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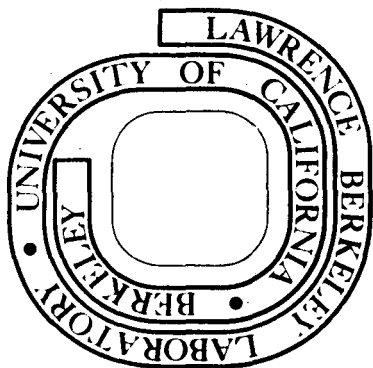
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Running Head: Activation of RuDPCase by Metabolites

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Activation of Ribulose 1,5-Diphosphate Carboxylase by Chloroplast
Metabolites¹

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²Abbreviations: RuDPCase, ribulose 1,5-diphosphate carboxylase (E.C. 4.1.1.39); RuDP, ribulose 1,5-diphosphate; FDP, fructose 1,6-diphosphate; PGA, 3-phosphoglycerate; F6P, fructose-6-phosphate; 6-PGluA, 6-phosphogluconate.

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ABSTRACT

Ribulose 1,5-diphosphate carboxylase, when activated by preincubation with 10 mM $MgCl_2$ and 1 mM bicarbonate in the absence of ribulose 1,5-diphosphate, can be further activated about 170% with 0.5 mM NADPH present in the preincubation mixture. $NADP^+$, NADH, and NAD^+ are ineffective. The activation by NADPH is comparable to that previously seen with 0.05 to 0.10 mM 6-phosphogluconate in that these specific preincubation conditions are required, but the effects of NADPH and 6-phosphogluconate are not additive. Moreover, where higher concentrations of 6-phosphogluconate inhibited the enzyme, higher concentrations of NADPH give a greater activation, saturating at about 1 mM and 200%. Under the specified conditions of preincubation, fructose 1,6-diphosphate has an activation curve similar to that of 6-phosphogluconate, peaking at 0.1 mM and 70%. Above this level, activation decreases, and inhibition is seen at still higher concentrations. Other metabolites tested produced smaller or no effects on the enzyme activity assayed under these conditions. When either reduced NADP or 6-phosphogluconate are present in the preincubation mixture, it becomes possible to determine the K_m for bicarbonate using a Lineweaver-Burke plot, and the K_m for bicarbonate under these conditions is 2.8 mM, corresponding to 0.3% CO_2 at pH 7.8 and 25°C.

Ribulose 1,5-diphosphate carboxylase (RuDPCase²), which catalyzes the carboxylation of ribulose 1,5-diphosphate (RuDP) to give two molecules of 3-phosphoglycerate (PGA) (11,12), exhibits more activity when assayed following preincubation with MgCl₂ and bicarbonate in the absence of RuDP (8,4) than when assayed under other conditions. The activity of the enzyme assayed following preincubation with 10 mM MgCl₂ and 1 mM bicarbonate is 6 to 10 times greater than the activity of the enzyme assayed under conditions in which CO₂ and bicarbonate are carefully excluded from the reaction mixture until the enzyme is in contact with RuDP (4), and this activation persists for at least 20 min. When 0.05 to 0.10 mM 6-phosphogluconate (6-PGluA) is present in the preincubation mixture with MgCl₂ and bicarbonate, a further large activation is seen. With increasing levels of 6-PGluA, the activation decreases, with still higher concentrations causing inhibition. All levels of 6-PGluA inhibit the enzyme if the 6-PGluA is added to the enzyme concurrently with or subsequent to the addition of RuDP, or, in any event, if the concentration of bicarbonate is 50 mM (3,4,10).

This behavior of the enzyme in the presence of 6-PGluA and specific assay conditions has prompted us to investigate the effects on enzyme

activity of other metabolites found in chloroplasts as a function of assay conditions. At the same time, we have reexamined the K_m of the enzyme under these conditions and in the presence of effectors.

EXPERIMENTAL

Materials. Dithiothreitol, α -ketoglutaric acid, l-glutamic acid dehydrogenase, and the sodium salts of FDP, F6P, PGA, NAD, NADH, NADP, and NADPH were purchased from Sigma Chemical Co., ATP from Calbiochem. Sources of other materials were as described previously (3,4).

