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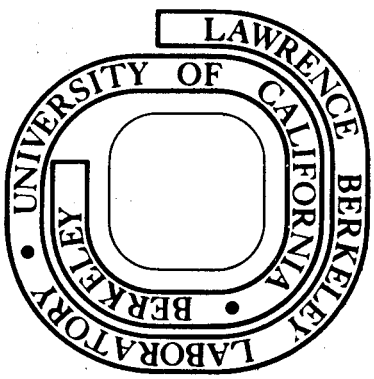

DISCLAIMER

Regulation of Ribulose 1,5-Diphosphate Carboxylase by Substrates and Other Metabolites: Further Evidence for Several Types of Binding Sites[1]

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ABSTRACT

Ribulose-1,5-diphosphate carboxylase (RuDPCase,[2] E.C. 4.1.1.39) isolated from spinach is metabolically regulated at 10 mM Mg^{+2} and low CO_2 concentrations by its substrates (RuDP and CO_2) and by effectors which include 6-phosphogluconate (6-PGluA), NADPH, and fructose-1,6-diphosphate (FDP), but not fructose-6-phosphate. Physiological concentrations of RuDP severely inhibit the enzyme activity when the enzyme has not been preincubated with HCO_3^- and Mg^{+2}, and this inactivity persists for 20 min or longer after HCO_3^- (1 mM) and Mg^{+2} (10 mM) are added. Maximum activity requires that the preincubation mixture also include either 0.01 mM 6-PGluA or 0.5 mM NADPH.

When the enzyme, preincubated with HCO_3^- and Mg^{+2}, is presented simultaneously with RuDP and either 6-PGluA or FDP, this latter compound gives simple competitive inhibition with RuDP, having K_i values of 0.020 and 0.190 respectively. NADPH or PGA at physiological concentrations do not have any effect when presented simultaneously with RuDP. Other studies on the order of addition of substrates and effectors, concentration effects, and kinetics provide additional information which serves as a basis for a proposed model of allosteric regulation combined with competitive inhibition.

In this model, there are catalytic sites at which the substrates and 6-PGluA and FDP can bind, and at least four allosteric regulatory sites, which we designate I, A_1, A_2, and A_3. RuDP binds very tightly to site (I)

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

(in the absence of Mg^{+2} or HCO_3^-), causing a conformational change in the protein to an inactive form which persists for as long as 20 min in the subsequent presence of Mg^{+2} and HCO_3^- (1 mM). Mg^{+2} and HCO_3^- (or CO_2) bind to site A_3 (in the absence of RuDP), holding the enzyme in an active form which has a much lower affinity for RuDP at site (I), so that when physiological levels of RuDP are then added, only part of the enzyme activity is lost. This active form of the enzyme can bind 6-PGluA or FDP at site A_1 and NADPH at site A_2 during preincubation with Mg^{+2} and HCO_3^-. With optimal levels of bound effectors, 6-PGluA or NADPH, enzyme activity is fully maintained, even when RuDP is subsequently added. Without one of these effectors present, addition of RuDP following preincubation reduces enzyme activity to about 40% at the levels of substrates and effectors studied. FDP is a much poorer effector, and this is ascribed to a possible binding of FDP at site (I), as well as at site A_1.

The physiological role of this regulation is discussed, particularly with respect to protection of "C-3" plants against oxidation of RuDP to phosphoglycolate.

When ribulose 1,5-diphosphate carboxylase (RuDPCase) is presented with physiological levels of one of its substrates, ribulose 1,5-diphosphate (RuDP), in a medium in which either CO_2 and bicarbonate, or Mg^{+2} ions have been kept at very low concentrations, the enzyme is inactivated. That is, its activity when 1.0 mM HCO_3^- and Mg^{+2} are then added is less than 10% of that of the fully active enzyme (10). This inactivity of the enzyme persists for more than 20 min with 1 mM HCO_3^- and Mg^{+2}, although the enzyme can be rapidly activated in the presence of high levels of HCO_3^- (e.g., 50 mM). In contrast, when the enzyme is preincubated with Mg^{+2} (10 mM) and 1 mM HCO_3^- for 5 min, the enzyme is fairly active, and remains so for 20 min or longer after the same levels of RuDP are added to start the reaction.

The activity of the HCO_3^-- and Mg^{+2}-preincubated enzyme can be further increased nearly threefold by the inclusion in the preincubation and assay mixture of either 0.10 mM 6-phosphogluconate (6-PGluA) or 0.5 mM NADPH. Smaller activations (about 50%) are seen if 0.1 mM fructose 1,6-diphosphate (FDP) or 0.5 mM 3-phosphoglycerate (PGA) are included in the preincubation and assay mixtures. None of these activations are additive when the concentrations giving the maximum effects are present. At concentrations above 0.1 mM, the activations due to 6-PGluA or FDP fall off, and at levels of these effectors above 1 mM they become inhibitory. Either 0.1 mM 6-PGluA or FDP are inhibitory when added after or simultaneously with the RuDP to the inactive form of the enzyme (the form of the enzyme when it has not been preincubated with HCO_3^- and Mg^{+2}) (9,10). Neither NADPH nor PGA cause less activation when present in preincubation

and assay mixtures at concentrations up to 2 mM, and neither is appreciably inhibitory when added to the non-preincubated enzyme.

By inclusion of either 0.5 mM NADPH or 0.05 6-PGluA, or both, in the preincubation and assay mixtures, it becomes possible to obtain a good plot of $1/v$ *vs.* $1/(HCO_3^-)$ which allows an accurate apparent K_m for HCO_3^- to be calculated as 2.8 mM. This concentration of HCO_3^- corresponds to a CO_2 pressure of about 0.3% at pH 7.8 and 25°C.

These effects of substrates and other metabolites on enzyme activity can be explained by a model assuming allosteric regulation by substrates and metabolites, as well as competitive inhibition by 6-PGluA and FDP. A preliminary description of this model was previously given (10,11). Some features of this model, such as the long-term inactivation of the enzyme by physiological concentrations of one of its substrates, appear to be unusual. In this report we present additional kinetic data on the enzyme activity and further discussion of the model and of the physiological role of this regulation.

EXPERIMENTAL

Enzyme isolation procedure and detailed assay methods were given in a previous report (10). Preincubation was for 5 or 10 min as specified with 10 mM Mg^{+2} and 1 mM $NaH^{14}CO_3$ at pH 7.8. These concentrations of Mg^{+2} and HCO_3^- were also used in all assays (except in K_m determinations for HCO_3^-). The specific radioactivity of ^{14}C is indicated with the data. Levels of RuDP and other metabolites are given with the data for each experiment.

RESULTS

Effect of RuDP Concentrations on Activation by Preincubation with Effectors. In previous studies, the level of RuDP in the assay mixture has usually been 0.5 mM, a level judged to be physiological on the basis of in vivo steady-state studies with Chlorella pyrenoidosa (4). When the level of RuDP is lowered, the activation due to preincubation of the enzyme with 6-PGluA or NADPH is reduced (Table I). As the concentration of RuDP in the assay is lowered, activation due to preincubation decreases less for NADPH as effector than for 6-PGluA as effector.

