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Author
Chu, Douglas K.

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Douglas K. Chu and J. A. Bassham

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INHIBITION OF RIBULOSE 1,5-DIPHOSPHATE CARBOXYLASE BY 6-PHOSPHOGLUCONATE

DOUGLAS K. CHU AND J. A. BASSHAM

Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory,

University of California, Berkeley, California 94720

ABSTRACT

6-Phosphogluconate is a much more effective inhibitor of the photosynthetic carboxylation enzyme, ribulose-1,5-diphosphate carboxylase, than other sugar phosphates and sugar acids of the reductive and oxidative pentose phosphate cycles. The inhibition appears to be non-competitive with ribulose 1,5-diphosphate. Since 6-phosphogluconate is unique to the oxidative cycle and inhibits at concentrations comparable to those found *in vivo* it is proposed that its inhibition of the carboxylase may be an additional regulatory factor.

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In the carboxylation reaction of the photosynthetic reductive pentose phosphate cycle (3,4) ribulose 1,5-diphosphate (RuDP)* reacts with CO_2 and water to give two molecules of 3-phosphoglycerate (PGA). The isolated enzyme catalyzing this reaction, 3-phospho-D-glycerate carboxylase (dimerizing) (E.C. 4.1.1.39), hereafter referred to as ribulose diphosphate carboxylase (RuDPCase), was found to require Mg^{++} or certain other divalent cations, but required no ATP, NADP, or other cofactors derived from the light (18,19). The specific activity of the isolated enzyme in early studies seemed to be too low for it to be able to catalyze CO_2 fixation at the rates observed for green cells _in vivo_ (13).

From analysis of steady-state levels of RuDP in the dark following photosynthesis, Pedersen _et al._ (14) concluded that the mechanism for the carboxylation reaction _in vivo_ becomes inactive after about 3 min of darkness, since the level of RuDP drops very slowly after this time, despite the fact that the free energy change for the carboxylation reaction is about 10 Kcal negative (4,6). Other studies showed that the carboxylation reaction is inactivated, even with the light on, by the addition of fatty acids (13) which are thought to interfere with ion pumping in the

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*Abbreviations: RuDP, ribulose-1,5-diphosphate; PGA, 3-phosphoglycerate; RuDPCase, ribulose 1,5-diphosphate carboxylase; 6-PGluA, 6-phosphogluconate.

thylakoids. Inactivation also occurred with addition of vitamin K_5, which may interfere with electron transport. Another effect of the addition of vitamin K_5 in the light was the immediate appearance of 6-phosphogluconate (6-PGluA) (11).

Jensen and Bassham (10) found that CO_2 fixation in isolated spinach chloroplasts ceases within about 2 min after the light is turned off, even though the level of RuDP does not drop more than 60% from its value in the light. If ATP was added to the chloroplasts, the level of RuDP was maintained as high in the dark as in the light, yet CO_2 fixation still stopped completely in the dark. Thus the dark inactivation of the carboxylation reaction apparently occurs in isolated chloroplasts as well as _in vivo_.

It has been proposed that the RuDPCase is activated in the light in chloroplasts by changes in the ionic content of the chloroplasts, and by the higher ratio of reduced to oxidized coenzymes (1,2,5). Both the changes in ionic content (especially Mg^{++} and H^{+}) and the higher ratio of reduced to oxidized cofactors would be consequences of the photoelectron transport reactions occurring in the thylakoid membranes, and it was proposed that these changes, as reflected in the stroma region, provide a general regulatory mechanism whereby RuDPCase and other key regulated enzymes are activated in the light. Such a light-activated mechanism does not necessarily obviate the need for another mechanism to turn off more completely the carboxylation in the dark.

From studies of the metabolites in _Chlorella pyrenoidosa_ in light and dark, it was found that the one metabolite which is most notably higher in concentration in the dark than in the light is 6-PGluA (5). This compound, which is considered by us to be an indicator of the operation of the oxidative pentose phosphate cycle, appears immediately in the

dark and disappears within 1 min in the light. The appearance of 6-PGluA, whether in the dark in vivo, or upon the addition of vitamin K_5 in the light to either Chlorella pyrenoidosa or isolated spinach chloroplasts (11) thus seems to be correlated with the inactivation of the carboxylation reaction (as well as with certain other regulated reactions). The present study shows that 6-PGluA is an effective inhibitor, at low levels, of the RuDPCase.