Experimental. The detailed assay methods were given in the previous report (4) (method V or method VI were used in this report for the assay of RuDP carboxylase activities). In the activation or inhibition studies, the final concentration of $\text{NaH}^{14}\text{CO}_3$ was 1 mM (4.14 $\mu\text{C}/\mu\text{mole}$). For the studies of K_m 's, the enzyme had been preincubated with the effector in the presence of MgCl_2 and different concentrations of $\text{NaH}^{14}\text{CO}_3$ (1.68 $\mu\text{C}/\mu\text{mole}$) for 5 min before the reaction was started by the addition of RuDP.

A control experiment was done to check the validity of NADPH activation. NADPH, which showed activation of RuDP carboxylase activity, was first oxidized in the reaction catalyzed by l-glutamic acid dehydrogenase in the presence of α -ketoglutarate and NH_4Cl . In other reactions, either α -ketoglutarate or NH_4Cl was omitted. The product mixture from each of the above reactions was used to see its effect on RuDP carboxylase activity.

RESULTS

When the RuDPCase is assayed following preincubation with 10 mM MgCl_2 and 1 mM bicarbonate, together with various metabolites present

in chloroplasts, many metabolites have little effect in either activating or inhibiting the enzyme (Fig. 1). Among those compounds that do affect the enzyme activity, two types of dependence of activity on effector concentration are seen. With 6-PGluA, as previously reported (4), a 180% activation is seen at 0.10 mM; above this concentration the activation decreases, with higher concentrations causing inhibition. The effect of FDP is similar, but the maximum activation at 0.10 mM is only 70%.

A different kind of dependence of enzyme activity on effector concentration is seen with NADPH and with PGA. Activation of the enzyme continues to rise with effector concentration, saturating at about 1 mM at nearly 200% for NADPH but only about 50% for PGA. The concentration of NADPH needed to give 100% (about half of the maximum) activation is 0.20 mM. Although both PGA and NADPH cause activation with a similar type of dependence on effector concentration, these two activation effects appear to be rather specific, since little or no activation or inhibition of the enzyme is seen with NADP^+ , NADH , NAD^+ (Figs. 1 and 2), or F6P (Fig. 1). Also without effect were reduced dithiothreitol and ATP (not shown). Earlier studies (4) showed glucuronic acid-1-phosphate and glucose-6-phosphate to be without effect.

If the enzyme was assayed by adding MgCl_2 -preincubated enzyme to the reaction mixture which contained all the required ingredients for the reaction [method V in previous report (4)], FDP caused less inhibition than 6-PGluA. Other metabolites, including NADPH, gave no significant effect.

A kinetic study showed that the enzyme reached its highest activity only after 6 min preincubation with NADPH (0.5 mM) in the presence of MgCl_2 and $\text{NaH}^{14}\text{CO}_3$ (Fig. 2).

When the enzyme was preincubated with MgCl_2 and different concentrations of $\text{NaH}^{14}\text{CO}_3$, a plot of enzyme activity vs. NaHCO_3 concentration gave a curve not in accordance with Michaelis-Menten kinetics. It was not possible to obtain an accurate determination of the K_m by a double reciprocal (Lineweaver-Burke) plot [$1/V$ vs. $1/(\text{NaH}^{14}\text{CO}_3)$] (Fig. 3). However, if the enzyme was preincubated with MgCl_2 and different concentrations of $\text{NaH}^{14}\text{CO}_3$ in the presence of 6-PGluA (0.05 mM), NADPH (0.5 mM), or both, the K_m for HCO_3^- could be determined and was about 2.8 mM. This corresponds to a K_m for CO_2 of about 0.3% at pH 7.8 and 25°C.

A control experiment indicated that NADPH, which gave the above activation on carboxylase activity, could not show any activation effect after it was oxidized in the reaction catalyzed by l-glutamic acid dehydrogenase in the presence of α -ketoglutaric acid and NH_4Cl . When either α -ketoglutaric acid or NH_4Cl was omitted in the reaction, the reaction mixture induced about the same activation effect on carboxylase activity as the original NADPH.

DISCUSSION

In our previous report (4), we suggested that RuDPCase can change between the more active and less active forms, depending on the amount of binding of RuDP and of bicarbonate at allosteric sites. We proposed that binding of RuDP at these sites, occurring in the absence of bicarbonate and Mg^{+2} or when added simultaneously with Mg^{2+} and 1 mM bicarbonate, converts the enzyme to the less active form which then can not bind bicarbonate at the allosteric sites unless the bicarbonate concentration is much higher, for example, 50 mM. In this model, binding 1 mM bicarbonate in the presence of 10 mM Mg^{2+} and the absence of RuDP

prevents much of the allosteric binding of the RuDP and activates, or keeps active, the enzyme.