Effects of RuDP Concentration on Kinetics With and Without Preincubation. When the enzyme was assayed following preincubation with HCO_3^- and Mg^{+2}, the reaction rate increased with increasing RuDP concentration up to 0.5 mM (Fig. 1). The fact that the rate appears to decrease more rapidly with time when the RuDP concentration is low (0.05 mM) is due to using up of this substrate, and thus is not in conflict with the model proposed later (as it would be otherwise). When the enzyme is not preincubated with Mg^{+2} and HCO_3^-, and thus starts out in the "inactive" form, the rate is actually higher with the lowest levels of RuDP, and increases more with time. This is strong evidence in support of the proposal for inhibition of the enzyme activity by allosteric binding of RuDP.

Inhibitor Binding Constants for 6-PGluA and FDP. As noted earlier, the activating effects of 6-PGluA and FDP (as well as of NADPH and PGA) are seen only when these effectors are present in the preincubation mixture. When they are added simultaneously with RuDP, inhibitions are observed with all concentrations of 6-PGluA and FDP. A series of assays were performed in which the concentrations of effectors added together

with RuDP were varied at each of several levels of RuDP. Plots of 1/v vs. 1/(RuDP) for 6-PGluA (Fig. 2) and for FDP (Fig. 3) show that each of these compounds is a competitive inhibitor of RuDP at the active site. Under these conditions FDP and 6-PGluA are not acting as effectors, so that their competitive inhibitory activities are unmodified. The K_i for 6-PGluA is 0.02 mM, while the K_i for FDP is 0.19 mM. The binding of 6-PGluA is thus about 10 times stronger than that of FDP. The K_m for RuDP in these experiments was calculated to be about 0.035 mM. This is of course much higher than the K_d reported by Wishnick et al. (25) to be 0.001 mM, which we now believe to be the binding constant for RuDP at the allosteric binding site for RuDP under non-preincubation conditions.

Kinetics of Preincubated Enzyme With Effector Present During Preincubation or Added With RuDP. As already mentioned, the effectors must be present during preincubation of the enzyme with HCO_3^- and Mg^{+2} to be effective. Even up to 18 min after the start of the reaction with RuDP, the presence of effectors added simultaneously with RuDP causes little or no effect (Fig. 4). The level of 6-PGluA used in this experiment (0.05 mM) caused very little inhibition in the presence of 0.05 mM RuDP, consistent with the results in Figure 2. The data in Figure 4, together with those in Figure 1, illustrate the persistence of the several levels of activity of the enzyme, once RuDP is added and the reaction is started.

Interaction of Effectors and Substrates. In order to learn more about the possible interaction of RuDP and of effectors with the enzyme, especially at the proposed regulatory sites, the combined effects of the effectors added at differing times during the preincubation was examined (Table II). As before, either 0.05 mM 6-PGluA or 0.5 mM NADPH increased

the activity of the preincubated enzyme nearly threefold (lines b,c). Also, 0.25 mM FDP in the preincubation mixture increased the activity by 1/2 (line n), but 1.0 mM FDP in the preincubation mixture with either 6-PGluA or NADPH abolished the stimulation due to either 6-PGluA or NADPH (lines d,e and f). However, when 1.0 mM FDP was added simultaneously with RuDP, only a slight inhibition of enzyme preincubated with Mg^{+2} and HCO_3^- alone was seen, and the activation due to 6-PGluA or NADPH when present in the preincubation mixture was only slightly inhibited (lines g,h and i). With either NADPH or 6-PGluA present during the entire 10-min preincubation, but with 1 mM FDP added for only the last 5 min of preincubation, the activation of the subsequent reaction was reduced to 74% or 88% (lines j and k). However, if the order of addition was reversed, and FDP was added first and either NADPH or 6-PGluA was added later, the activation due to effectors was entirely abolished (line l and m). Finally, most of the 50% activation seen with 0.25 mM FDP present in the preincubation period is retained, even if another 0.75 mM FDP is added simultaneously with RuDP (lines n and o). Thus, although the presence of 1.0 mM FDP in the assay mixture is slightly inhibitory, as seen previously, the activation due to 0.25 mM FDP being present in the preincubation mixture is not significantly and immediately negated by addition of 0.75 mM FDP once the reaction is started.

When the enzyme is first preincubated with 0.05 mM 6-PGluA for 5 min, and 1.0 mM FDP is then added, inacativation due to the FDP occurs slowly over a period of many minutes (Fig. 5). Similarly, when the enzyme is first preincubated with 1 mM FDP and 0.05 mM 6-PGluA is then added, there is a slow, small activation of the enzyme.

DISCUSSION

Although RuDPCase has been known for 20 years and has been extensively studied and its properties reviewed (12,15,23), the specific type of allosteric behavior described in this and our two previous reports (10,11) has largely escaped notice. Probably this is due to the fact that, although Pon et al. (19) first reported the increased activity of RuDPCase following preincubation with Mg^{+2} and HCO_3^- in 1963, many workers have paid little attention to this effect in their studies. Furthermore, the enzyme has often been assayed with very high and unphysiological levels of HCO_3^- such as 50 mM. With 50 mM HCO_3^-, the inactivation of the enzyme by RuDP is quickly overcome (10), so that the effects we have described would not be seen.

Mention of the activation of the enzyme when 6-PGluA is present in the preincubation mixture was made by Buchanan and Schurmann (8) in their report on activation of the enzyme by fructose-6-phosphate (F6P) and inactivation by FDP. They also reported substantial activation by ribulose-5-phosphate, ribose-5-phosphate, xylulose-5-phosphate and various other compounds. We are unable to find any significant activation (more than 20%) with F6P or ribose-5-phosphate, either with or without preincubation under our assay conditions which include 10 mM Mg^{+2} and 1 mM HCO_3^- (10,11). While we do find competitive inhibition with FDP when the level is above 1 mM and it is not included in the preincubation mixture, we also find activation when the level of FDP is 0.25 mM (which we believe to be in the physiological range or above it), provided the FDP is included in the preincubation mixture. The differences between the results of Buchanan and Schurmann and the results obtained by us and

by others (2) are difficult to evaluate, since Buchanan and Schurmann have not provided certain details of their experimental conditions, especially the critically important preincubation times, and times of addition of the other components in the reaction mixture. The only kinetic data in their report suggest that the apparent stimulatory effect of F6P is a short-lived effect, with a drastic decline in rate after 5 min. Since the activity of the enzyme can vary greatly during several minutes of preincubation with Mg^{+2} and HCO_3^- and with effectors, widely varying results can be obtained if these preincubation times are not precisely controlled.

The RuDPCase molecule, with a molecular weight of 560,000 daltons and 16 subunits (8 at 55,000 daltons and 8 at about 15,000 daltons) (21,22,24,25) clearly appears to have the structural complexity which could accommodate complex metabolic regulation. Nishimura and Akazawa (16) have been able to dissociate the molecule into its subunits and demonstrate that the catalytic activity resides in the larger subunit, while one type of regulatory activity, a shift in pH optimum with Mg^{+2} concentration (5), resides in the smaller subunit. Moreover, a ternary complex of enzyme, CO_2, and Mg^{+2} has been demonstrated (1). Since the regulatory activities we report are dependent on preincubation with both Mg^{+2} and HCO_3^-, they might also be located on the smaller subunit, but we have no independent evidence on this location.