METHODS

Enzyme Purification. Carboxylase was purified from spinach leaves. The purification procedures basically follow methods of others, with some modifications (12,16,17). Briefly, the purification procedures include homogenization in a Waring blender for 3 min, heating at 50°C for 20 min, Sephadex G-25 column chromatography, precipitation by 30-60% saturation with ammonium sulfate, DEAE-cellulose column chromatography with a linear gradient of NaCl (0-1 M), and Sephadex G-200 column chromatography. The purified enzyme had a specific activity of about 0.5 units/mg protein (one unit is defined as 1 mole of carbon incorporated into acid-stable compound per min under assay condition).

Materials. RuDP and 6-PGluA were purchased from Sigma Chemical Co. as the barium and tri-monocyclohexylammonium salts, respectively. The acid forms of these compounds were obtained by treating the salt solutions with Dowex-50. The scintillation fluid, "Aquasol", was purchased from New England Nuclear.

Enzyme Assay. In a final 200 µl, the reaction mixture contains the following components and their concentrations: Tris-HCl, 175 mM; $MgCl_2$, 10 mM; pH 7.8; RuDP; $NaH^{14}CO_3$; 6-PGluA; and enzyme in different amounts as shown in each figure. The reaction was started with the addition of

enzyme solution to the reaction mixture contained in a tube sealed with a serum cap. After 5 or 10 min incubation in the water bath at 23°C, 100 μl of 6 N acetic acid was added to stop the reaction. An aliquot (250 μl) of this mixture was transferred to a scintillation counting vial and was dried in the oven at 90°C for 1 hr. Then 250 μl of water was added to the vial, followed by 18 ml of "Aquasol". The radioactivity was measured by scintillation counter with a counting efficiency around 90%.

Spinach chloroplasts were isolated and allowed to photosynthesize with $H^{14}CO_3^-$ as described previously (10). Rates of ^{14}C uptake into acid-stable compounds were determined, and analysis was made of these compounds by paper chromatography and radioautography (14). These rates and patterns were compared for chloroplast suspensions with and without additions of 6-PGluA to concentrations of 0.34 mM and 0.68 mmM.

RESULTS

With a constant concentration of $NaHCO_3$ of 50 mM, with RuDP concentration varied from 0.5 to 0.02 mM, and with the addition of 0, 25, 50, 100, 150, and 200 μM of 6-PGluA, the plot of 1/v vs. 1/[RuDP] curves shows a non-competitive inhibition pattern (Fig. 1). Replots of the intercepts and slopes of Fig. 1 vs. (6-PGluA) indicate this is a linear non-competitive inhibition with K_{ii} = 298 μM and K_{is} = 44 μM (Fig. 2), according to the nomenclature and theory by Cleland (8).

With a constant concentration of RuDP at 0.5 mM and a varying $NaHCO_3$ concentration (15.8 - 0.32 mM) with the addition of 0, 25, 50, 100, and 200 μM of 6-PGluA, the plot of 1/v vs. 1/[HCO_3^-] also shows a non-competitive inhibition (Fig. 3). However, the replots of the intercepts and slopes of Fig. 3 vs. (6-PGluA) indicate a hyperbolic non-competitive inhibition (Fig. 4). Replots of 1/$slope_I$-$slope_o$ or 1/$intercept_I$-$intercept_o$ of Fig. 3

vs. 1/(6-PGluA) give $K_{ii} = K_{is} = 417$ μM (Fig. 5), according to Cleland (8).

Comparison of the inhibitory effect of 6-PGluA with the effects of other metabolites (Table I) shows that 0.5 mM 6-PGluA inhibited the activity 75%, whereas fructose-1,6-diphosphate, the next most effective inhibitor tested, inhibited only 14%. At 1.0 mM concentrations, these compounds inhibited 85% and 23% respectively. Neither gluconate nor glucose-6-phosphate significantly inhibited the activity at these concentrations. Thus 6-PGluA is a much more effective inhibitor than fructose-1,6-diphosphate, a metabolite of the reductive pentose phosphate cycle previously found to inhibit the isolated enzyme. Other compounds tested which caused less than 10% inhibition when added at 0.5 mM concentration included fructose-1-phosphate, fructose-6-phosphate, glucose-1-phosphate, glucose-6-phosphate, galactose-1-phosphate, and 3-phosphoglycerate.