According to this hypothesis, RuDP can still bind to some allosteric RuDP-binding sites, even when the enzyme is preincubated with Mg^{2+} and 1 mM bicarbonate. Thus, such preincubation does not give maximum activation with 10 mM Mg^{2+} and 1 mM bicarbonate alone. The further activation of the enzyme by the presence of 6-PGluA during the preincubation was explained as resulting from 6-PGluA competing for the allosteric RuDP-binding sites, but without inactivating the enzyme or preventing bicarbonate binding. The similar, though lesser, activating effects of FDP seen in the present study could be explained by a similar hypothesis.

The inhibition caused by higher levels of 6-PGluA or FDP may be a manifestation of competitive inhibition (with RuDP at the active site). Such inhibition is seen with all levels of 6-PGluA and FDP with the less active form of the enzyme.

The activation caused by the presence of NADPH and by PGA during preincubation of the enzyme might be ascribed also to prevention of the inactivation by RuDP resulting from NADPH or PGA binding at the RuDP allosteric binding sites. However, given the structural dissimilarity between NADPH and RuDP, and the specificity of the NADPH effect (no effect by $NADP^+$, $NADH$ or NAD^+), it may be more reasonable to expect that there is a different allosteric binding site for NADPH. If so, binding of NADPH at this site would somehow activate, or keep active, the enzyme, perhaps by preventing RuDP binding at the RuDP allosteric sites, or by facilitating bicarbonate or CO_2 binding. In the cases of

PGA and of NADPH there is little or no inhibition of the enzyme in its inactive form, and presumably no competitive inhibition of the enzyme in its active form either. Thus, higher concentration so these effectors do not result in loss of the activation or in inhibition.

In earlier studies of RuDPCase, the K_m for bicarbonate has been reported as 11 mM (12) and 20 mM (9). With the non-preincubated enzyme, with very high Mg^{2+} concentration (45.5 mM) and with very low RuDP concentration (0.136 mM), the K_m was determined to be 2.5 mM (2). However, V_{max} was not reported and was probably considerably lower than V_{max} seen with the preincubated enzyme.

The fact that in the present study with the preincubated enzyme, Michaelis-Menten kinetics are not observed when only 1 mM bicarbonate and 10 mM Mg^{2+} are present in the preincubation mixture seems consistent with the proposal that RuDP, when added to the preincubated enzyme, binds to some of the RuDP allosteric binding sites. Higher concentrations of bicarbonate overcame the inhibiting effect of RuDP, making the enzyme active after a short time lag even without preincubation (4). If, as we suggest, the presence of 6-PGluA or NADPH in the preincubation mixture is able to prevent RuDP binding at the allosteric RuDP binding sites, then the enzyme would tend to stay fully active at all levels of bicarbonate following preincubation. Thus, Michaelis-Menten dependence of rate on bicarbonate concentration can be observed.

The further activation of preincubated enzyme by NADPH seems likely to play some physiological role, given the specificity of this activation (lack of activation by $NADP^+$, NAD^+ and NADH). One possibility arises during the time when the plant cells are going from dark to light.

During the dark, the level of RuDP will have become very low or zero, but CO_2 will be present due to respiratory reactions. When the light is turned on, or the sun rises, the level of Mg^{2+} ions in the chloroplasts are expected to increase (6,7). During the dark, and during the first seconds of bright light, or longer in dim light, 6-PGluA is present due to operation of the oxidative pentose phosphate cycle in the chloroplasts (1,5). This combination of preincubation of the enzyme with CO_2 , Mg^{2+} , and 6-PGluA can cause maximum activation of the RuDPCase, as suggested earlier (4). However, as the light brightens, or is on longer, the 6-PGluA will disappear (1) and NADPH will appear. Thus, the role of keeping the RuDPCase fully activated can be taken over by NADPH.

