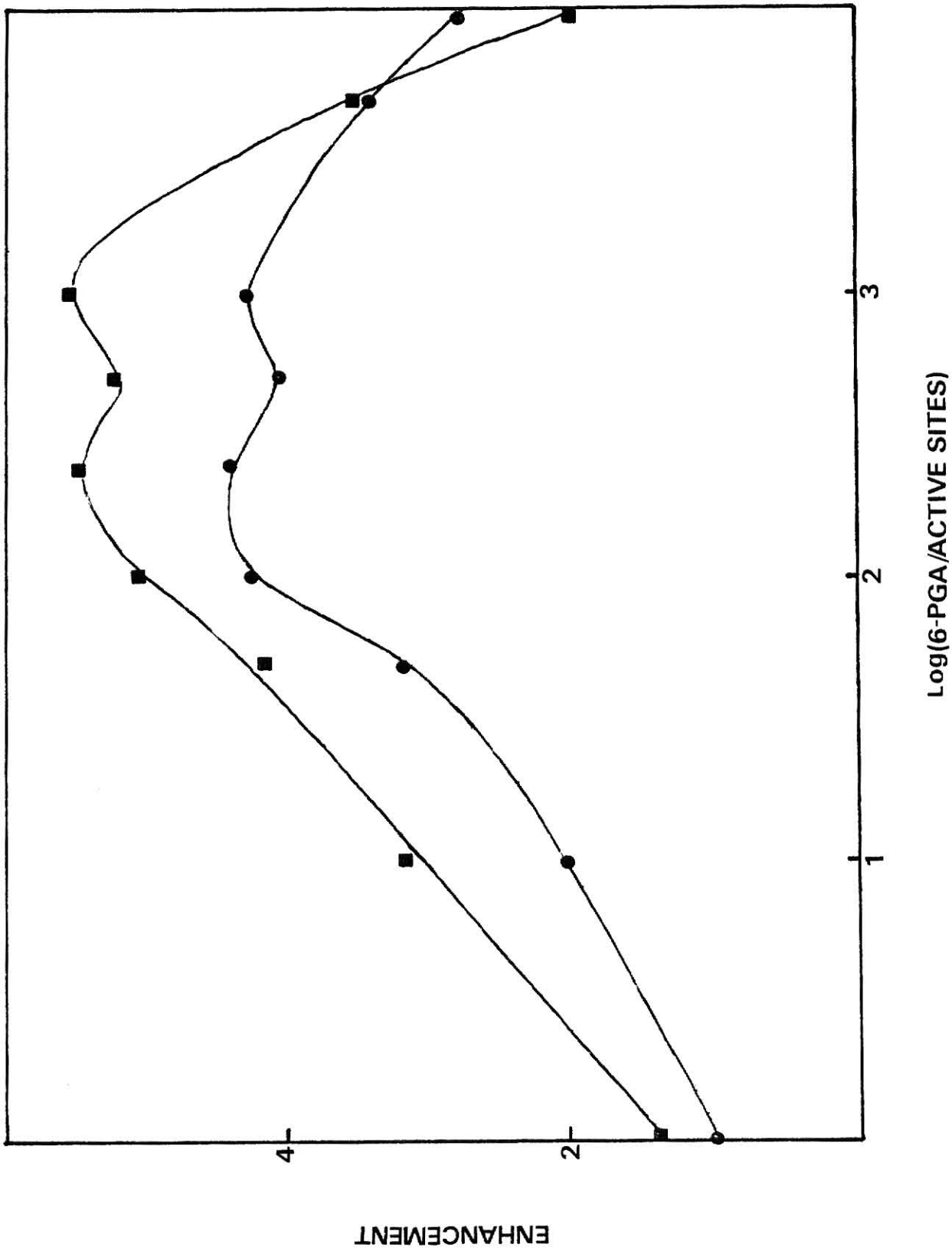

ENHANCEMENT
4
2
1
2
3
Log(6-PGA/ACTIVE SITES)

Figure 17. Enhancement plot of the effects of 3-PGA on RuBPCase activity. Enhancement is defined as in Fig. 16. A log scale is used arbitrarily to display all the data on one plot.

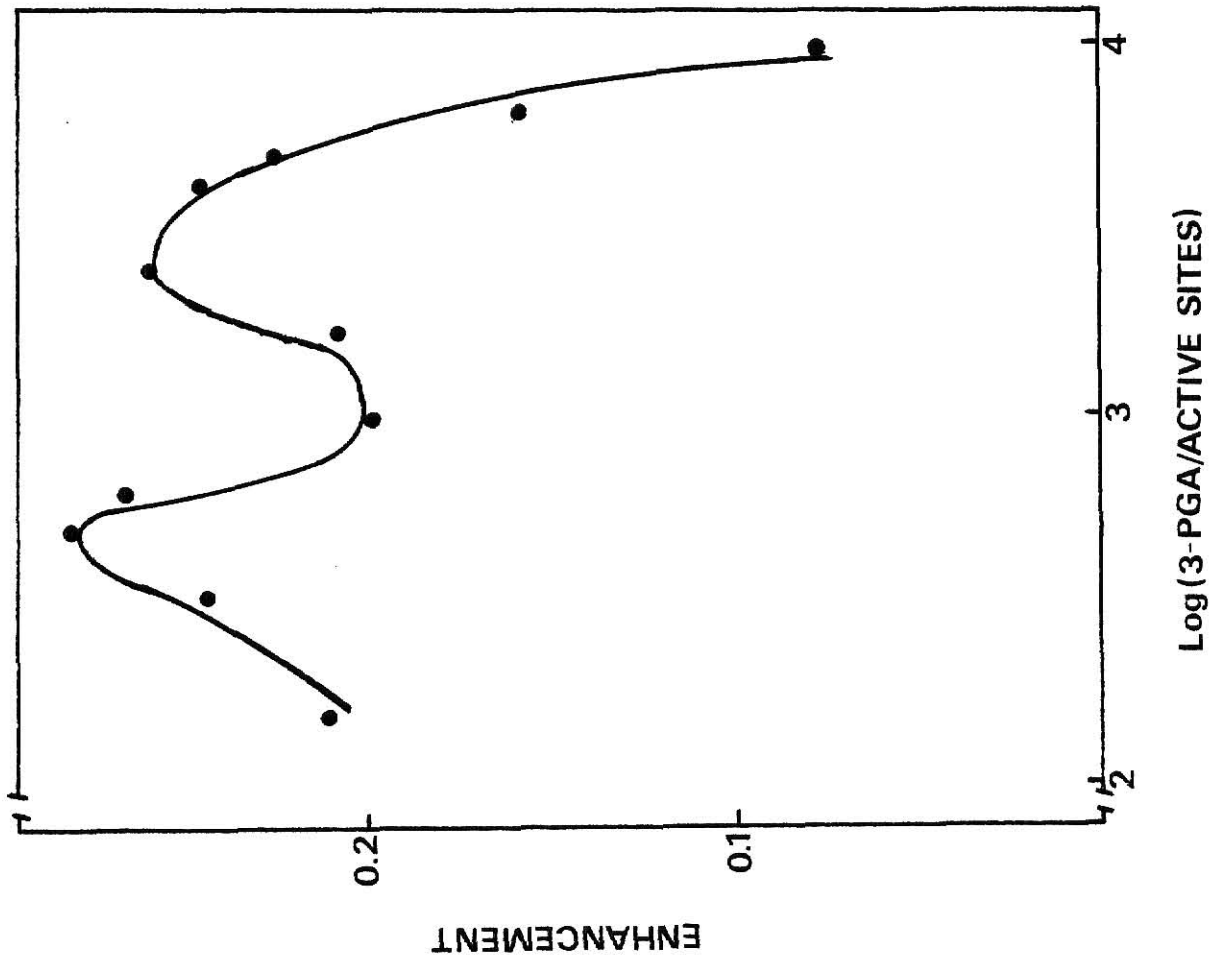

ENHANCEMENT
0.2
0.1
4
3
2
Log(3-PGA/ACTIVE SITES)

Table III

Apparent Maximum Activation (%) from Optimum Preincubation Times

Effector concentration at preincubation	% Activation	
	Maximum	"Steady State" (20 min)
4.0 mM 6-PGA	190	190
8.0 mM 3-PGA	162	91
8.0 mM 3-PGA[a]	133	100
8.0 mM 3-PGA[b]	22	8
0.8 mM 3-PGA[c]	41	14
0.16 mM FBP	79	96
0.16 mM 2-PG	72	19
0.16 μM CHBP	17	inhibition

[a]Enzyme and 3-PGA both incubated at 1 mM $NaH^{14}CO_3$ and assayed for 90 s.

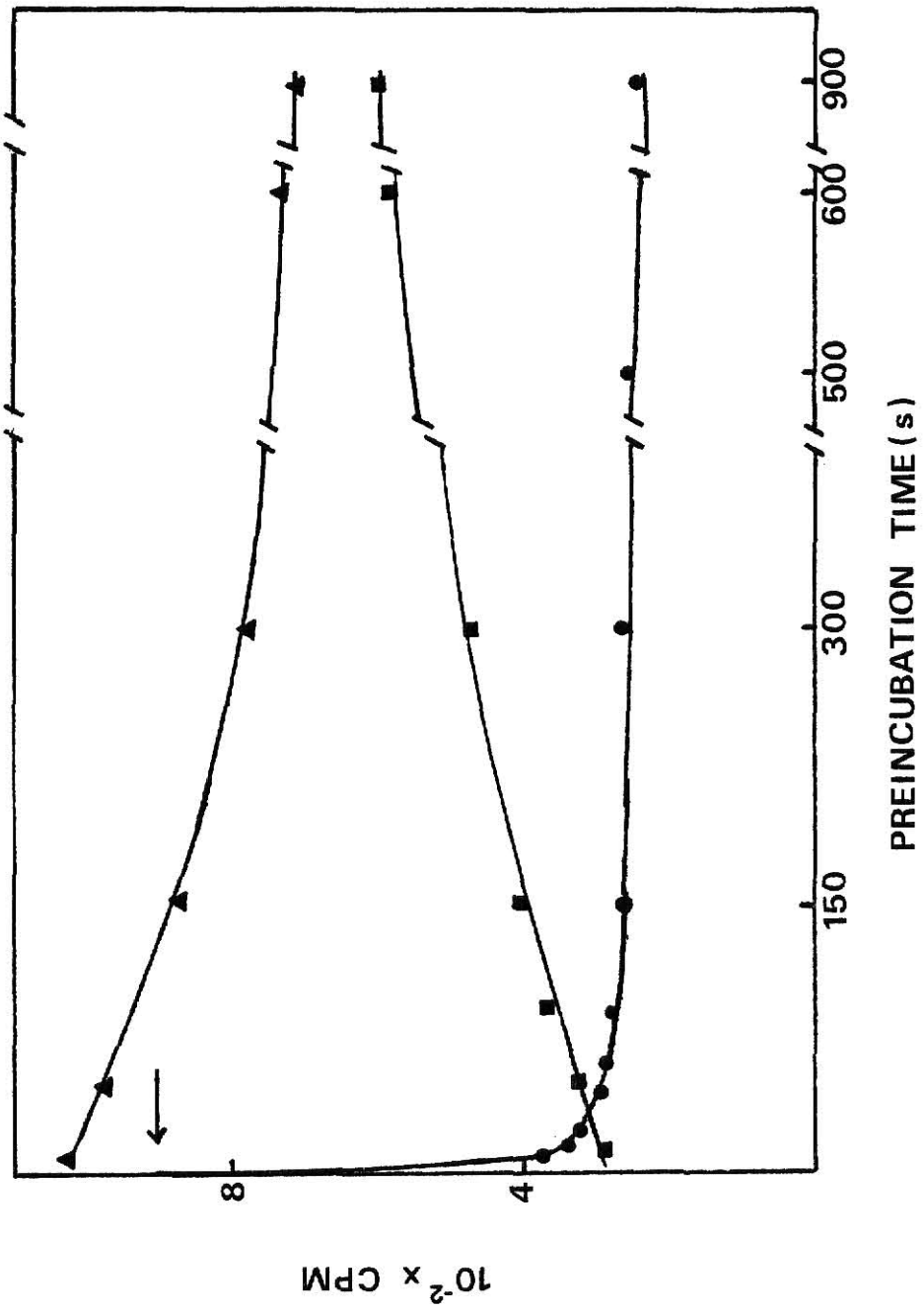
[b]Enzyme and 3-PGA both incubated at 1 mM $NaH^{14}CO_3$ and assayed for 20 s.