The rate of CO_2 fixation and the metabolic pattern of ^{14}C incorporation by isolated spinach chloroplasts were found to be unaffected by the addition of 0.5 mM 6-PGluA to the suspending medium.

DISCUSSION

The K_{ii}'s and K_{is}'s observed in this study may be compared with reported in vivo concentrations of metabolites. These were estimated by using the saturating ^{14}C label of metabolites in Chlorella pyrenoidosa during steady-state photosynthesis in the light and during respiration in the dark to determine the gram atoms of carbon in each compound per cm^3 of packed algae cells used in making up the algal suspension (6). The arbitrary assumption was made that the metabolically active space containing the metabolites was 1/4 of the packed cell volume. This gave a RuDP concentration of 2.04 mM in the light, and a 6-PGluA concentration of 0.047 mM in the dark. The dark RuDP concentration was not reported, but the level

of RuDP in the comparable study of Pedersen et al. (14) was 1.36 mM in the light and 0.20 mM in the dark, based on the same assumptions. Thus, the several values for K_i are slightly higher, but in the worst case within an order of magnitude of the estimated concentration of 6-PGluA in the dark. It should be noted that, in the dark, the oxidative phosphate cycle is in operation in the chloroplasts (11), and CO_2 is being liberated but not consumed in the chloroplasts. Thus the K_i values obtained with 50 mM HCO_3^- (Figs. 1 and 2) are not necessarily unrealistic in terms of in vivo metabolism. However, the in vivo concentrations would include 6-PGluA in both cytoplasm and chloroplasts. In the only reported case of the appearance of 6-PGluA in isolated chloroplasts, the amount of ^{14}C label was smaller than in the in vivo experiments.

In any event, these comparisons between estimated K_i values and 6-PGluA concentration in the dark in vivo suggest some possibility that non-competitive inhibition of RuDPCase by 6-PGluA in the dark plays a role in the inactivation of carboxylation reaction required by the switch from the reductive pentose phosphate cycle during photosynthesis to the oxidative pentose phosphate cycle in the dark. The enzymes characteristic of the oxidative pentose phosphate cycle have been found to be present in previously isolated spinach chloroplasts, although there were larger amounts of such enzymes in the cytoplasm (9).

The need for a light-dark switch in metabolism of chloroplasts has been discussed elsewhere (1,2,5). Turning off the reductive cycle in the dark would conserve ATP and sugar phosphates, while turning on the oxidative cycle in the chloroplasts would produce NADPH which could be utilized for biosynthesis. Other parts of the mechanism for this switch to oxidative metabolism in the dark should include the inactivation of fructose

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1,6-diphosphatase (E.C. 3.1.3.11) and phosphoribulokinase (E.C. 2.7.1.19) and the activation of glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49).

Evidence for some of these changes can be seen upon either addition of vitamin K_5 to Chlorella pyrenoidosa in the light (11), or without additions when the light is turned off (14). It has been proposed that vitamin K_5 in its oxidized state diverts electrons from the reduction of ferredoxin and $NADP^+$, and that the resulting increased ratio of $NADP^+$/NADPH or of $ferredoxin_{ox}/ferredoxin_{red}$ activates glucose-6-phosphate dehydrogenase. Similar changes in activities occur when the light is turned off, without any additions.

Data in the present study suggest that the increased level of 6-PGluA may then further inactivate the RuDPCase, thus completely stopping the carboxylation reaction. The lack of inhibition of photosynthesizing spinach chloroplasts by 6-PGluA added to the medium is presumed to be due to lack of penetration of the limiting double membrane of the intact chloroplasts.

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Table I. Inhibition of Ribulose 1,5-Diphosphate Carboxylase by 6-Phosphogluconate and Related Compounds

Compound	^{14}C total fixed cpm	% of Control (H_2O)
Control	14,990	100
1.0 mM		
Fructose 1,6-diphosphate	11,540	77
6-Phosphogluconate	2,265	15
Glucose-6-phosphate	14,524	97
Gluconate	14,754	98
0.5 mM		
Fructose-1,6-diphosphate	12,900	86
6-Phosphogluconate	3,763	25
Glucose-6-phosphate	14,775	99
Gluconate	14,716	98

Assay as shown in Methods. Protein, 10.0 μg; RuDP, 0.5 mM; $NaH^{14}CO_3$, 50 mM (0.26 μc/μm); incubation time, 10 min.