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

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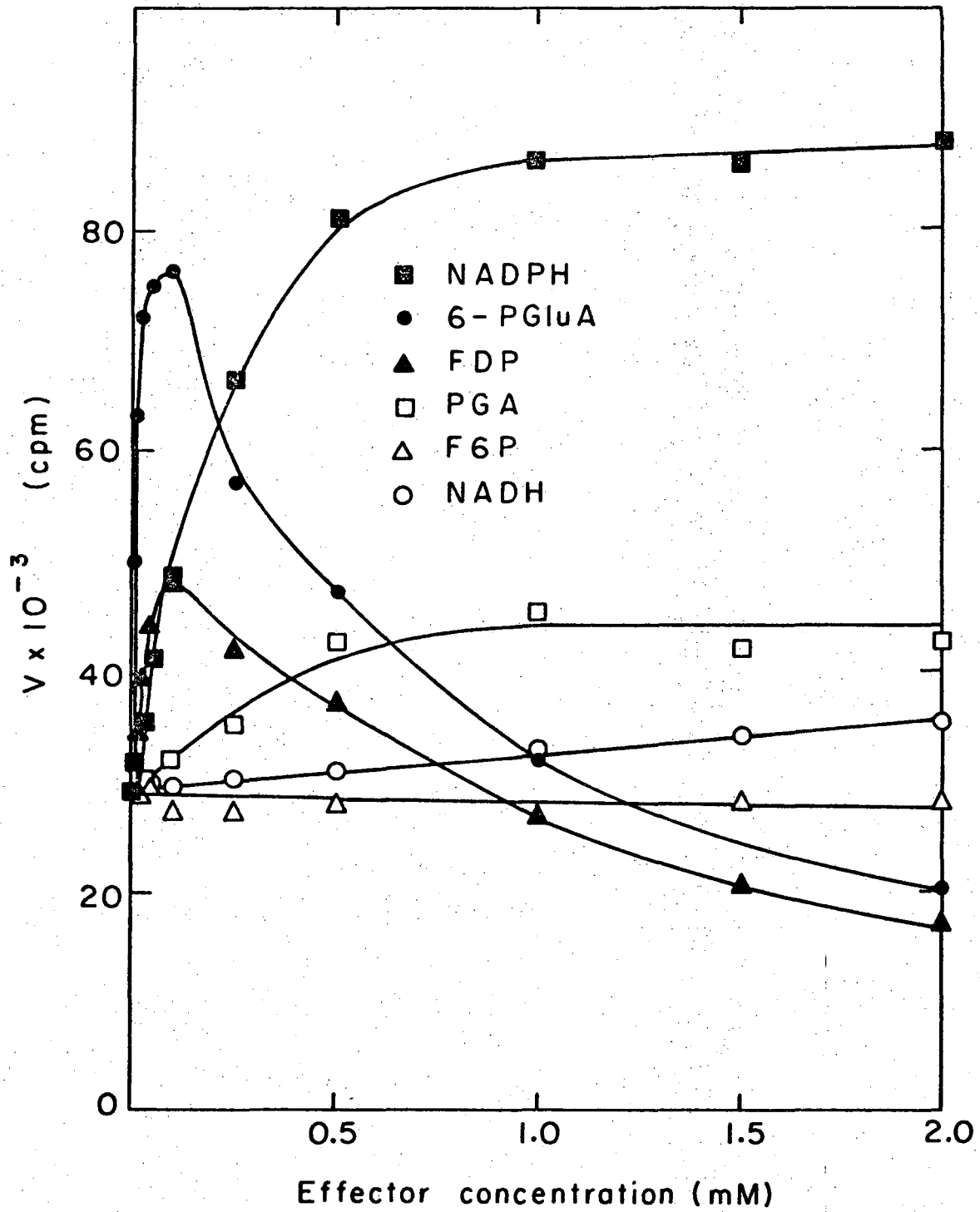
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FIGURE CAPTIONS

Fig. 1. The effects on RuDPCase activity caused by various concentrations of effectors with the preincubation assay method. The enzyme had been preincubated with effector in the presence of 10 mM MgCl_2 and 1 mM $\text{NaH}^{14}\text{CO}_3$ for 5 min and the reaction was started by the addition of RuDP. Reaction time: 5 min.