[c]The apparent optimum when assayed as in b.

Stabilization of the CO_2-Activated Complex of RuBPCase by 6-PGA in Low Mg^{2+}

For these studies concentrated enzyme was incubated in the normal 50 mM Tris buffer which was made 2 mM in added $NaH^{14}CO_3$ and 10 mM in Mg^{2+} with an equal volume of TME-DTE or 3.2 mM 6-PGA for 25 min at 25 ± 1°. A 20 ug aliquot was added to ~ 520 ul of TM-DTE buffer made 1 mM in $NaH^{14}CO_3$ and when appropriate contained 1.6 mM 6-PGA and incubated again for various times, whereupon 0.7 mM RuBP was added to initiate the reaction. In the absence of 6-PGA, approximately 60% of the carboxylase activity was lost in the first 40 s following addition of 20 ug of activated enzyme to a larger volume of buffer containing 1 mM $NaH^{14}CO_3$ and Mg^{2+} (Fig. 18). Enzymatic activity leveled off following 2 min of incubation in 1 mM Mg^{2+}. The net loss of activity at this and later times was approximately 72%. In the presence of 1.6 mM 6-PGA at activation and catalysis, only 32% of the carboxylase activity was lost when diluted into 1 mM Mg^{2+} (Fig. 18). However, the average activation due to the presence of 6-PGA was about 300%. When 1.6 mM 6-PGA was present only at catalysis, carboxylase activity increased almost 100% (Fig. 18). In both experiments containing 6-PGA, the enzymatic activities appeared to be converging toward a common value. Thus as the incubation time with 6-PGA in low Mg^{2+} increased, more activation occurred and at long enough times (~ 20 min) would no doubt have given the same result as preincubation in high Mg^{2+} and subsequent dilution into the same low Mg^{2+} concentration.

Figure 18. Stabilization of the CO_2-activated complex by 6-PGA in low Mg^{2+}. A concentrated sample of RuBPCase made 2 mM in $NaH^{14}CO_3$ was preincubated for 25 min with an equal volume of TME-DTE or 3.2 mM 6-PGA. A 20 ug aliquot (20 ul) was then added to 523 ul of TM-DTE buffer (1 mM Mg^{2+}) made 1 mM in $NaH^{14}CO_3$ and where appropriate 1.6 mM in 6-PGA and preincubated again for various times. 10 ul of 39 mM RuBP were added to initiate the reaction. The reactions were stopped after 20 s. (●), absence of 6-PGA; (■), 1.6 mM 6-PGA present at catalysis; (▲), 1.6 mM 6-PGA present at activation and catalysis. The arrow indicates the normal assay in 10 mM Mg^{2+}.


PREINCUBATION TIME (s)
150
300
500
600
900
4
8
10^{-2} x CPM

Effect of Assay pH and Mg^{2+} Concentration on the Carboxylase Activity when Activated in the Presence and Absence of 6-PGA

For these studies, concentrated enzyme (2 mg/ml) was incubated in the normal 50 mM Tris buffer which was 1 mM in added $NaH^{14}CO_3$, 10 mM in Mg^{2+} and contained the desired amounts of 6-PGA. Aliquots of this solution were used to initiate the reaction in the same buffer except that it contained 0.7 mM RuBP, varying amounts of Mg^{2+}, and had been adjusted to the indicated pH values. Data for the comfrey enzyme showed that the pH optimum for carboxylase activity was highly dependent of Mg^{2+} and 6-PGA concentration when assayed in 1 mM $NaHCO_3$. Increasing the 6-PGA concentration at a fixed Mg^{2+} concentration shifted the pH optimum for carboxylase activity to lower values (Fig. 19). At 10 mM Mg^{2+} and no 6-PGA, the apparent pH optimum was monophasic and found to be about 7.4, whereas at 0.8 mM 6-PGA at activation, the main apparent pH optimum was near 7.0 but was biphasic with a second smaller optimum at 7.5 (Fig. 19). It should be noted that the (500/1) 6-PGA (8 mM) data had lower activity than the (50/1) data (0.8 mM) due to the inhibition at the higher 6-PGA concentration. Increasing the Mg^{2+} concentration at a constant 6-PGA concentration tended to broaden and shift the pH optimum back toward the normal pH optimum of ~ 7.4 (Fig. 20). The data plotted in Fig. 20 also indicate that in the absence of 6-PGA, carboxylase activity is very low, when assayed in 1 mM $NaHCO_3$ and low Mg^{2+}. At pH 7.5, the specific activities in 1, 2 and 5 mM Mg^{2+} are .01, .02, and .05 umoles $^{14}CO_2$ fixed/min/mg protein, respectively, whereas under fully saturating conditions of 20 mM HCO_3^- and 10 mM Mg^{2+} a typical value of 1.5 umoles

Figure 19. Effect of assay pH on RuBPCase activity when activated in the presence of 6-PGA at pH 7.5. For these experiments, 6-PGA was incubated with the enzyme made 1 mM in $NaH^{14}CO_3$ for 50 min at 25 ± 1°. A 20 ug aliquot was used to initiate the reaction in T(MOPS)ME-DTE made 1 mM in $NaH^{14}CO_3$ and 0.7 mM in RuBP and adjusted to the indicated pH. (●), no 6-PGA; (▲), 0.8, mM; (■), 8.0 mM 6-PGA at activation.