Incorporating the data from our previous report (10,11) and the present study, we propose that in the absence of Mg^{+2} plus bicarbonate ions, the enzyme RuDPCase binds RuDP very tightly at an allosteric site or sites [Site (I)]. This binding of RuDP may have a constant of less than 0.001 mM (25). The enzyme undergoes a conformational change to a form

in which the K_m for CO_2 is high (corresponding to an apparent K_m for HCO_3^- of 20 mM) and the enzyme is reduced in activity to a small fraction of its potential activity in the presence of 1 mM HCO_3^-. Moreover, this form of the enzyme is persistent in the presence of 1 mM HCO_3^- and 10 mM Mg^{+2}. Under these conditions, the already small activity of the enzyme is further inhibited (competitively) by 6-PGluA and FDP. Other chloroplast metabolites are largely without effect when present in physiological levels. The inactive form of the enzyme, with RuDP on the allosteric binding sites, is unable to bind 6-PGluA, NADPH, FDP or PGA at allosteric binding sites. Over a long period of time with 1 mM HCO_3^- and Mg^{+2}, the enzyme would gradually regain activity (see Fig. 4). This would come about presumably by the gradual conformational change to the more active form as CO_2 and Mg^{2+} bind to more sites on the enzyme through a sort of "bootstrap" operation, in which each binding of CO_2 and Mg^{+2} would increase subsequent binding of more CO_2 and Mg^{+2}.

When the enzyme is preincubated with Mg^{+2} and 1 mM HCO_3^- for 5 min or more (10), the ternary complex of Mg^{+2}, enzyme, and CO_2 is assumed to form at one or more sites (A_3) on the enzyme leading to a conformational change to an active form with a low K_m for CO_2. Since the higher activity of the enzyme is persistent in the presence of RuDP, this conformation must have a smaller tendency to bind RuDP at site (I). Nevertheless, once 0.5 mM RuDP is added and the reaction started, the activity of the enzyme falls off a little with the first few minutes, and in any case is much less than the maximum achieved when an effector, 6-PGluA or NADPH, is included in the preincubation mixture. Also, as reported earlier (11), the K_m for HCO_3^- is higher at low HCO_3^- concentrations if the effector is

not present. These effects indicate that some RuDP does bind at allosteric binding site (I) when it is added following preincubation, and some conformational change in the direction of the inactive form does occur in the absence of effector. We conclude, therefore, that an effector, 6-PGluA or NADPH, can bind at some allosteric binding sites (A_1,A_2) in such a way as to prevent RuDP binding at site (I), and that the effectors, in the presence of 1 mM HCO_3^- and Mg^{+2}, prevent inactivation by RuDP of the most active form of the enzyme. Also, FDP and PGA can bind at a site such as A_1, but are less effective than 6-PGluA or NADPH.

Another observation is important to an understanding of the various effects of substrates and effectors. The two compounds bearing structural similarities to RuDP, namely, 6-PGluA and FDP, exhibit typical competitive inhibition with RuDP at the catalytic site. The K_i's for 6-PGluA and FDP are 0.020 mM and 0.19 mM respectively, while the K_m for RuDP is 0.035 mM. Presumably each binding is rapidly reversible and without significant conformational effect on the enzyme molecule. In contrast, the effectors, 6-PGluA, NADP, and FDP, and the substrate, RuDP, bind tightly and somewhat irreversibly to the allosteric binding sites. The concentrations of NADPH, 6-PGluA, and FDP which give half the maximum activation when present in the preincubation mixture, are 0.20 mM, 0.007 mM, and 0.015 mM, respectively.

Data in Table II are particularly illustrative of the nature of binding at the allosteric sites. Although 0.25 mM FDP in the preincubation mixture stimulates activity about 45%, a level of 1.0 mM FDP gives about the same activity as preincubation without effector, for reasons discussed below. If 1.0 mM FDP is added in the preincubation mixture at the same time as

0.05 mM 6-PGluA, the resulting activity (equal to the preincubation without effector) shows that FDP was bound to the allosteric sites and prevented binding by 6-PGluA at its 20 times lower concentration. Similarly, 0.5 mM NADPH in the preincubation mixture is without effect in the presence of 1.0 mM FDP due to the higher binding constant for NADPH. However, if the same amounts of these effectors are used, but the FDP is added 5 min later than the 6-PGluA or the NADPH, over half of the activation seen without FDP addition is retained. Finally, when the FDP is added only at the same time as RuDP (after 10 min preincubation with 6-PGluA or NADPH), none of the activation is abolished, although a small amount of competitive inhibition results in a slight decrease in activity. From these results, it is clear that among the three effectors, the first one bound onto the enzyme tends to stay there, and is not readily displaced even by excesses of a potential displacing effector. The final two lines of Table II show that FDP bound at the allosteric site remains there and is not displaced when RuDP is added; also, the subsequent addition of additional FDP merely causes the small expected competitive inhibition.

Looking more closely at these data in Table II, we see an anomoly (with respect to the model so far presented) which bring us to the question of the number and nature of the allosteric binding sites. We have explained the fact that 1.0 mM FDP in the preincubation mixture (line d) gives approximately the control activity. When only 0.25 mM FDP is present in the preincubation mixture, we see an activation of 55% (line n). One would predict that the addition of the remaining 0.75 mM FDP to give a total FDP concentration of 1.0 mM in the assay mixture would cause enough competitive inhibition to bring the activity back to the level seen with 1.0 mM FDP from the

start. In actuality, the enzyme is still activated 45% (line o). Thus we are forced to the conclusion that FDP in the preincubation mixture has an additional inhibitory effect beyond that expected from competitive inhibition. One way of explaining this is to recall that the allosteric binding site for RuDP (binding of RuDP leading to inactivation) is different from the allosteric binding sites A_1 and A_2 for 6-PGluA and NADPH (binding of these effectors leading to activation). FDP may bind to the activating site (A_1) at lower concentrations, but as the concentration increases could also bind to the inactivating site (I), thus negating some of its activating effect. Since we have already proposed that the RuDP binds very tightly to the allosteric binding site (I), and preincubation inhibition due to FDP is only seen at very high concentrations of FDP, it is clear that FDP binds very poorly to site (I). In fact, this probably is not an important physiological mechanism since the concentrations of FDP required are higher than physiological. With this more complex model, it is important to remember that binding at A_1 or A_2 decreases binding at (I), and binding at (I) decreases binding at A_1 or A_2. These stipulations are required to explain the long duration of preincubation activation and inactivation effects.