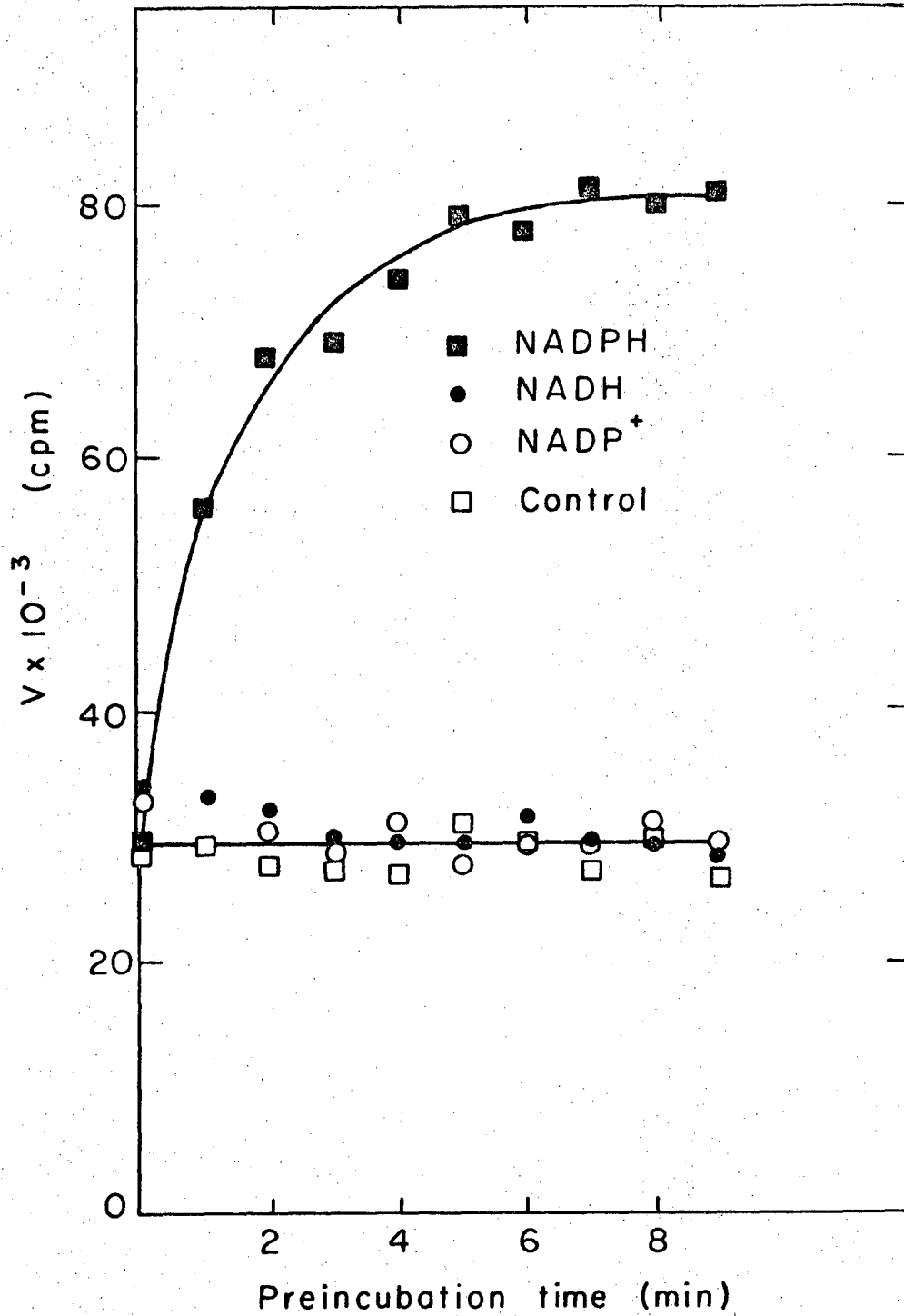
Fig. 2. Dependence of the carboxylation reaction on the time of preincubation of RuDPCase with effectors (0.5 mM) in the presence of 10 mM MgCl_2 and 1 mM $\text{NaH}^{14}\text{CO}_3$. The assays were conducted in separate vials. Effector or buffer solution was added to the enzyme which had been preincubated with MgCl_2 and $\text{NaH}^{14}\text{CO}_3$, so that when RuDP was added, the preincubation time with the effector in each vial was different but the total preincubation time with MgCl_2 and $\text{NaH}^{14}\text{CO}_3$ was kept the same. Total preincubation time with MgCl_2 and $\text{NaH}^{14}\text{CO}_3$: 5 min; reaction time: 5 min.