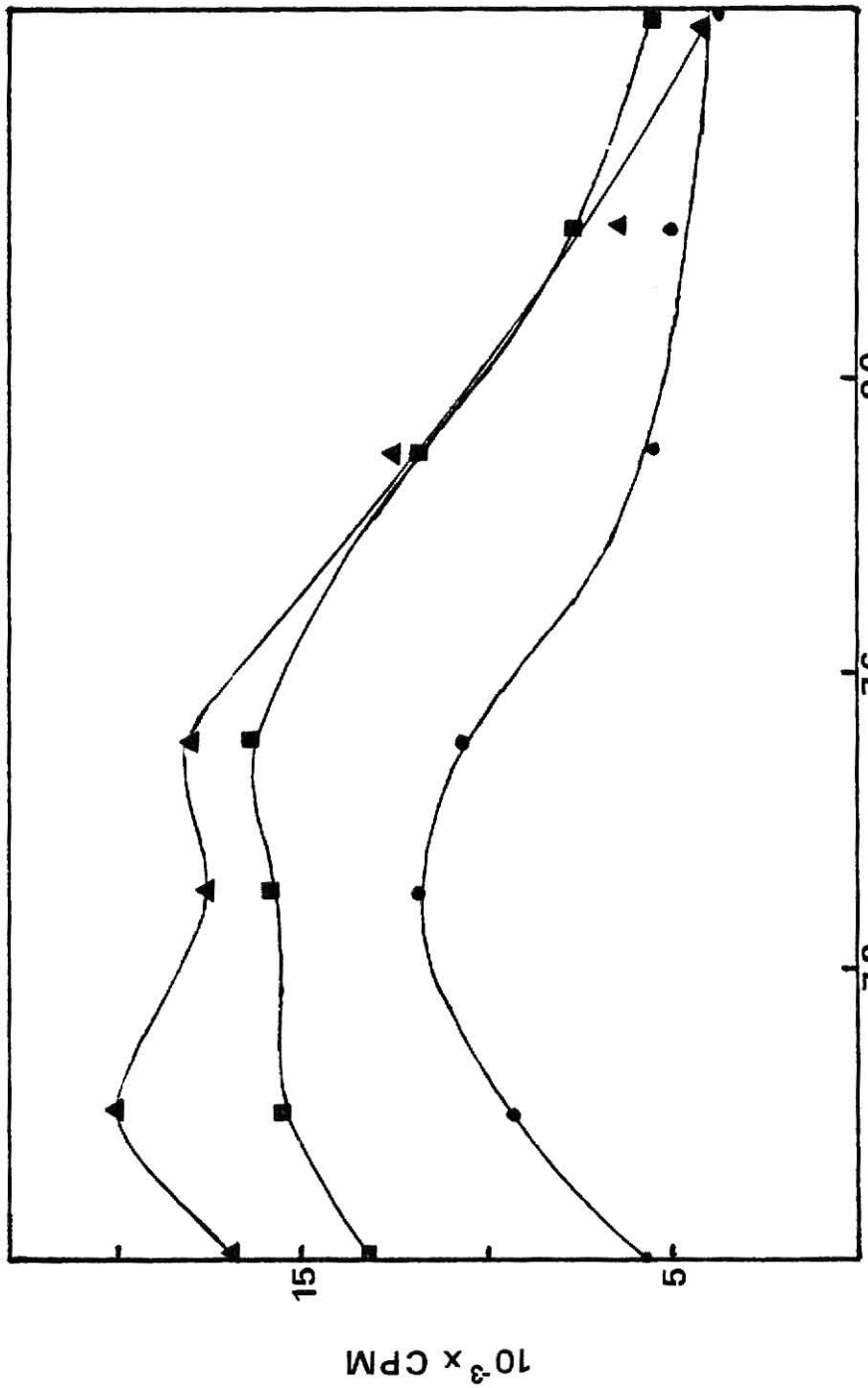
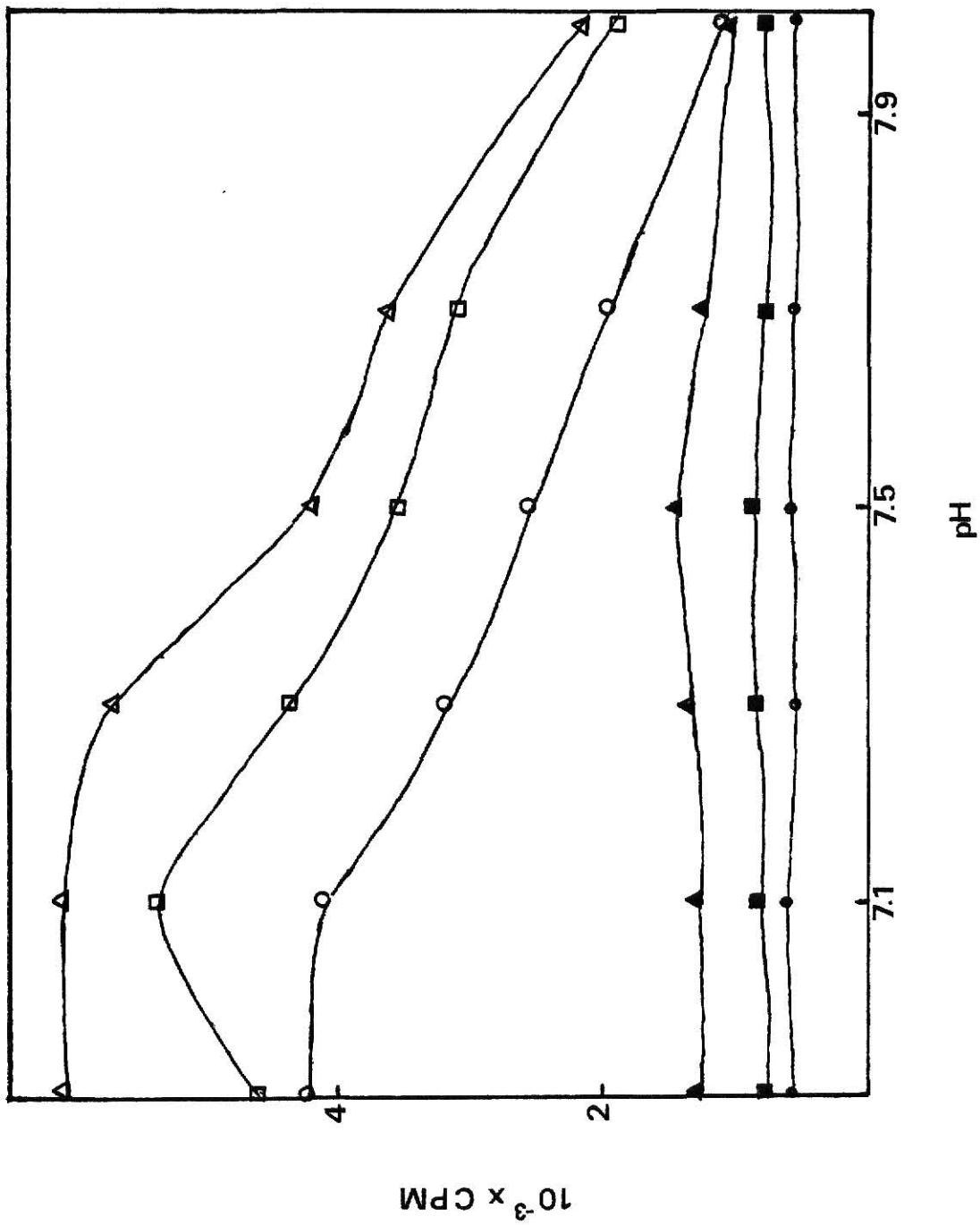

15
5
7.2
7.6
8.0
pH
10⁻³ x CPM

Figure 20. Effect of assay pH and Mg^{2+} concentration on RuBPCase activities when activated with 0.8 mM 6-PGA at pH 7.5 and 10 mM Mg^{2+}. For these experiments, 6-PGA was incubated with the enzyme in TME-DTE made 1 mM in $NaH^{14}CO_3$ for 50 min at 25 ± 1°. A 20 ug aliquot was used to initiate the reaction in T(MOPS)ME-DTE adjusted to the desired Mg^{2+} concentration and indicated pH. Filled symbols, no 6-PGA. Open symbols, 0.8 mM 6-PGA. Circles, 1 mM Mg^{2+}; squares, 2 mM Mg^{2+}; triangles, 5 mM Mg^{2+}.


10^{-3} x CPM
4
2
7.1
7.5
7.9
pH

$^{14}CO_2$ fixed/min/mg protein is seen. In addition, this particular data show that there is virtually no pH dependence on activity in the absence of 6-PGA when assayed at 1 mM $NaHCO_3$ and low Mg^{2+}. Only when the Mg^{2+} concentration begins to approach optimum levels (5 - 10 mM) is there evidence of a pH effect.

Effect of pH and Mg^{2+} Concentration on Activation by 6-PGA when Assayed in 10 mM Mg^{2+} at pH 7.5

For these studies concentrated enzyme (2 mg/ml) was incubated in the normal 50 mM Tris buffer made 1 mM in $NaH^{14}CO_3$ and 8.0 mM in 6-PGA and which contained varying amounts of Mg^{2+} and had been adjusted to the indicated pH values. Aliquots of this solution were used to initiate the reaction in the same buffer containing 1 mM $NaH^{14}CO_3$, 0.7 mM RuBP, 10 mM Mg^{2+} and adjusted to pH 7.5. Activation of RuBPCase by 6-PGA also was highly dependent on the level of Mg^{2+}, increasing by a factor of nearly 5 when the Mg^{2+} concentration during activation was raised from 0.5 mM to 10 mM at pH 7.3 and incubated with 8.0 mM 6-PGA (Table IV). In the absence of 6-PGA at pH 7.3, activity increased by about 15% when raising the Mg^{2+} concentration at activation from 1 mM to 10 mM (Table IV). In other words, the effect of Mg^{2+} on activity at any pH is smaller in the absence of 6-PGA because they promote the same overall reaction; holding a greater fraction of RuBPCase molecules in the activated state.

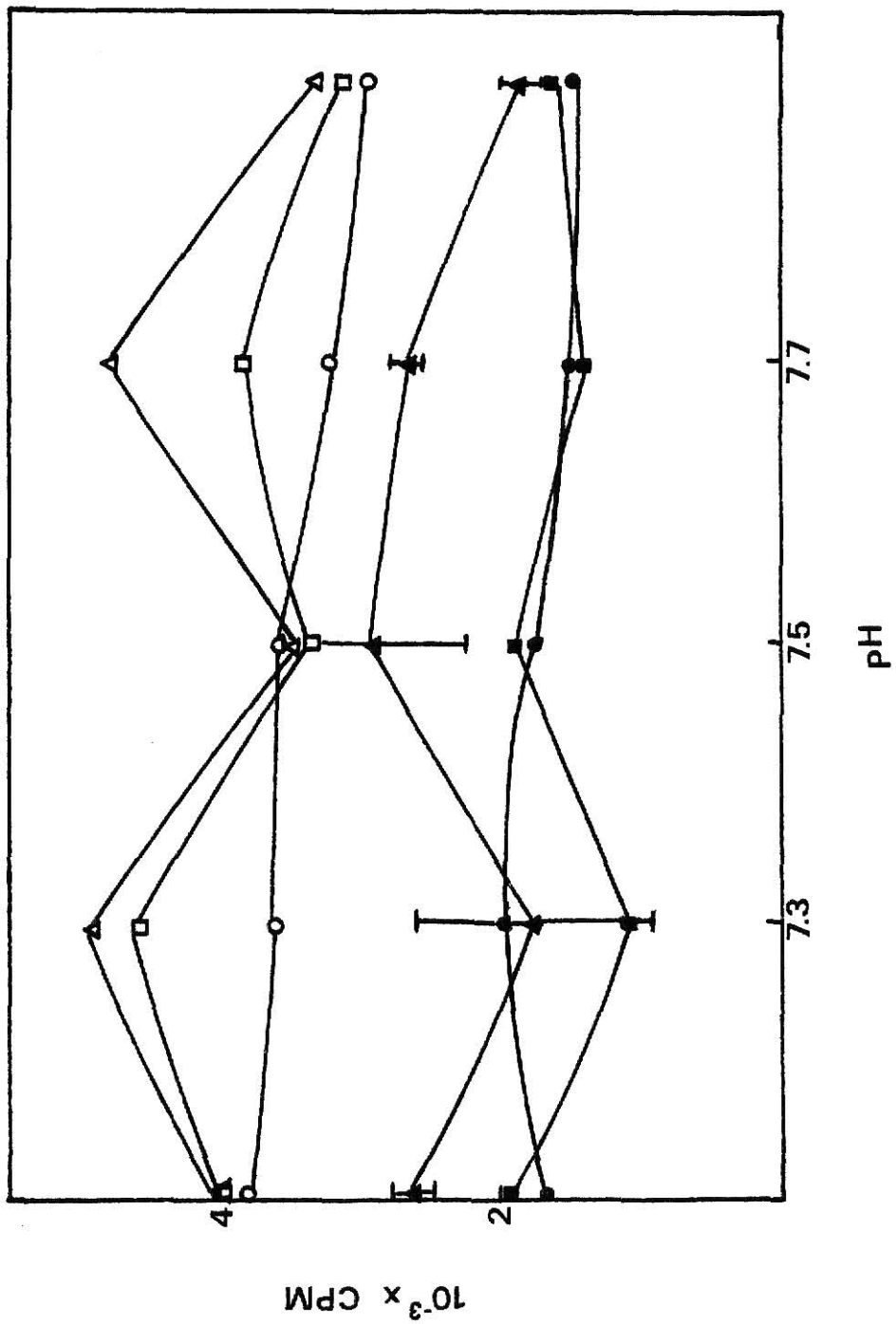
The pH effects on activation in the presence of 6-PGA is also very dependent on Mg^{2+} concentration. In the presence of 8.0 mM 6-PGA at activation and low Mg^{2+} (0.5, 1, and 2 mM) there is virtually no pH

Table IV

Enhancement of RuBPCase Activity at pH 7.3 by 8.0 mM 6-PGA at preincubation [500/1 (6-PGA)/(active sites)] and at various Mg^{2+} concentrations

mM Mg^{2+}	Activity (CPM)	
	NO 6-PGA	8.0 mM 6-PGA
0.5		1090
1	1655	1795
2		3571
5	1845	4525
10	1910	4827

Figure 21. Effect of activation pH and Mg^{2+} concentration in the presence of 8.0 mM 6-PGA on RuBPCase activity when assayed at 10 mM Mg^{2+} and pH 7.5. For these experiments, 6-PGA was incubated with the enzyme in T(MOPS)ME-DTE made 1 mM in $NaH^{14}CO_3$ at various Mg^{2+} concentrations and pH's for 50 min at 25 ± 1°. A 20 ug aliquot was used to initiate the reaction in TME-DTE, pH 7.5, made 1 mM in $NaH^{14}CO_3$ and 0.7 mM in RuBP. (●), normal assay in 10 mM Mg^{2+} without 6-PGA; the remaining data contain 8.0 mM 6-PGA at activation. Mg^{2+} concentrations (mM), (■), 0.5; (▲), 1.0; (○), 2.0; (□), 5.0; and (△), 10. Error bars represent extremes of duplicate measurements.