Considering the structural dissimilarities between 6-PGluA and NADPH, and the specificity for these compounds ($NADP^+$, NADH, glucuronic-6-phosphate, and other metabolites resembling 6-PGluA and NADPH are inactive), we find it reasonable that there are at least two different activation catalytic sites, A_1 and A_2. However, just as the inactivating and activating sites are interdependent, with binding at one decreasing binding at the other, or at least abolishing the effect of binding at the other, we must also suppose that sites A_1 and A_2 are interdependent in that the

maximum effects due to binding of NADPH and 6-PGluA are not additive. In this model, we suggest that FDP can bind at A_1 (6-PGluA site) as well as binding poorly at site (I). Possibly PGA also binds at site A_1. NADPH would bind at site A_2. Saturation binding at either site produces the full activating effect, and when one activation site is already fully bound, binding at the other activation site causes no additional activation. Since FDP may be able to bind at either A_2 or poorly at (I), its activating effect is less than that of the other activators at lower concentrations, while at higher concentrations its activating effect is abolished.

The activation effects due to effectors can only be seen if the enzyme is preincubated with HCO_3^- and Mg^{+2}. We suggest that the preincubation with only 1.0 mM HCO_3^- and Mg^{+2} fully activates the enzyme, but that addition of 0.5 mM RuDP to start the reaction partially inactivates the enzyme due to some binding of RuDP at site (I). The rate falls off during the first 5 min, and this could be due to further binding of RuDP at site (I). In other words, activation with 1.0 mM HCO_3^- and Mg^{+2} alone does not completely prevent binding of RuDP at the inactivation site (I). Binding of 6-PGluA, or to a lesser extent of FDP or PGA, at site A_1, prevents this binding of RuDP, as does binding of NADPH at site A_2. Consequently, once the enzyme has been fully activated in this way, it becomes possible to obtain linear $1/v$ vs. $1/(HCO_3^-)$ kinetics since the enzyme is no longer partially inactivated by RuDP at low concentrations of HCO_3^- and reactivated by high concentrations of HCO_3^-.

From Figure 1, it is clear that even 0.05 mM RuDP is enough to severely inactivate the enzyme, and this is not surprising if the dissociation constant from the site (I) is indeed lower than 0.001 mM.

However, once the enzyme is activated by preincubation with Mg^{+2} and 1 mM HCO_3^- (but without other effectors) even such high levels of RuDP as 0.5 mM cause only partial inhibition. We conclude therefore that as a result of the conformational change whereby the activation occurs there is a large increase in the binding constant for RuDP at site (I). Thus, over a very wide range of concentrations, HCO_3^- and RuDP can exert opposing influences on the activity of the enzyme. Probably the reported substrate inhibition by levels of RuDP above 0.7 mM in the presence of 50 mM HCO_3^- (18) is an extension of these effects into the non-physiological range.

The model we have proposed here is designed to explain the various characteristics described in this and our previous reports. Despite its complexity, it appears to be the simplest model we can devise consistent with these characteristics. It may not be the only model satisfying these conditions.

Physiological Significance. As suggested in our previous report, activation of the RuDPCase by Mg^{+2}, HCO_3^-, 6-PGluA, and NADPH can play an *in vivo* role in activating and keeping the enzyme active at the end of a period of darkness and during an ensuing transition in the light (11). We have estimated the level of NADPH in illuminated, photosynthesizing spinach chloroplast to be in the range of 0.3 to 0.5 mM, assuming the stroma or aqueous space inside the chloroplasts to be about half the total volume. Studies with *Chlorella* indicate the concentration of 6-PGluA to be around 0.05 mM during periods of darkness (4). These are ranges shown to be capable of activating the enzyme in our previous report. There remains the important question of why the RuDPCase should be inactivated when presented with RuDP, one of its substrates, in the absence of HCO_3^-.

When the isolated enzyme is exposed to atmospheric O_2 and RuDP at low levels of CO_2, O_2 binds to the enzyme competitively with CO_2. Following this binding of O_2, there is an oxidative attack on the RuDP leading to formation of phosphoglycolic acid and PGA (7,13,14); In whole cells, phosphoglycolate is converted to glycolate which is lost from chloroplasts and in higher plants serves as a substrate for photorespiration. In Chlorella glycolate may be mostly excreted into the medium. While there is some controversy over the importance of RuDP oxidation as a route to glycolate in photorespiration, it seems likely that at least a part of the glycolate formed in vivo is made this way (3). Certain tropical plants which exhibit "C-4" metabolism have a mechanism for maintaining a higher level of CO_2 inside chloroplasts, thus perhaps avoiding a part of photorespiration due to this mechanism. It has not been known what defense, if any, other plants might have against this type of oxidation of RuDP. Data in this and our previous report indicate that the inactivation of RuDPCase at very low levels of HCO_3^- and in the presence of RuDP is accompanied by an increase in the binding constant for CO_2. Since O_2 binds competitively with CO_2 (6,17), it is reasonable to suppose that the binding of O_2 is also affected in the direction of less binding by the conformational change, and that this is in fact the primary reason for the regulatory inactivation. Recently, it has been reported that the oxygenase activity of RuDPCase is in fact enhanced through preincubation of the enzyme with 6-PGluA, and is inhibited when 6-PGluA is added to the enzyme along with RuDP (20). This is of course exactly what would be predicted if the active form of RuDPCase binds O_2 as well as CO_2 more tightly than the inactive form of the enzyme. We have shown that the apparent K_m for HCO_3^-

is lowered at low concentrations of HCO_3^- by preincubation with Mg^{+2}, HCO_3^-, and 6-PGluA. Moreover, Bassham and Kirk (3) found that when photosynthesizing Chlorella were suddenly exposed to 100% O_2 and no CO_2, the rate of formation of phosphoglycolate from RuDP was rapid during the first minute and then dropped rapidly, as would be expected if the O_2 binding of the RuDPCase were decreasing (the binding constant increasing). Probably this mechanism for minimizing the oxygenase effect on RuDPCase is of great importance to "C-3" plants, and is one reason why the larger part of glycolate formed in such plants may come from another source than oxidation of RuDP.

LITERATURE CITED

1. AKOYUNOGLOU, G. AND M. CALVIN. 1963. Mechanism of the Carboxydismutase Reaction. II. Carboxylation of the Enzyme. Biochem. Z. 338: 20-30.

2. AVRON, M. AND M. GIBBS. 1974. Carbon Dioxide Fixation in the Light and in the Dark by Isolated Spinach Chloroplasts. Plant Physiol. 53: 140-143.

3. BASSHAM, J. A. AND M. KIRK. 1973. Sequence of Formation of Phosphoglycolate and Glycolate in Photosynthesizing Chlorella pyrenoidosa. Plant Physiol. 52: 407-411.

4. BASSHAM, J. A. AND G. H. KRAUSE. 1969. Free Energy Changes and Metabolic Regulation in Steady-state Photosynthetic Carbon Reduction. Biochim. Biophys. Acta 189: 207-221.

5. BASSHAM, J. A., P. SHARP AND I. MORRIS. 1968. The Effect of Mg^{++} Concentration on the pH Optimum Michaelis Constants of the Spinach Chloroplast Ribulosediphosphate Carboxylase. Biochim. Biophys. Acta 153: 898-900.

6. BOWES, G. AND W. L. OGREN. 1972. Oxygen Inhibition and Other Properties of Soybean Ribulose 1,5-Diphosphate Carboxylase. J. Biol. Chem. 247: 2171-2176.