Fig. 3. Lineweaver Burke plot for the effects of $\text{NaH}^{14}\text{CO}_3$ concentration on the activity of RuDPCase in the presence of effector(s). In each case the enzyme had been preincubated with the effector in the presence of MgCl_2 and different concentrations of $\text{NaH}^{14}\text{CO}_3$ (1.68 $\mu\text{C}/\mu\text{mole}$) for 5 min before starting the reaction by the addition of RuDP. Reaction time: 5 min.



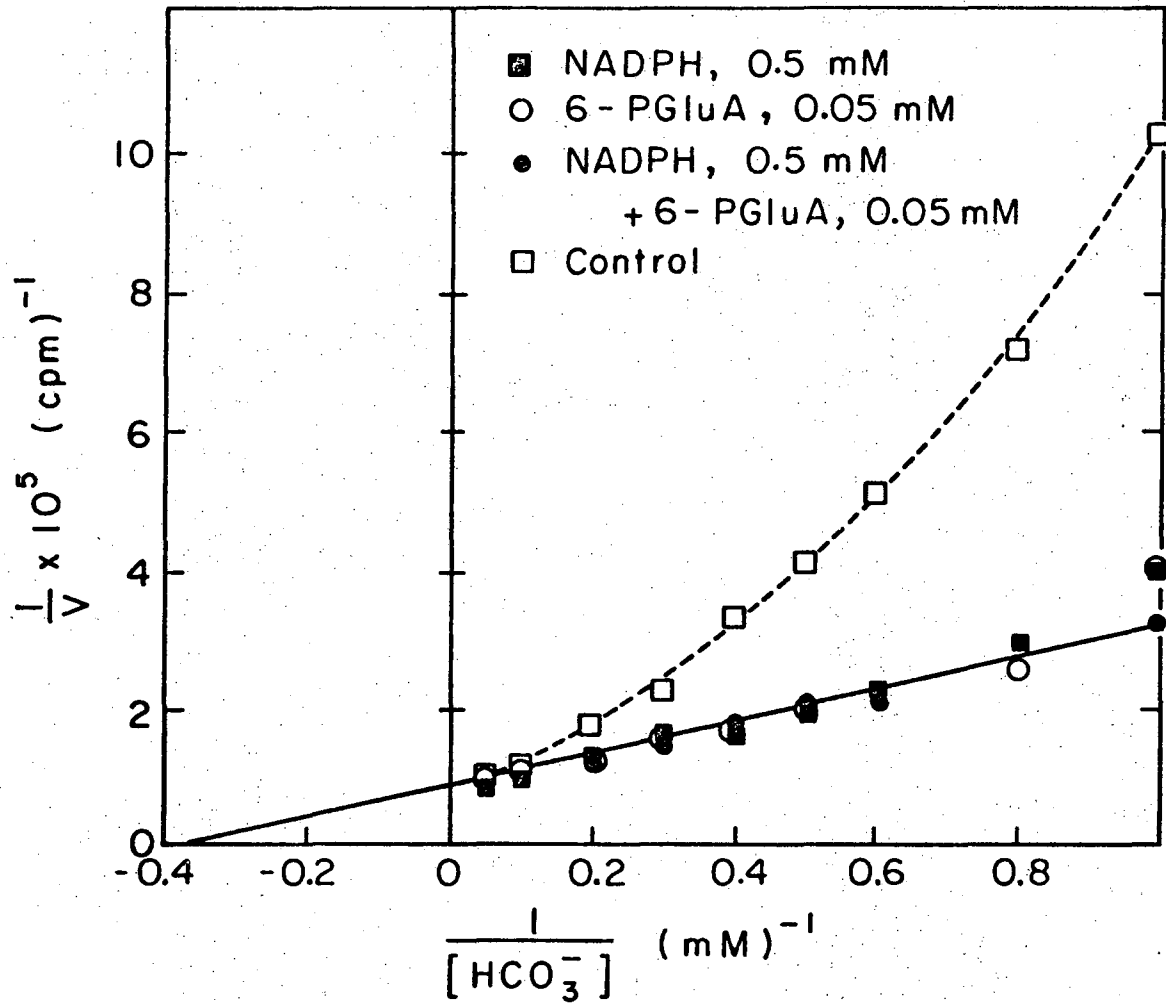
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Fig. 1



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Fig. 2



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Fig. 3

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