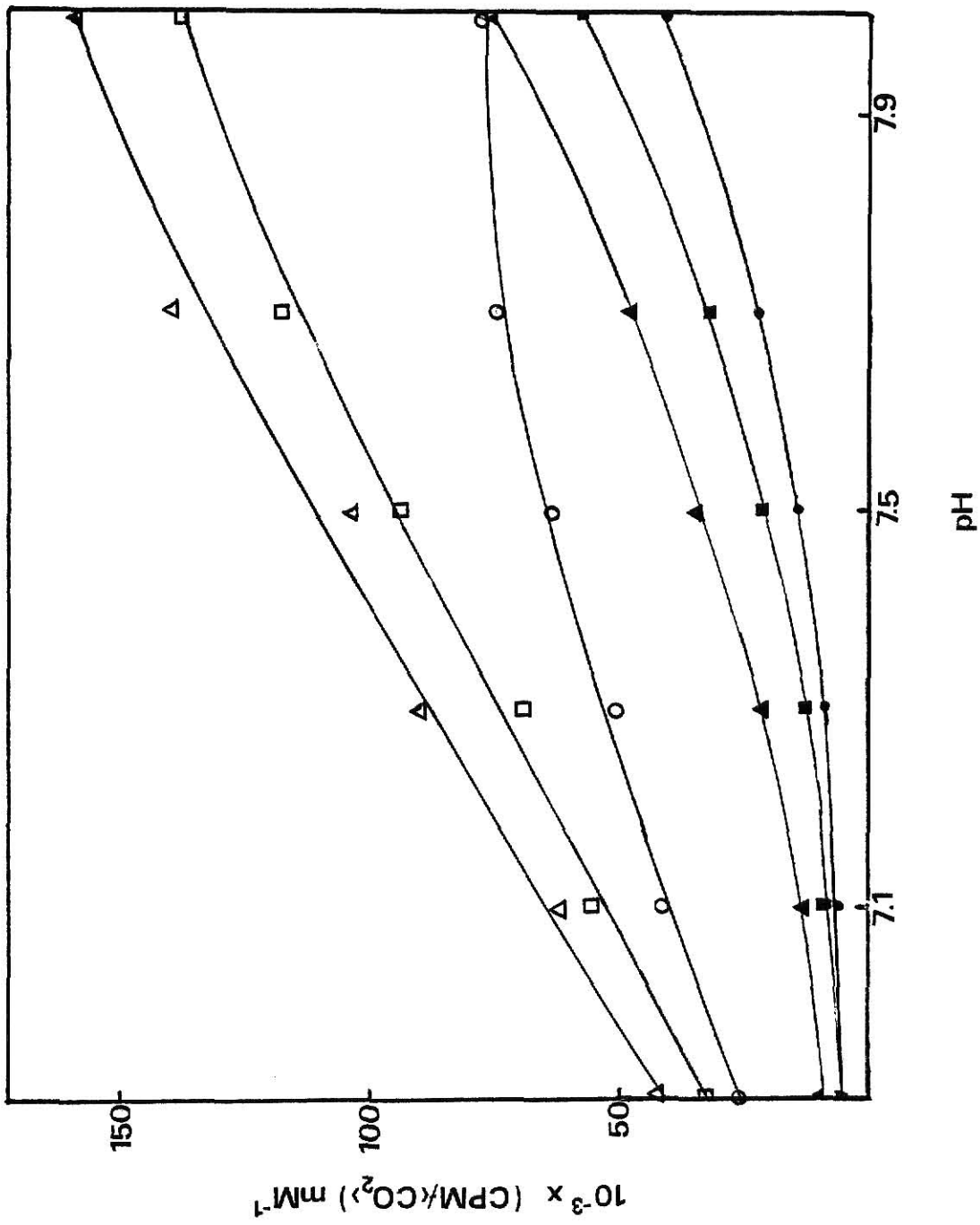

4
2
7.3
7.5
7.7
pH
10⁻³ x CPM

dependence on activation (Fig. 21). However, when the Mg^{2+} concentration at activation approaches optimum levels (5 - 10 mM) there is a pronounced pH effect with apparent optima at pH 7.3 and 7.7 (Fig. 21).

Effect of pH and Mg^{2+} at a Constant Concentration of CO_2

There is an inherent problem in measuring the pH dependence of reactions requiring CO_2, such as the carboxylase and activation processes of RuBPCase in that the CO_2 concentrations in equilibrium with a fixed amount of HCO_3^- varies with pH. There are at least two ways to alleviate this problem. First the concentration of bicarbonate could be raised as the pH is raised to give a constant amount of CO_2. This would be possible up to the point at which bicarbonate itself becomes inhibitory (~ 30 mM) (Bonsall, 1981). Another way is to calculate the concentrations of CO_2 *in equilibrium* with a fixed amount of HCO_3^- at each pH and use these values to put the activity data on a constant CO_2 basis. The later approach has been used in this study because it appeared more practical and straight-forward. The converted pH dependencies are shown in Fig. 22 for catalysis and Fig. 23 for activation. In the absence of 6-PGA, optimum activity for catalysis appears to be somewhere above pH 8.0 (Fig. 22) with overall activity increasing when the Mg^{2+} is raised from 1 mM to 5 mM. However, in the presence of a fixed 6-PGA concentration, optimum activity is seen at about pH 8.0 in 1 mM Mg^{2+} and above pH 8.0 when the Mg^{2+} concentration is raised to 5 mM (Fig. 22).

Figure 22. Effect of assay pH and Mg^{2+} concentration on RuBPCase activity when activated with 0.8 mM 6-PGA at pH 7.5 and 10 mM Mg^{2+}. The same data shown in Fig. 20 are replotted at a constant CO_2 level by dividing the CPM by the mM concentration of CO_2 in equilibrium with 1 mM HCO_3^- at the indicated pH values. Filled symbols, no 6-PGA; open symbols, 0.8 mM 6-PGA. Circles, 1 mM Mg^{2+}; squares, 2 mM mMg^{2+}, triangles, 5 mM Mg^{2+}.


150
100
50
10^{-3} x (CPM/CO_2) mM^{-1}
7.1
7.5
7.9
pH

Although the pH dependence on activation appeared complex, the addition of 6-PGA at 10 mM Mg^{2+}, tended to lower the apparent pH optimum for activation from above 8 to the pH 7.9 - 8.0 range (Fig. 23).[2] Again raising the Mg^{2+} concentration at a fixed 6-PGA level lowered the pH optimum for activation below 8 (Fig. 23). Based on the 1 mM concentration of EDTA present in our assays, the free Mg^{2+} concentrations were calculated from the equilibrium constants for the protonation and Mg^{2+} chelation of EDTA (Portzehl et al., 1964). At 1 mM EDTA, the following amounts of Mg^{2+} were complexed: pH 6.8, 0.012 mM; pH 7.9, 0.19 mM; pH 8.5, 0.2 mM when the total Mg^{2+} was 0.5 mM.

Km for CO_2 in the Presence and Absence of 6-PGA

For these studies concentrated enzyme (2 mg/ml) was incubated in the normal 50 mM Tris buffer pH 8.0 which was purged with N_2 for 30 min prior to use and made 20 mM in $NaH^{14}CO_3$ and where necessary 1.2 mM in 6-PGA. Aliquots of these solutions were used to initiate the reaction in the same buffer, pH 7.5, made 0.7 mM in RuBP and containing varying amounts of CO_2 from $NaH^{14}CO_3$. Five CO_2 concentrations were done ranging from 26.9 uM to 118.4 uM and points within the first 15 s were used to determine the initial velocities (Fig. 24). Reciprocal plots gave Km values of 130 uM without 6-PGA (Fig. 25) and 140 uM with 1.2 mM 6-PGA (data not shown). These data indicate that the Km for CO_2 both with and without 6-PGA are not significantly different, showing that 6-PGA although a good activator, does not change the affinity for the binding of CO_2 to the active site.

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[2]The pH optimum for activation has been looked for at higher pH values and appears to be above 9.0. 6-PGA did lower the pH optimum for activation but not into an accesible region (< 9.0).

Figure 23. Effect of activation pH and Mg^{2+} concentration in the presence of 8.0 mM 6-PGA on RuBPCase activity when assayed at 10 mM Mg^{2+} and pH 7.5. The data are a replot of that in Fig. 21 at a constant CO_2 level. Dashed line (●), normal assay in 10 mM Mg^{2+}; solid lines, enzyme incubated with 8.0 mM 6-PGA for 50 min at 25 ± 1°. Mg^{2+} concentration (mM), (■), 0.5; (▲), 1.0; (○), 2.0; (□), 5.0; and (△), 10.