7. BOWES, G., W. L. OGREN AND R. H. HAGEMAN. 1971. Phosphoglycolate Production Catalyzed by Ribulose Diphosphate Carboxylase. Biochem. Biophys. Res. Commun. 45: 716-722.

8. BUCHANAN, B. B. AND P. SCHURMANN. 1973. Regulation of Ribulose 1,5-Diphosphate Carboxylase in the Photosynthetic Assimilation of Carbon Dioxide. J. Biol. Chem. 248: 4956-4964.

9. CHU, D. K. AND J. A. BASSHAM. 1972. Inhibition of Ribulose 1,5-Diphosphate Carboxylase by 6-Phosphogluconate. Plant Physiol. 50: 224-227.

10. CHU, D. K. AND J. A. BASSHAM. 1973. Activation and Inhibition of Ribulose 1,5-Diphosphate Carboxylase by 6-Phosphogluconate. Plant Physiol. 52: 373-379.

11. CHU, D. K. AND J. A. BASSHAM. 1974. Activation of Ribulose 1,5-Diphosphate Carboxylase by Reduced Nicotinamide Dinucleotide Phosphate and Other Chloroplast Metabolites. Plant Physiol. In press.

12. KAWASHIMA, K. AND S. G. WILDMAN. 1970. Fraction I Protein. Ann. Rev. Plant Physiol. 21: 325-358.

13. LORIMER, G. H., T. J. ANDREWS AND N. E. TOLBERT. 1973. Ribulose Diphosphate Oxygenase. I. Synthesis of Phosphoglycolate by Fraction I Protein of Leaves. Biochemistry 12: 11-18.

14. LORIMER, G. H., T. J. ANDREWS AND N. E. TOLBERT. 1973. Ribulose Diphosphate Oxygenase. II. Further Proof of Reaction Products and Mechanism of Action. Biochemistry 12: 18-23.

15. McFADDEN, B. A. 1973. Autotrophic CO_2 Assimilation and the Evolution of Ribulose 1,5-Diphosphate Carboxylase. Bacteriol. Rev. 37: 289-319.

16. NISHIMURA, M. AND T. AKAZAWA. 1974. Studies on Spinach Leaf Ribulose-diphosphate Carboxylase. Carboxylase and Oxygenase Reaction Examined by Immunochemical Methods. Biochemistry 13: 2277-2281.

17. OGREN, W. L. AND G. BOWES. 1971. Ribulose Diphosphate Carboxylase Regulates Soybean Photorespiration. Nature New Biol. 230: 159-160.

18. PAULSEN, J. M. AND M. D. LANE. 1966. Spinach Ribulose Diphosphate Carboxylase. I. Purification and Properties of the Enzyme. Biochemistry 5: 2350-2357.

19. PON, N. G., B. R, RABIN AND M. CALVIN. 1963. Mechanism of the Carboxydismutase Reaction I. The Effect of Preliminary Incubation of Substrates, Metal Ion and Enzyme on Activity. Biochem. Z. 338: 7-19.

20. RYAN, F. J. AND N. E, TOLBERT. 1974. Properties and Regulation of Ribulose Diphosphate Oxygenase. Fed. Proc. 33: 1290.

21. RUTNER, A. C. 1970. Estimation of the Molecular Weight of Ribulose Diphospahte Carboxylase Sub-units. Biochem. Biophys. Res. Commun. 39: 923-929.

22. RUTNER, A. C. AND M. D. LANE. 1967. Nonidentical subunits of Ribulose Diphosphate Carboxylase. Biochem. Biophys. Res. Commun. 28: 531-537.

23. SIEGEL, M., M. WISHNICK AND M. D. LANE. 1972. Ribulose 1,5-Diphosphate Carboxylase. In The Enzymes. P. D. Boyer, ed. Acacemic Press, New York and London. pp. 169-192.

24. SUGIYAMA, T. AND T. AKAZAWA. 1970. Subunit Structure of Spinach Leaf Ribulose 1,5-Diphosphate Carboxylase. Biochemistry 9: 4499-4504.

25. WISHNICK, M., M. D. LANE AND M. C. SCRUTTON. 1970. The Interaction of Metal Ion with Ribulose 1,5-Diphosphate Carboxylase from Spinach. J. Biol. Chem. 245: 4939-4947.

Table I. Effect of RuDP Concentrations on Activation by Preincubation with Effectors

The enzyme was preincubated with the effector in the presence of $NaH^{14}CO_3$ (1 mM, 4.1 μc/μmole) and 10 mM $MgCl_2$ for 5 min before the addition of RuDP to initiate the reaction.

RuDP, mM	Activity* x 10^{-3}, cpm Effector H_2O	6-PGluA, 0.05 mM	NADPH, 0.05 mM
0.25	26.5	74.1	39.0
0.125	24.3	60.7	35.4
0.05	21.9	35.9	29.4
0.025	15.5	18.6	20.0

*The activity was measured in 5 min of reaction.

Table II. Combined Effects of Two Effectors on RuDPCase Activities

The enzyme activities were assayed according to the following scheme:

Line	$NaH^{14}CO_3$, $MgCl_2$, enzyme and first effector	After 5 min, second effector added	After 10 min, reaction started with	relative activity, %
a	H_2O	---	RuDP*	100**
b	6-PGluA, 0.05 mM	---	RuDP	275
c	NADPH, 0.5 mM	---	RuDP	265
d	FDP, 1 mM	---	RuDP	95
e	6-PGluA, 0.05 mM + FDP 1 mM	---	RuDP	100
f	NADPH, 0.5 mM + FDP, 1 mM	---	RuDP	104
g	H_2O	---	RuDP+FDP, 1 mM	85
h	6-PGluA, 0.05 mM	---	RuDP+FDP, 1 mM	250
i	NADPH, 0.5 mM	---	RuDP+FDP, 1 mM	240
j	6-PGluA, 0.05 mM	FDP, 1 mM	RuDP	188
k	NADPH, 0.5 mM	FDP, 1 mM	RuDP	174
l	FDP, 1 mM	6-PGluA, 0.05 mM	RuDP	101
m	FDP, 1 mM	NADPH, 0.5 mM	RuDP	104
n	FDP, 0.25 mM	---	RuDP	155
o	FDP, 0.25 mM	---	RuDP+FDP, 0.75 mM	145

*RuDP concentration was 0.5 mM.

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

$NaH^{14}CO_3$ = 1 mM (1.5 μc/μmole); $MgCl_2$ = 10 mM.

Figure Captions

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

Fig. 2. Inhibition of RuDPCase by 6-PGluA. The reactions were started by additions of the mixtures of RuDP and 6-PGluA to the enzyme which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$ (1 mM, 1.4 μc/μmole). Preincubation time: 5 min; reaction time: 5 min; concentrations of 6-PGluA are indicated in the figure.

Fig. 3. Inhibition of FuDPCase by FDP. The reactions were started by additions of the mixtures of RuDP and FDP to the enzyme which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$ (1 mM, 1.4 μc/μmole). Preincubation time: 5 min; reaction time: 5 min; concentrations of FDP are indicated in the figure.