FIGURE CAPTIONS

Fig. 1. Inhibition of RuDPCase by 6-PGluA. Concentrations of 6-PGluA are indicated in the figure. Protein, 5.0 μg; $NaH^{14}CO_3$, 50 mM (2.6 μc/μm); incubation time, 10 min.

Fig. 2. Replots of intercepts and slopes of Fig. 1 vs. 6-PGluA concentration.

Fig. 3. Inhibition of RuDPCase by 6-PGluA. Concentrations of 6-PGluA are indicated in the figure. Protein, 5.0 μg; RuDP, 0.5 mM; incubation time, 5 min; $NaH^{14}CO_3$, 13.9 μc/μm.

Fig. 4. Replots of intercepts and slopes of Fig. 3 vs. 6-PGluA concentration.

Fig. 5. Replots of $1/(\text{intercept}_I - \text{intercept}_0)$ and $1/(\text{slope}_I - \text{slope}_0)$ of Fig. 3 vs. 1/(6-PGluA concentration).

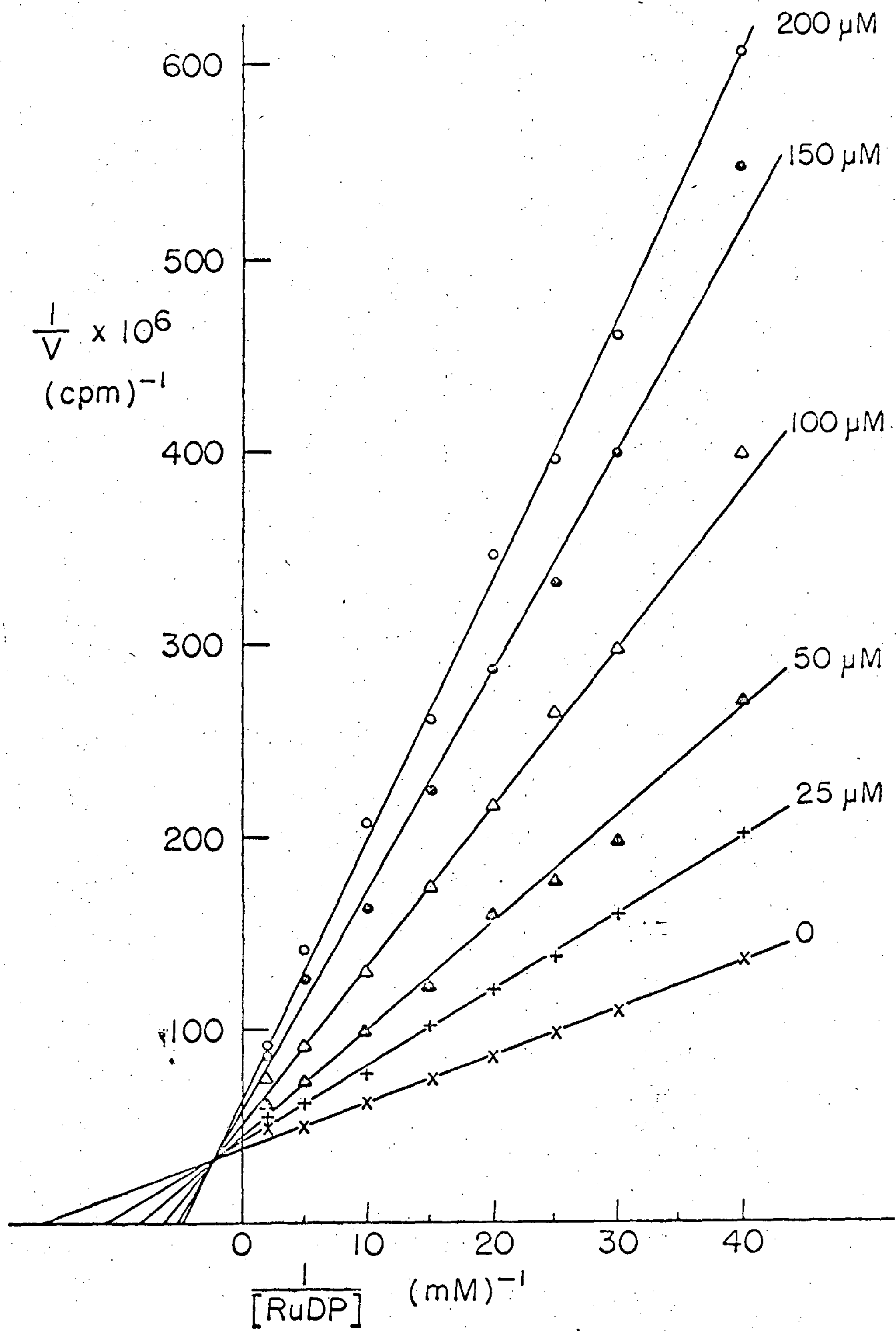

Fig. 1

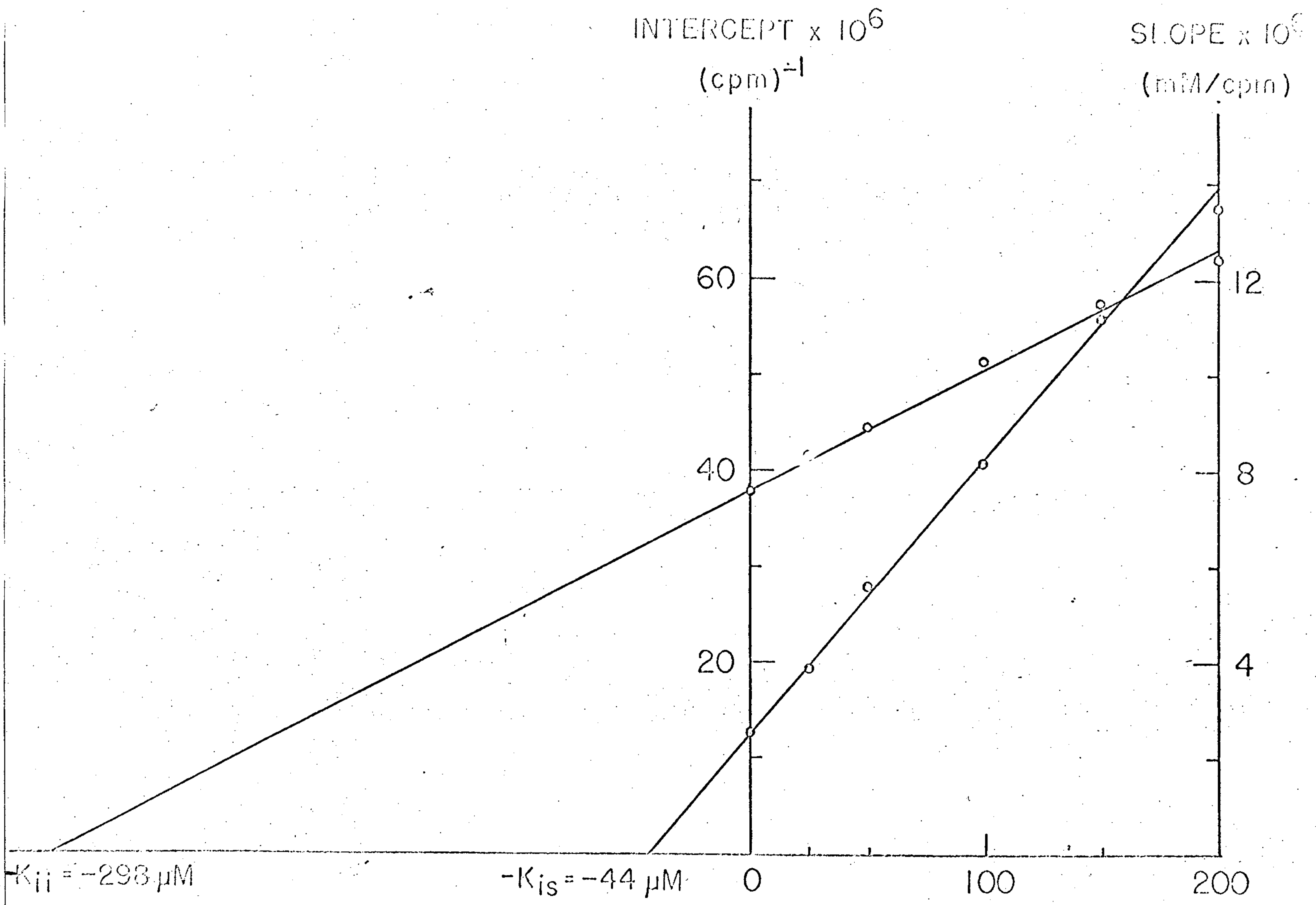

Fig. 2