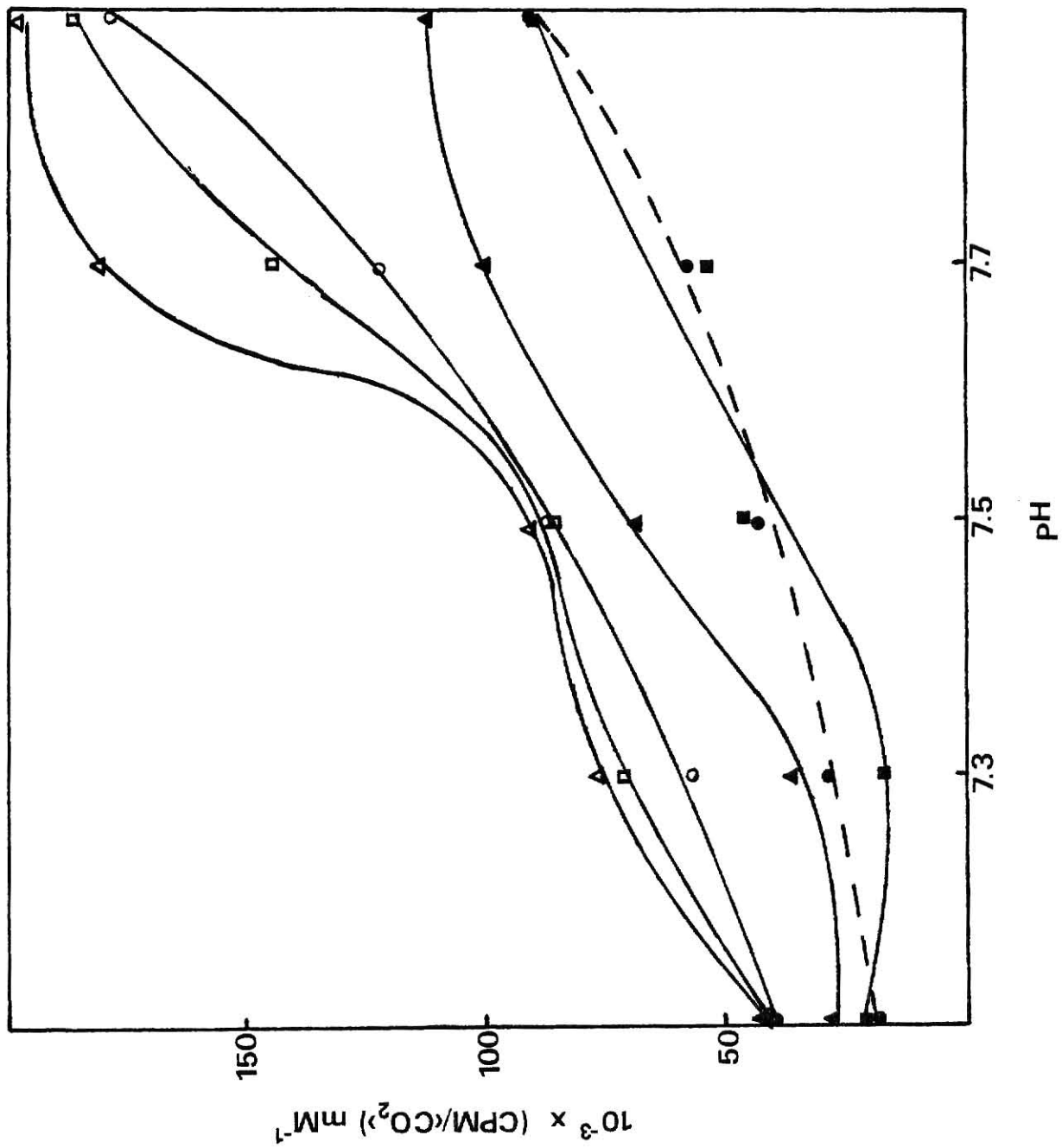

150
100
50
10^{-3} x (CPM/‹CO$_2$›) mM^{-1}
7.3
7.5
7.7
pH

Figure 24. Initial velocities at low concentration of CO_2 in the absence of 6-PGA. RuBPCase was preincubated for 45 min in a sealed vial containing N_2 purged TME-DTE (pH 8.0) made 20 mM in $NaH^{14}CO_3$. A 20 ug aliquot (12 ul) was added to an appropriate volume of N_2 purged TME-DTE (pH 7.5) made 0.7 mM in RuBP and in which the $NaH^{14}CO_3$ was adjusted to give the following final CO_2 concentrations (μM); (●), 26.9; (□), 37; (Δ), 47; (■), 67 and (▲), 116.8. The assays were quenched at the indicated times.

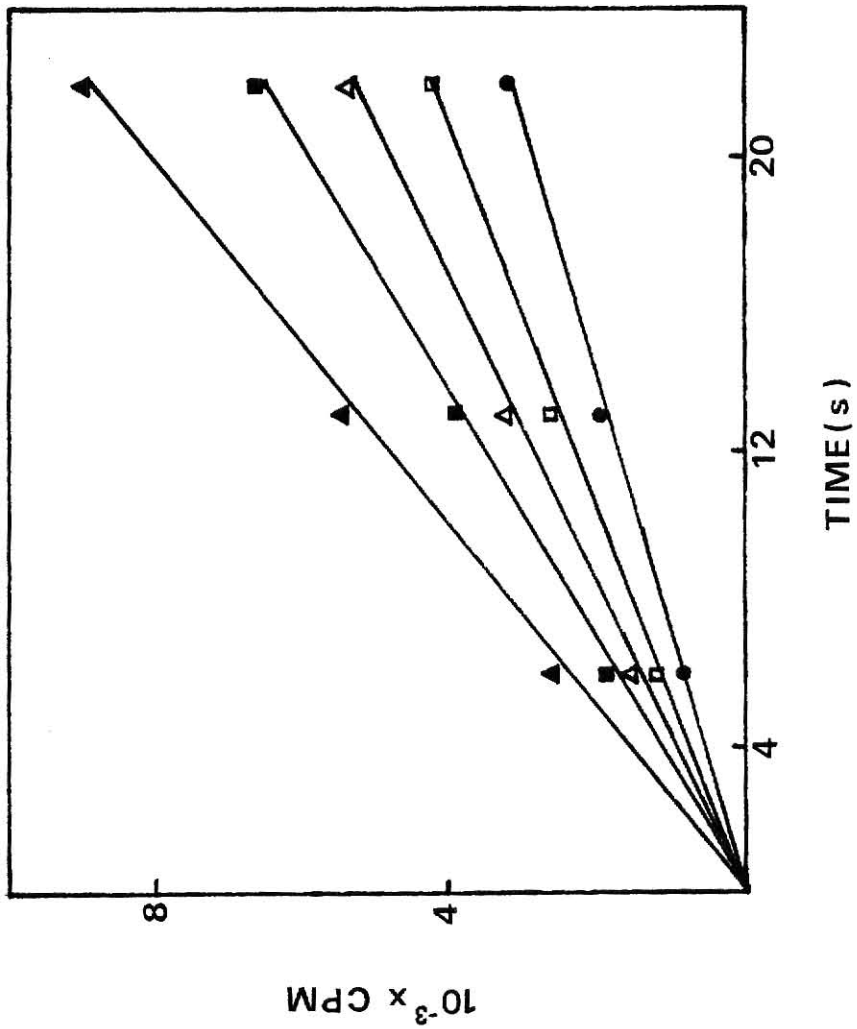
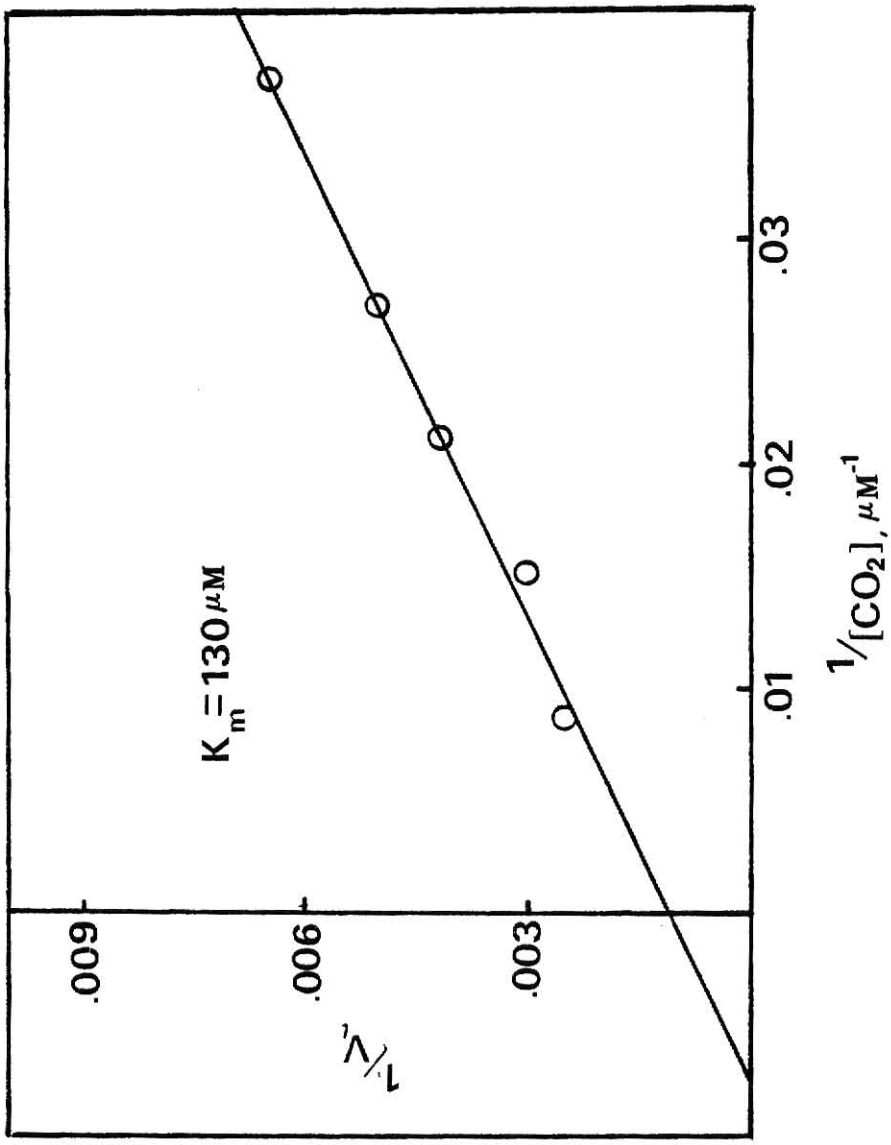

8
4
10^{-3} x CPM
4
12
20
TIME(s)

Figure 25. Lineweaver-Burke plot for the Km of CO_2 in the absence of 6-PGA. Slopes taken from the first 15 s of the data plotted in Fig. 24 were used to determine the initial velocities (V_i).


.009
.006
.003
1/v
$K_m = 130\,\mu M$
.01
.02
.03
$1/[CO_2], \mu M^{-1}$

DISCUSSION

Stabilization of the CO_2-Activated Complex by 6-PGA in Low Mg^{2+}

Other investigations have established that for activation of RuBPCase to occur there must be an ordered addition of CO_2 and then Mg^{2+} with CO_2 addition being the rate determining step (Mizioko and Mildvan, 1974; Lorimer et al., 1976, 1977, 1981). For the stabilization experiments the enzyme was activated in 1 mM $NaH^{14}CO_3$ and 10 mM Mg^{2+} and diluted almost 28-fold into a larger volume containing 1 mM $NaH^{14}CO_3$ and 1 mM Mg^{2+} for various times before assay. Following dilution the enzyme was in a suboptimum Mg^{2+} concentration, and an immediate drastic decrease in activity in the absence of 6-PGA (Fig. 18) was observed. This was due to the fact that the Mg^{2+} concentration was not sufficient to maintain the enzyme-carbamate complex in the active ternary state. Instead CO_2 probably began to come off the enzyme leaving it in a partially activated form leading to a decrease in activity. On the other hand, when 6-PGA is present at activation and catalysis, there was a higher activity both initially and at the end of the experiment (Fig. 18).