Fig. 4. Fixation of $^{14}CO_2$ via the carboxylation reaction with different assay methods in the presence of effector vs. the time of reaction. The reactions were started by adding RuDP to the enzyme which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$ in the presence of either 6-PGluA (o—o) or NADPH (Δ—Δ). Other reactions were started by adding the mixture of RuDP and 6-PGluA (●—●) or RuDP and NADPH (▲—▲) to the enzyme which had

Figure Captions (Cont.)

been preincubated with $MgCl_2$ and $NaH^{14}CO_3$). The control reaction is shown by x—x. Preincubation time: 5 min; $NaH^{14}CO_3$: 1 mM, 1.08 μc/μmole; NADPH: 0.5 mM; 6-PGluA: 0.05 mM; RuDPCase: 15 μg.

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

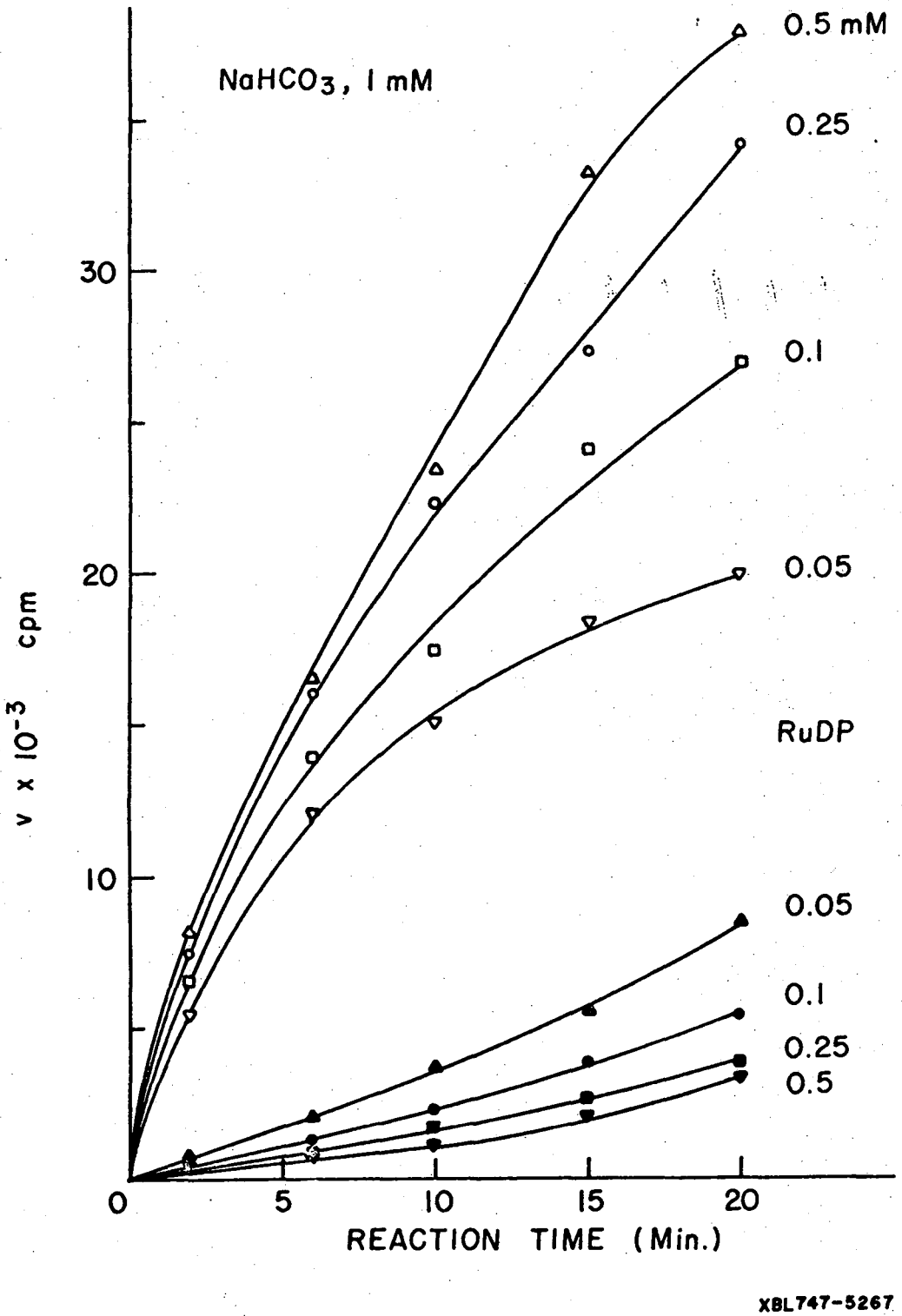

NaHCO3, 1 mM
0.5 mM
0.25
0.1
0.05
RuDP
0.05
0.1
0.25
0.5
v x 10-3 cpm
0
10
20
30
0
5
10
15
20
REACTION TIME (Min.)
XBL747-5267

Fig. 1.