6-PGA and presumably other effectors as well act by holding Mg^{2+} and CO_2 on the enzyme and thereby stabilizing the activated complex and allowing RuBPCase to stay in the active form longer. Again, since the enzyme is diluted into a large volume of 1 mM Mg^{2+} and it is preactivated with 6-PGA, activity declines, but not nearly as rapidly as seen in the absence of the stabilizing 6-PGA. When 6-PGA is present

only at catalysis, the enzyme begins with low activity but once diluted into 1 mM Mg^{2+} in the presence of added 6-PGA, the activity begins to increase due to 6-PGA's stabilizing effects on the active ternary complex. In addition as would be expected in both experiments with 6-PGA, the final activities are converging toward a common value (Fig. 18).

Km for CO_2 in the Presence and Absence of 6-PGA

In these studies, the enzyme was fully activated in the normal 50 mM Tris buffer (purged with N_2) made 20 mM in $NaH^{14}CO_3$, 10 mM in Mg^{2+}, and where necessary 1.2 mM in 6-PGA and incubated for 45 min. Assays were run at five different low CO_2 concentrations and data from the first 15 s of the assays were used to determine initial velocities. Double reciprocal plots were used to determine Km for CO_2 with and without 6-PGA (130 μM). In the presence of 6-PGA, the Km for CO_2 was ~ 140 μM with a V_{max} of 2.6 units, which denoted no significant change with added 6-PGA. The data indicate that 6-PGA, although a good activator, does not change the affinity for the binding of CO_2 to the active site. Apparently 6-PGA acts strictly on the carbamylation product of the lysine amino groups involved in activation. The reported Km (CO_2) for other RuBPCases range from 16-20 μM at a pH of 7.8, 8.0 and 8.4 (Badger and Andrews, 1974; Bahr and Jensen, 1974). Since at the higher pH's (7.8 - 8.4), the amount of CO_2 present is about a factor of 4 to 6 less than that present for comfrey at pH 7.5, these RuBPCases must bind CO_2 tighter to get the same fraction of active site binding. Comparing the Km (CO_2) to the amount of CO_2 present, one can see that the Km's differ by approximately the same factor as does the amount of CO_2 present at these various pH's.

Effect of pH, Mg^{2+} and 6-PGA Concentration on the Catalytic Activity of RuBPCase

The effects of pH on catalytic activity has been studied by other investigators and the optimum was found to be near pH 8.0 for both spinach and tobacco (Lorimer, et al., 1976; Hatch and Jensen, 1980). Data from the comfrey enzyme indicate an apparent pH optimum for catalytic activity of 7.4 (Fig. 19). In general, the pH effects on catalysis result from different states of ionization of certain groups in the active site. Since the apparent pH optimum for comfrey RuBPCase is over half a pH unit lower than those of other RuBPCases the pK_a's of critical ionizable side chains in the active site must also be lower. A possible explanation for the lower apparent pH optimum for comfrey, lies in the fact that the comfrey enzyme binds CO_2 less effectively than does either tobacco or spinach; therefore, at pH 7.5 there is approximately 5 times as much CO_2 present than at 8.2 based on the calculations from the equilibrium constants between HCO_3^- and CO_2 (Gibbons and Edsall, 1963). Thus the comfrey enzyme saturates at about the same concentration of HCO_3^- at its pH optimum as do the others at their optima.

The effect of 6-PGA when present at activation on the catalytic activity of comfrey RuBPCase is to lower the apparent pH optimum for catalysis and probably that of activation as well. 6-PGA acts to stabilize the CO_2-activated complex by holding Mg^{2+} and CO_2 on the enzyme which may effectively lower the pK_a's of certain groups in the active site. In vitro this appears advantageous to the enzyme and could give additional activation in vivo provided the pH optimum is the same in

the chloroplast. The effect of 6-PGA on catalysis, however, appears biphasic with apparent optima at pH 7.0 and 7.5 (Fig. 19). This biphasic nature can be explained by the fact that 6-PGA, as stated before, lowers the pH optimum for activation. Consequently additional activation can occur during catalysis as the assay pH approaches that of optimal activation.

The effects of Mg^{2+} on activation and catalysis appear to be opposite. For activation, Mg^{2+} acts similar to 6-PGA in that it lowers the apparent pH optimum (Figs. 21, 23). Mg^{2+}, as does 6-PGA, helps maintain higher levels of carbamylated lysines which are essential to RuBPCase activity (Miziorko and Lorimer, 1980) and when its concentration increases more ternary complex is formed at a given pH. However, in catalysis, Mg^{2+} appears to shift the pH optimum to higher values. A possible explanation for this is that 6-PGA which contains three negative charges at pH 7.5, may lower the pK_a's of basic groups (His, Lys, or Arg) in the active site through the secondary effect of a conformational change. Therefore, it's reasonable to propose that Mg^{2+} with two positive charges may counteract the effects of 6-PGA and thus shift the pH optimum back toward more normal values (Figs. 20, 22). It is known that the peptide containing the active site of the spinach enzyme contains all of these basic groups. In any event, the effects of pH, Mg^{2+} and 6-PGA concentration present during activation and catalysis are critical to the overall catalytic activity of RuBPCase.

Available evidence suggests that the role of effectors in the activation of the carboxylase reaction of RuBPCase is a stabilization of the active ternary complex (CO_2·Mg·enzyme) through binding at the

catalytic site (Badger and Lorimer, 1981; McCurry, et al., 1981). Evidence from this study generally supports this model and indicates that slow conformational changes are also involved in the activation of RuBPCase.

Time Dependence of RuBPCase Activation by Several Phosphometabolites

In the presence of 4 mM 6-PGA [250/1 (6-PGA)/(active sites)] during preincubation, one sees a continual increase in activity up to approximately 10 min of preincubation (Fig. 3). This time dependence profile is thought to occur by essentially instantaneous binding of 6-PGA to the enzyme which slowly enhances activation by CO_2. The evidence for instantaneous binding of 6-PGA to the enzyme is based on the fact that when 6-PGA and RuBP are added simultaneously, inhibition is seen immediately. In the presence of carbonic anhydrase (15 units/assay), the time required to reach maximal activation is the same (Fig. 4), which suggests that the slow activation by 6-PGA is not due primarily to the slow establishement of equilibrium between HCO_3^- and CO_2 which is needed for activation. However, in the presence of carbonic anhydrase there appears to be an approximately 30% increase in activity between the 5 and 10 min times following addition of 6-PGA to the enzyme. This increase can be explained by the fact that 6-PGA shifts the equilibrium to the right in the following equation.

$$ENZ - NH_2 + CO_2 \rightleftharpoons ENZ - NHCOO^- + Mg^{2+}$$

6-PGA in essence pulls the free CO_2 out of the solution and facilitates

its complexation to the enzyme. At the early times following 6-PGA addition to the enzyme, the activities both with and without carbonic anhydrase are quite comparable due to the fact that the effects of 6-PGA are slow and the equilibrium has not been shifted that far to the right in the above equation. However, at the later times, the concentrated 6-PGA-enzyme complex in the preincubation mixture has shifted the equilibrium substantially to the right, thereby, temporarily depleting the free CO_2 available for activation. Since the equilibrium between HCO_3^- and CO_2 is established only slowly (Gibbons and Edsall, 1963), replacement of free CO_2 is not fast enough to compensate for the loss due to activation. On the other hand, in the presence of carbonic anhydrase over the same times, free CO_2 is replaced quicker ($HCO_3^- + H^+ \overset{\text{fast}}{\rightleftharpoons} CO_2 + H_2O$), thus allowing for the fastest possible activation by 4 mM 6-PGA which explains the ~ 30% increase in activity at 10 min of preincubation in the presence of carbonic anhydrase. At longer times, the two curves appear to approach one another which would be expected as activation slowed and the free CO_2 regained its equilibrium value.

The time dependence profiles in the case of the other effectors are quite different (Figs. 5, 6, 7, 8). In each of these cases there is an initial rise in activity (10-110 s) with increasing preincubation time followed by a period of decline. After the period of sharp decline (approximately 3 min except for CHBP), the behavior of the effectors becomes more individualistic either reaching a "steady state" level of activity (3-PGA in most cases, 2-PG at low concentrations, FBP at high concentrations), continuing to decline in activity (CHBP

and 3-PGA in one case), or increasing again (FBP at low concentrations, 2-PG at high concentrations). The amount of initial rise, degree of decline and final (20 min) activity depended on the concentration of effector. For less than 20 mM effector at activation, the maximum occurred between 40 and 110 s of preincubation. At very high concentrations of effector during incubation, for example, 80 mM 3-PGA (Fig. 5), activation is seen only at the shortest times followed by a slow-decline to very low activities.

When the enzyme is incubated in 2 mM $NaH^{14}CO_3$ and diluted to 1 mM with effector, a greater amount of activation is observed than when incubated in 1 mM $NaH^{14}CO_3$ and diluted with 1 mM $NaH^{14}CO_3$-effector. When the enzyme is incubated in 2 mM and assayed in 1 mM $NaH^{14}CO_3$, one generally sees a continual decrease in activity at the later preincubation times, suggesting a slow continuous deactivation of the enzyme in the reduced bicarbonate concentration, probably reflecting loss of CO_2. However, when the enzyme is incubated in 1 mM $NaH^{14}CO_3$ and assayed at 1 mM $NaH^{14}CO_3$ there seems to be a leveling off of activity at the later preincubation times (compare Figs. 5 and 9 to 10 and 12). This accounts for the differences at longer preincubation times for the 3-PGA data at moderate concentrations but does not account for the steep decrease at high concentrations of effector (Fig. 5).

Since activation by effectors probably results from binding at the active site (Badger and Lorimer, 1981), such occupancy would be by definition inhibitory. Therefore, the interpretation of the activation data must include a consideration of the degree of inhibition present at assay. The method of preincubation at high concentrations

of effectors and enzyme followed by dilution into a saturating solution of RuBP for assay introduced by Hatch and Jensen (1980), also has been employed in the present study to minimize the inhibitory effects. In the absence of other complicating factors, it should be possible to predict the degree of inhibition at assay for any effector concentration from the active site binding constants for the effectors and substrate molecules. Fortunately this information is available for the interactions of 3-PGA and 2-PG with the comfrey enzyme (Bolden, 1981), as well as the Km values for RuBP (Simpson, et al., 1980) and CO_2 (Fig. 25). Using the values of 0.99 and 0.50 mM for the active site binding constants of 3-PGA and 2-PG, respectively, Km (RuBP) = 0.030 mM, and a concentration of 0.7 mM for RuBP, relative inhibitions, i, were calculated from the following equation (Segel, 1975).

$$i = \frac{(I)}{(I) + K_i(1 + \frac{(S)}{Km})}$$

These calculations assumed no effect on the Km for CO_2 which at least for 3-PGA is reasonable since the available evidence suggests that the 0.99 mM constant reflects binding at the opposite end of the active site from where the CO_2 site must be located. From the relative inhibition values listed in Table V, it can be seen that inhibition of RuBP binding is negligible after dilution except at the very highest preincubation concentration (80 mM). Even in this case only a 13% inhibition is predicted from the competition of 3-PGA with RuBP for the active site. However, since the other effectors may block the

Table V

Relative inhibition (i) at assay in the presence of various concentrations of effector

mM 3-PGA (at preincubation)	i
0.8*	0.0015
8.0	0.015
16.0*	0.023
80.0*	0.130
mM 2-PG (at preincubation)	**i**
0.016	0
0.16	~ 0
0.8	0.002
2.0	0.006
8.0	0.023

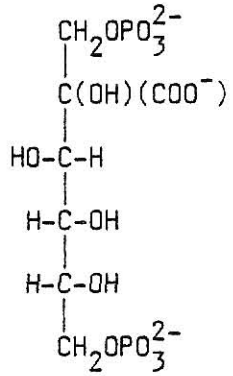
*dilution factor = 21.8. Dilution factor for all other values was 27.7.

CO_2 sites as well (especially CHBP), the overall inhibition maybe greater than that predicted in Table V. This would compromise the selection of the optimal effector concentration for activation, but the data needed to estimate overall inhibition, even assuming that they would be additive, are not currently available. Attempts to measure the degree of inhibition directly would be similarly compromised by the amount of activation occurring during the assay. Chu and Bassham (1973) attempted to overcome this problem by using the enhancement approach described earlier, but this method does not correct for activation during assay either. Consequently, highly accurate relationships between effector concentrations and degree of activation can not be obtained by the present methods. Therefore, they will be referred to as concentrations giving "apparent maximal" activation.

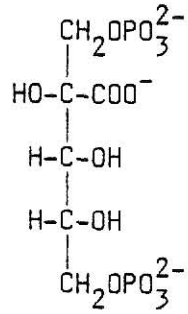
In regard to the time course of the activation process, it is clear from the early rise in activity with preincubation time that activation by 3-PGA, FBP, 2-PG and to a certain extent CHBP is not instantaneous but does reach apparent maxima much faster than 6-PGA. The faster activation times do not result simply from smaller molecules occupying the active site, since FBP and CHBP are equally as large as 6-PGA. Neither is the time course of activation simply related to charge, or even charge density of the molecules, since 2-PG is essentially triply negatively charged at pH 7.5 and would have the greatest charge density of the group but is no faster than 3-PGA which is also triply charged at this pH. FBP (-4), on the other hand is a differently charged, sized and shaped (largely in the furanose form)

molecule but still shows the same similar early rise in activity between 40 and 110 s. 6-PGA (-3), on the other hand, acts much slower (10 min), but is a linear molecule like CHBP which on the other hand seems to act very rapidly.

The extents of apparent activation for the best concentrations and optimum times were quite different for the effectors studied (Table III) ranging from a high of 190% for 6-PGA to a low of 17% for CHBP. Despite the structural similarities between 6-PGA and CHBP they have very different activation properties. The carboxylate group on CHBP apparently makes it a very effective inhibitor and hence, a poorer measurable activator, because of its similarity with the proposed transition state analog (Wishnik et al., 1970), 2-carboxyarabinitol-1,5-bisphosphate (CABP), which is known to be an extremely potent inhibitor ($K_I \sim 10^{-11}$M)



CHBP



CABP

and which after about 20 min forms an irreversible complex with spinach RuBPCase in the presence of Mg^{2+} ions (Lorimer and Miziorko, 1980). Apparently the carboxylate group on 6-PGA does not fill the active site in the same way as that of CHBP which suggests that it is the relationship between the phosphate group in position 1 and the carboxylate group attached to carbon 2 that makes CHBP and CABP such potent

inhibitors. This view is supported by the fact that the diastereomer of CABP (the ribitol) is not nearly as effective an inhibitor and does not form irreversible complexes (Pierce et al., 1980). It is also known that the two diastereomers of CHBP have very different affinities for RuBPCase (Roach et al., 1982) but neither forms an irreversible complex with RuBPCase even in the presence of Mg^{2+} (Bolden, 1982). Furthermore, the presence of two phosphates at either end of a six carbon molecule is not sufficient to make it a good effector or a potent inhibitor as shown by the more or less intermediate results for FBP. However, it has a different conformation which makes comparisons difficult here. Indeed the smaller molecule, 3-PGA, is a better activator of RuBPCase activity. 2-PG on the other hand, appears to be too small and raises the activity only about the same as does phosphate (Bhagwat, 1981).

In the absence of any effector, standard assays (saturating conditions of substrate and cofactors) result in relatively high specific activities, whereas normal assays in 1 mM HCO_3^- and 10 mM Mg^{2+} give only about one-third the full activity. However, in the presence of 1 mM HCO_3^-, 10 mM Mg^{2+} and an appropriate effector concentration during preincubation (4 mM 6-PGA or 8 mM 3-PGA), the specific activity is raised to almost the same level present for the standard assays (Table VI).

The fairly sharp decline in activity immediately following the maximum was unexpected and must reflect a competing reaction. It appears that the onset of increased activation brought about by the binding of certain ligands to the active site induces a slow conformational change resulting in reduced activity. This reduction in

Table VI

Specific activities (umoles CO_2 fixed/min/mg protein) under various states of activation

Activation Conditions	Specific Activity
1 mM HCO_3^-, 10 mM Mg^{2+} (normal assay)	0.5
1 mM HCO_3^-, 10 mM Mg^{2+} and 4 mM 6-PGA	1.4 - 1.7
1 mM HCO_3^-, 10 mM Mg^{2+} and 8 mM 3-PGA	1.2
20 mM HCO_3^-, 10 mM Mg^{2+} (standard assay)	1.5

activity could arise from several possible factors: tighter binding of the effector, weaker binding of the substrates, less effective orientations of the catalytic groups in the active site, or tighter binding of the products. No information is currently available to allow a selection of the factor(s) responsible for this reduction. However, since the potent inhibitor CABP slowly forms an irreversible complex with Mg-RuBPCase, it is possible that prolonged incubation with an effector may initiate a similar response which could make it harder for RuBP to chase it even after dilution.

Evidence for a slow conformational change resulting in decreased activity can be seen in the data for 3-PGA at very high activation concentrations (80 mM) in which a small initial increase is seen due to the instantaneous binding of effector and concomitant activation, immediately followed by a slow decline in activity to very low levels. Since the assay time and concentration of RuBP were constant and only the preincubation time varied in these experiments, the data strongly suggests that 3-PGA slowly (over 10 min for completion) induces a conformational change which makes it almost impossible for RuBP to chase it during a 90 s assay. This conformational change must persist for several minutes even after the 3-PGA is diluted out during assay.

The very different behavior among the effectors at longer times seems to result from slow conformational changes as well. In the case of CHBP at very low concentrations 0.032 and 0.16 μM, the decline was substantial and continuous for the duration of the study, whereas with 1.6 μM it was less steep, but still declined suggesting that

conformational equilibrium was not reached even in 20 min. 2-PG at less than 4 mM and 3-PGA at less than 20 mM appeared to level off after 5 min in most cases indicating that conformational equilibrium had been attained in that time period. At 80 mM 3-PGA at preincubation, however, activity continued to decline for up to 10 min. This probably means that the half-life time for the conformation change induced by this effector is concentration dependent. With FBP at low levels or 2-PG at high concentrations (> 4 mM), the activity began to rise again after the initial decline. Apparently the slow conformational change induced by low concentrations of FBP or high concentrations of 2-PG eventually results in a form capable of greater activation or less inhibition than that produced at other concentrations.

The initial increase in each case is believed, again, to be due to an essentially instantaneous binding of the effector which initiates activation by CO_2. This process requires ~ 2 min for 3-PGA, FBP and 2-PG, about 10 min for 6-PGA, and less than 10 s for CHBP. Therefore, based on the arguments put forth earlier, the maximum point results from two competing reactions, a slow activation by CO_2, leading to increased activity and a slower conformational change which reduces activity. With 6-PGA, the slower conformational change is not apparent in the data up to 20 min. Consequently, if present, it must be nearly instantaneous or very slow. With 3-PGA, 2-PG at low concentrations and FBP at high concentrations, the conformational change appears complete in 3 - 5 min, but for CHBP at very low concentrations, the activity is still decreasing after 20 min. It should be noted that at reasonable concentrations of effector (< 10 mM), the "steady state" point is still above the normal values despite this conformational change.

When RuBPCase and 3-PGA both are incubated in 1 mM $NaH^{14}CO_3$ instead of 2 mM and 0 mM $NaH^{14}CO_3$, respectively, and assayed for 90 s and 20 s, one sees the same time dependence profile but a reversal in the degree of activation by 0.8 and 8.0 mM 3-PGA (compare Figs. 11 and 12). For 90 s assays, the 8.0 mM 3-PGA had the higher activity, whereas, with 20 s assays, the 0.8 mM 3-PGA was higher. A possible explanation for this data lies in the fact that when the 8.0 mM effector (500/1 [3-PGA]/[active sites]) is diluted approximately 25-fold into the final assay mixture, there remains about 20/1 3-PGA to active sites which maybe close to the optimum concentration for activation. Thus in the 90 s assay there would be additional time for activation (Fig. 12). To the contrary with the 20 s reaction time there is less time for activation during assay, therefore 0.8 mM gives the higher activity which probably represents the truer effects on activation and therefore closer to the actual optimum concentration (Fig. 11).

Evidence from other investigators indicate that the lower the K_i or K_{eq} values the better the compound is as an activator (Badger and Lorimer, 1981). Data in this study indicate that this hypothesis is not necessarily true as illustrated in Table VII. This study proposes that it is not necessarily how well a compound binds but how it specifically binds to the enzyme. As the nature of the interactions change, the extent and half-life time of the induced conformational change can also be altered.

Through the evidence provided in the time dependence studies on activation, it is apparent that the traditional enhancement approach

Table VII

Comparison of apparent maximum activation (%) to K_i or K_{eq} for several effectors

% Activation	K_i or K_{eq} (μM)
190 (6-PGA)	8
162 (3-PGA)	990
79 (FBP)	40
72 (2-PG)	500
17 (CHBP)	1[a]

[a]Roach et al., 1982. Mannitol diastereomer.

(Chu and Bassham, 1973) does not give the best comparisons. Although it does take into account inhibition, this can also be largely eliminated by dilution, however, it does not take into account effects of preincubation time when comparing effectors.