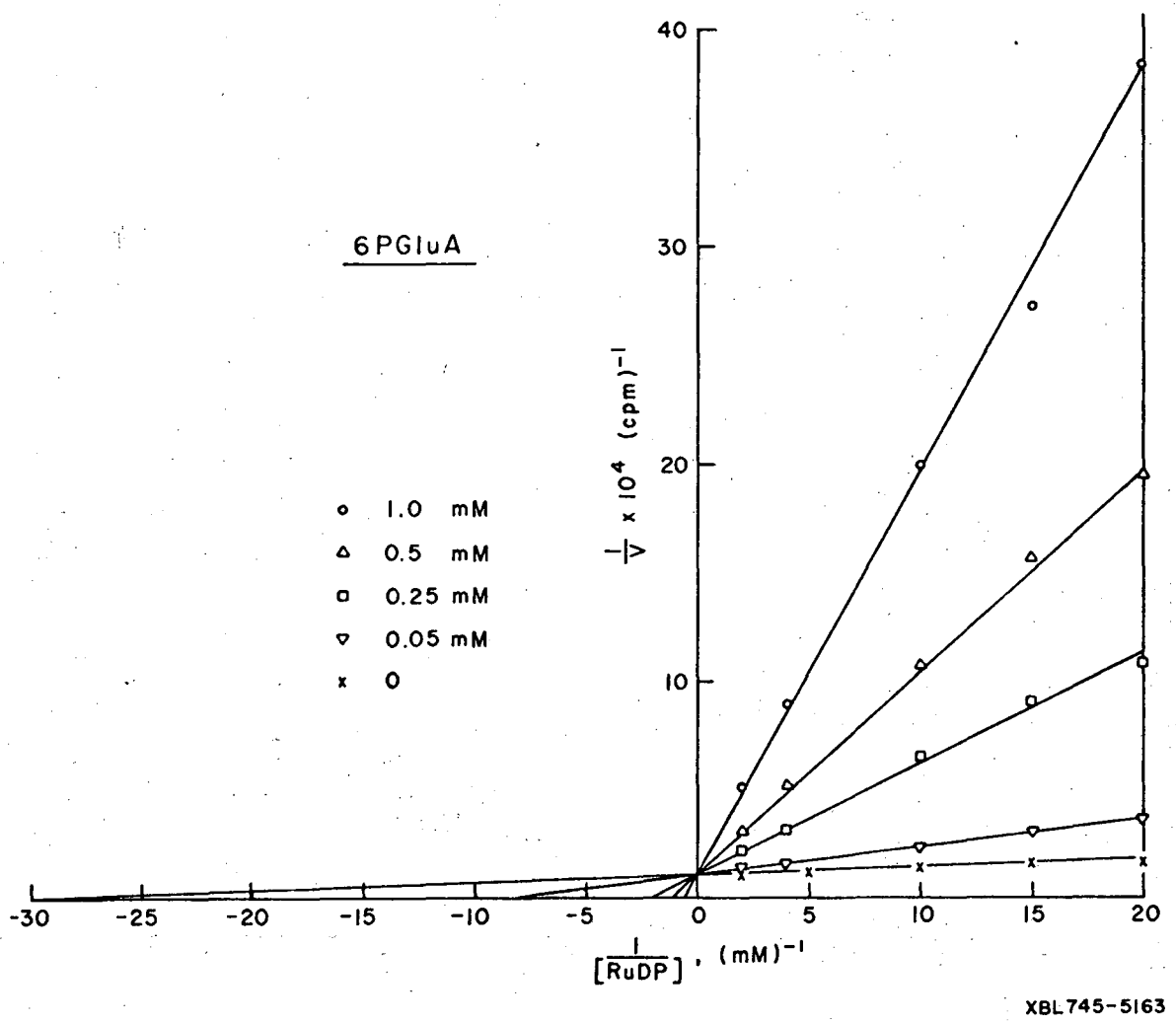

6PGluA
1.0 mM
0.5 mM
0.25 mM
0.05 mM
0
$\frac{1}{V} \times 10^4$ (cpm)$^{-1}$
40
30
20
10
-30
-25
-20
-15
-10
-5
0
5
10
15
20
$\frac{1}{[RuDP]}$, (mM)$^{-1}$
XBL745-5163

Fig. 2.

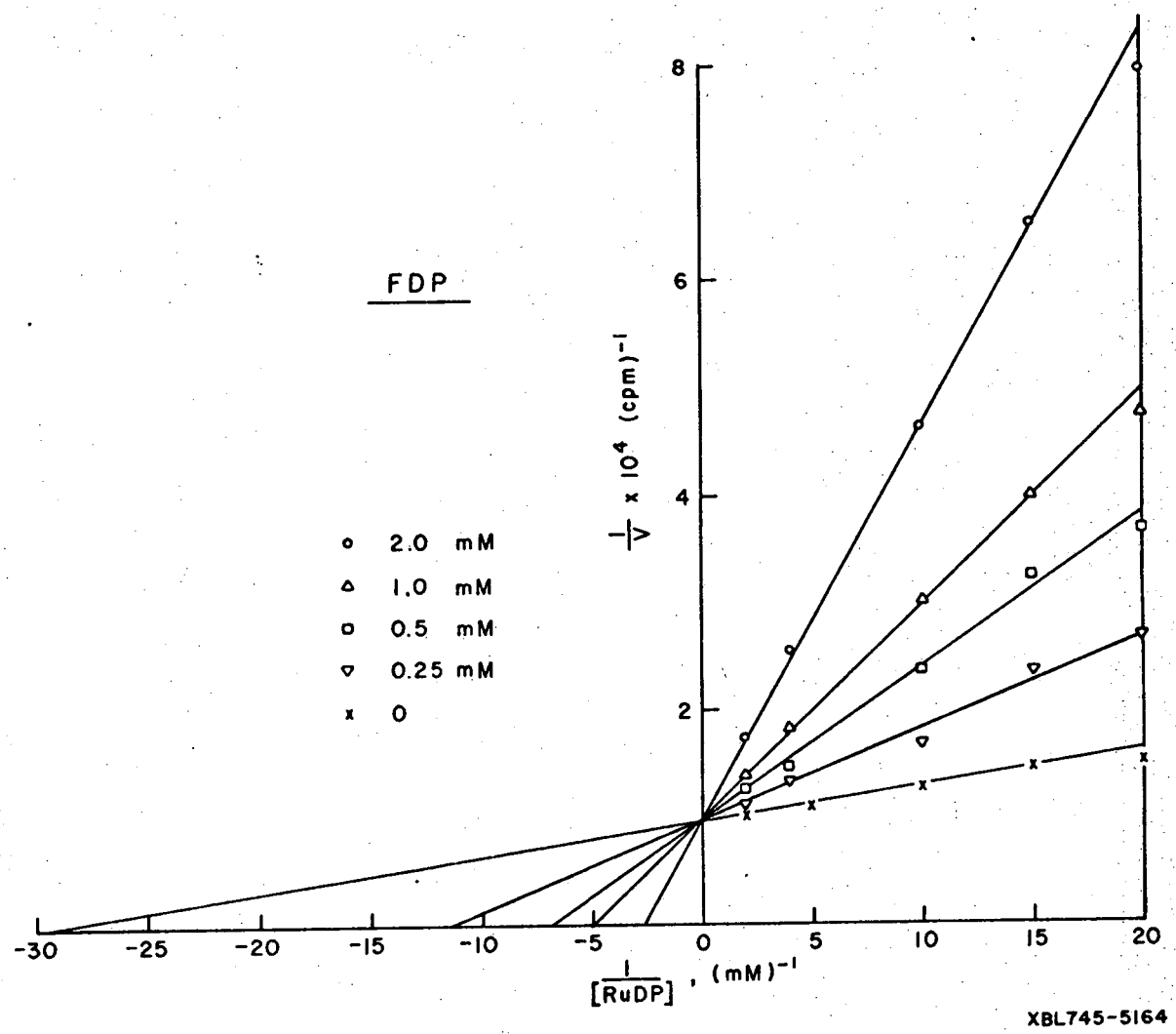
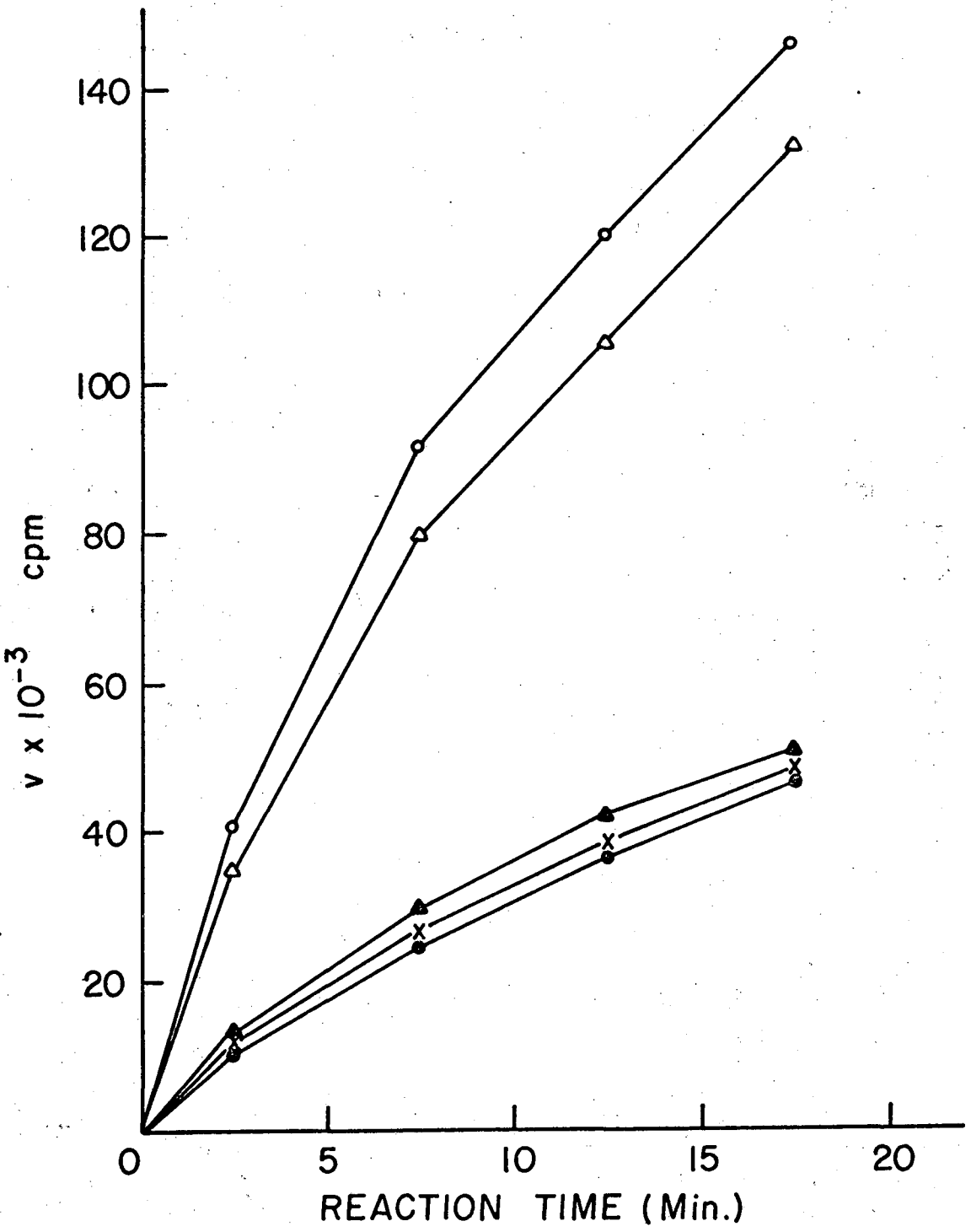