In conclusion, activation of RuBPCase is not only concentration dependent but time dependent as well (Figs. 3, 5, 6, 7, 8). The range of concentrations shown in most cases to bring about apparent maximal activation varied greatly with the phosphometabolite under investigation. They ranged from less than 0.16 mM, to moderate concentrations of 0.16 - 8.0 mM and high concentrations of greater than 16 mM. Most effectors, except for CHBP and to a certain extent 2-PG, activate RuBPCase at moderate concentration levels. In the case of CHBP, activation is seen only at very low concentrations ($\leq$ 0.16 µM). 2-PG on the other hand, activates both at moderate (0.8 and 4.0 mM) and reasonably low concentrations (0.16 mM). At high concentrations (> 16 mM), all the effectors studied except 3-PGA initially inhibit RuBPCase activation. The *in vivo* levels of 3-PGA, FBP and P_i probably are above 1 mM under most conditions (Stitt *et al.*, 1980). Of these, 3-PGA is the highest, apparently attaining levels in excess of 3 mM in the light activated chloroplasts of intact leaves (Stitt *et al.*, 1980) and even higher levels during the dark period. The other concentrations are not known as accurately but 6-PGA has been estimated to reach 0.05 mM in the dark (Chu and Bassham, 1973) compared to a value of 0.01 mM or less for RuBP in spinach protoplasts (Stitt *et al.*, 1980). Under illumination, however, 6-PGA levels decline and RuBP concentrations rise to 0.5 mM. Consequently, based on the relationship between effector

concentration and activation of RuBPCase found in this study, it can be predicted that 3-PGA and 6-PGA would exert the greatest *in vivo* activation and that this would occur in the dark where competition with RuBP is greatly reduced. The actual *in vivo* degree of activation by these effectors is harder to predict since many other factors are involved such as, prior binding of RuBP which is known to prevent activation by Mg^{2+}, CO_2 and effectors (Paulsen and Lane, 1966; Wishnick *et al.*, 1970; Chu and Bassham, 1973; Laing and Christeller, 1976).

SUMMARY

1. Substantial enhancement of RuBPCase activity by preincubation with 6-PGA and other phosphometabolites is seen only at low concentrations (1 mM) of $NaHCO_3$, suggesting that these effectors act by promoting activation by CO_2.

2. The Km for CO_2 is not significantly changed in the presence of added 6-PGA, suggesting that 6-PGA does not alter the affinity of CO_2 binding to the active site. In other words different CO_2 molecules are involved in activation and catalysis.

3. Positive effectors activate the enzyme by stabilizing the $Enz \cdot CO_2 \cdot Mg^{2+}$ ternary complex.

4. Activation of RuBPCase is not only concentration dependent but time dependent as well. Activation in the presence of 3-PGA, FBP, 2-PG and to a certain extent CHBP shows an initial increase between 10-110 s due to initial binding and promotion of CO_2 activation by the effector followed by a period of declining activity which we propose is due to a slow conformational change. At longer preincubation times, activity either continues to decrease (CHBP, 3-PGA in one case), reaches a "steady state" level (3-PGA in most cases, 2-PG at low concentrations, FBP at high concentrations) or increases again (2-PG at high concentrations, FBP at low concentrations).

5. For 6-PGA, this conformational change, if present, is not apparent in the data, implying that it either does not occur, is immediate, or very slow.

6. Table VIII

Comparison of Enhancement by 6-PGA to other RuBPCases

Enzyme Source	Activating Concentration (mM)			Molar Ratios (active sites)	Enhancement Factor
	$NaHCO_3$	Mg^{2+}	6-PGA		
Comfrey	1	10	4.0	(250/1)	5.5[a] (2)[a,*]
Spinach	1	10	0.05	(140/1)	3[b]
Spinach	1	2	0.5	(10/1)	3[c]
Tobacco	1	5	0.05	(350/1)	2[d]
Pseudomonas oxalaticus	5	10	1.0	(145/1)	2.4[e]

*Time dependence study

[a]This study

[b]Chu and Bassham (1973)

[c]Badger and Lorimer (1981)

[d]Hatch and Jensen (1980)

[e]Lawlis, et al., (1978)

7. The pH dependence of catalysis is monophasic with a maximum at pH 7.3 in the presence of 10 mM Mg^{2+}. For activation it also appears monophasic with no clear maximum up to pH 7.9 in 10 mM Mg^{2+}.

8. The presence of 6-PGA lowers the pH optimum for activation on a constant CO_2 basis to about 8 in 10 mM $Mg.^{2+}$. The variation in the extent of activation by 6-PGA with pH, however, appears biphasic. This biphasic nature probably arises from the fact that activation

and catalysis have closer pH optimum in the presence of 6-PGA. Near pH 7.3 on a constant total HCO_3^- basis, catalysis is optimal and activation somewhat suboptimal. At higher pH's the optimum for activation is approached and activity appears to increase due to additional activation during assay. A biphasic pH dependence on activation in the presence of 6-PGA apparently has not been noted with the enzyme from other sources.

9. The presence of 6-PGA during activation appears to lower the pK_a of certain groups in the active site but this does not seem to be a likely direct effect for basic (His, Lys, Arg) or acidic (SH, COOH) groups, since 6-PGA is a highly negatively charged ion. It seems more likely that 6-PGA either promotes a conformational change or lowers pKa through another secondary effect such as, holding Mg^{2+} on the enzyme which could lower the pKa of neighboring ionizable groups.

10. The level of Mg^{2+} present at activation and catalysis had a dramatic effect on RuBPCase activity and pH optimum. Mg^{2+} acts similarly to 6-PGA during activation, in that it lowers the pH opitimum for activation as well as holding more CO_2 onto the enzyme. Increasing Mg^{2+} broadens and shifts the pH optimum for catalysis to higher values. Mg^{2+} with its two positive charges may be counteracting the action of 6-PGA (present during activation) with its three negative charges during catalysis but not activation.

11. The effects of concentration and time dependence on activation alter the degree of activation. Thus, since some of these effectors are found in the chloroplast at millimolar concentrations or higher, they could be used to activate the enzyme prior to illumination which could lead to increased plant productivity. In fact, activation by these effectors in vivo may also be time dependent.

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REGULATION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE FROM COMFREY BY SEVERAL PHOSPHOMETABOLITES

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Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase, EC 4.1.1.39) catalyzes the addition of CO_2 to, and cleavage of, ribulose-1,5-bisphosphate to form two molecules of 3-phosphoglycerate. The same protein, functioning as an oxygenase, catalyzes the oxygenation of ribulose-1,5-bisphosphate to yield one molecule each of 2-phosphoglycolate and 3-phosphoglycerate. This enzyme is present in large amounts in virtually all photosynthetic organisms and some chemosynthetic bacteria in high concentrations making it the world's most abundant protein. RuBPCase from higher organisms is a large complex enzyme composed of eight large and eight small subunits giving the native structure a molecular weight in excess of 500,000 daltons. The enzyme from spinach is activated by CO_2 through carbamylation of a non-active site lysine residue on the large subunit and the product stabilized by interactions with magnesium ions. Prior activation by CO_2 and Mg^{2+} apparently is an absolute requirement for either carboxylase or oxygenase activity. At subsaturating levels of CO_2, such as that resulting from a 1 mM $NaHCO_3$ solution near neutral pH, activation is augmented by millimolar levels of certain phosphometabolites. For this study the effects of several phosphometabolites on the activation process have been investigated using the enzyme purified to electrophoretic homogeneity from comfrey leaves.

In the presence of 6-phosphogluconate (6-PGA), the most potent positive effector of comfrey RuBPCase carboxylase activity investigated, the Km for CO_2 (140 uM) at optimal levels of effector was not significantly different than that in its absence (130 uM), suggesting

that the CO_2 molecule used for carboxylation of ribulose-1,5-bisphosphate is different from that used for activation and that 6-PGA exerts its effect on activation not on catalysis. The effect of 6-PGA, is to stabilize the ternary enzyme-CO_2-Mg^{2+} complex, since dilution of 6-PGA-activated enzyme into a low Mg^{2+} solution containing the same amount of 6-PGA resulted in a *much* slower loss of activity than that observed in the absence of 6-PGA. In other words, positive effectors shift the equilibrium between inactive and active enzyme toward the active form by retarding the dissociation process.

Changes in pH, Mg^{2+} and 6-PGA concentration at either the activation or catalytic step have dramatic effects on the observed carboxylase activity. When activated with 6-PGA at various pH values and Mg^{2+} concentrations and assayed at constant 10 mM Mg^{2+}, pH 7.5, increasing levels of Mg^{2+} lowered the pH optimum for activation from well above 8 to 7.9. Conversely, holding the activation conditions constant at 10 mM Mg^{2+}, 0.8 mM 6-PGA, pH 7.5, while changing the Mg^{2+} concentration and pH of the assay mixture gave the opposite effect. At 0.8 mM 6-PGA, which in 10 mM Mg^{2+} lowers the pH optimum to about 7.0, raising the Mg^{2+} concentration broadened and moved the pH optimum for catalysis toward more normal values (7.4). It appeared that Mg^{2+} counteracted the pH dependent effect of the negatively charged 6-PGA. At any level of Mg^{2+}, the pH optima for activation and catalysis in the absence of 6-PGA is higher than in the presence of 6-PGA. In addition, the pH dependence was *biphasic* when activated by 6-PGA. This has not been noted for any other source of RuBPCase. A biphasic pH dependence probably resulted from 6-PGA lowering the pH optimum for activation to values closer to that of the assay (7.5).

Activation of RuBPCase by effectors was not only concentration dependent but time dependent as well. When the enzyme was preincubated with 3-phosphoglycerate (3-PGA), fructose-1,6-bisphosphate (FBP), 2-phosphoglycolate (2-PG) and to a certain extent 2-carboxyhexitol-1, 6-bisphosphate (CHBP) an inhibitor of RuBPCase there was an initial increase in activity between 10 - 110 s. At intermediate times (2 - 5 min) there was a decline in activity possibly due to a slow conformational change. After 5 min the behavior of the effectors were different. With CHBP (and 3-PGA in one case) there was a continuous decline in activity whereas with 3-PGA (in most cases), 2-PG at low concentrations and FBP at high concentrations activity remained level for up to 20 min. With 2-PG at higher concentrations and FBP at lower , the activity continued to rise even after 20 min. In the case of 6-PGA, this conformational change, if present, was not apparent since the activity rose steadily before leveling off between 10 and 20 min. Consequently it is not possible to compare degrees of activation by different effectors at a constant preincubation time as usually done. Apparent optimal activation of RuBPCase by 6-PGA and 3-PGA was nearly 200% and the corresponding concentrations were such that they could activate <u>in vivo</u> but changes in pH and Mg^{2+} concentration could also be involved.