FDP
2.0 mM
1.0 mM
0.5 mM
0.25 mM
0
$\frac{1}{V} \times 10^4$ (cpm)$^{-1}$
$\frac{1}{[RuDP]}$, (mM)$^{-1}$
XBL745-5164

Fig. 3.



XBL747-5265

Fig. 4.

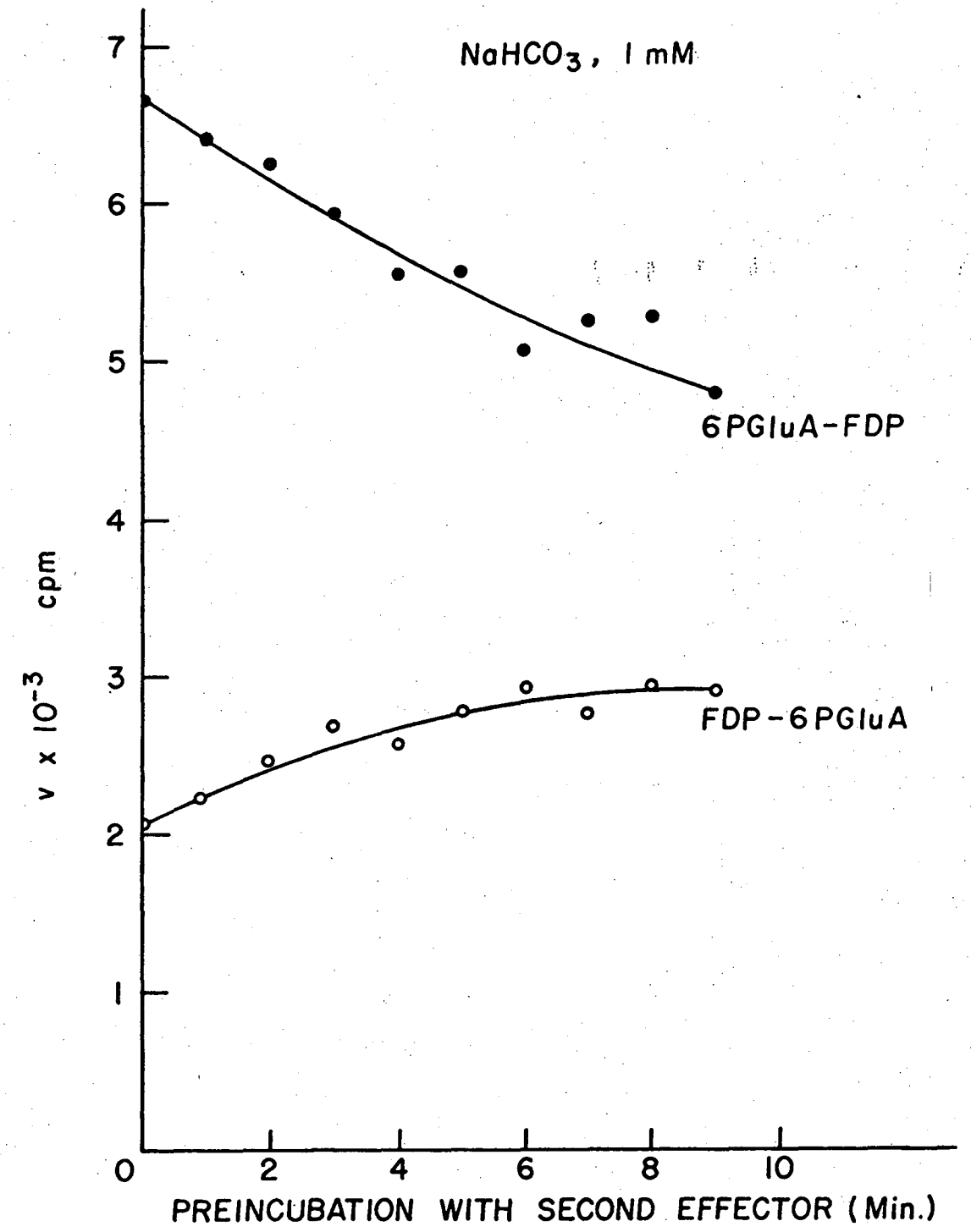

NaHCO3, 1 mM
6PGluA-FDP
FDP-6PGluA
v x 10-3 cpm
0
1
2
3
4
5
6
7
2
4
6
8
10
PREINCUBATION WITH SECOND EFFECTOR (Min.)
XBL747-5266

Fig. 5.

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