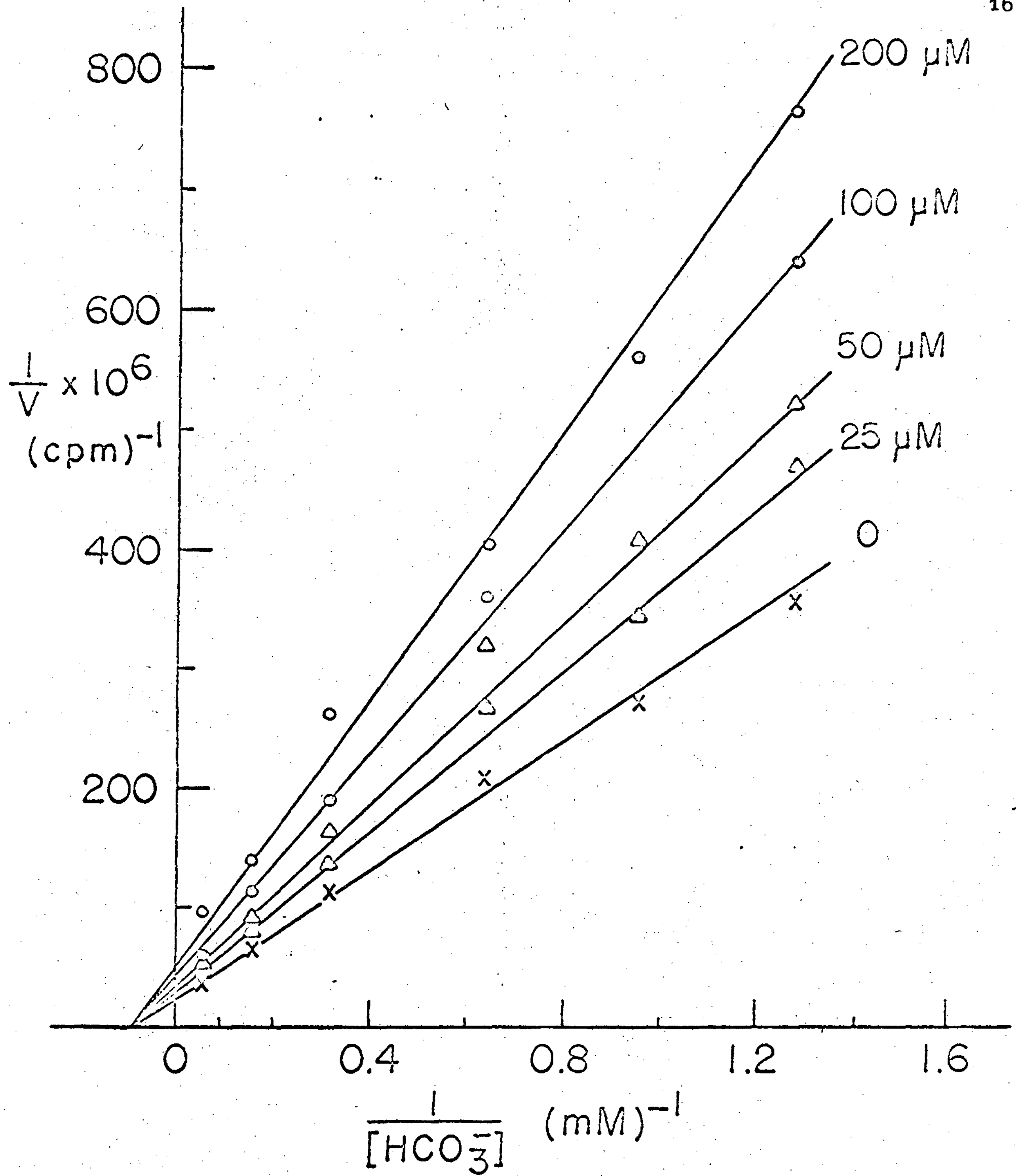

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Fig. 3

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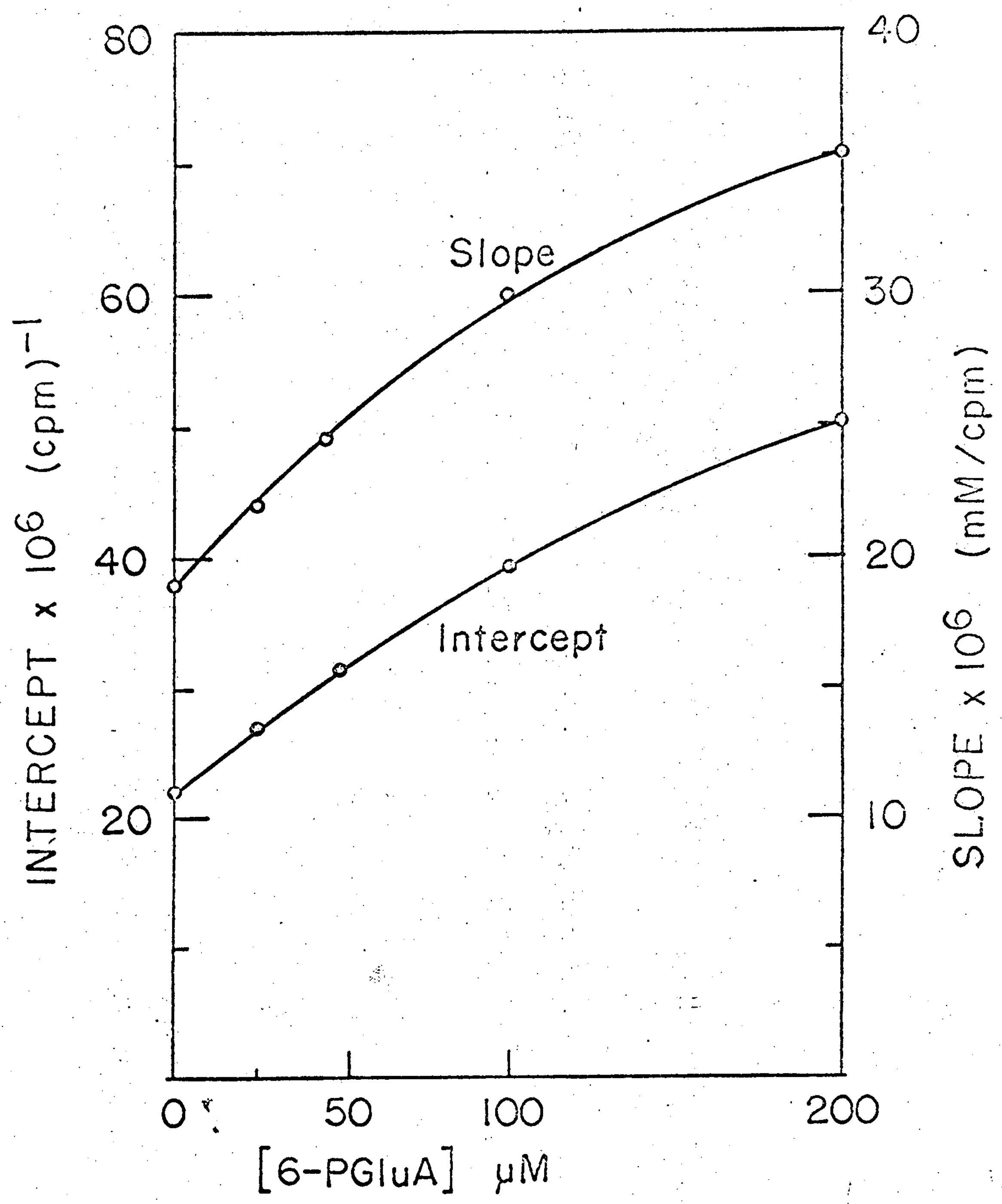

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Fig. 4

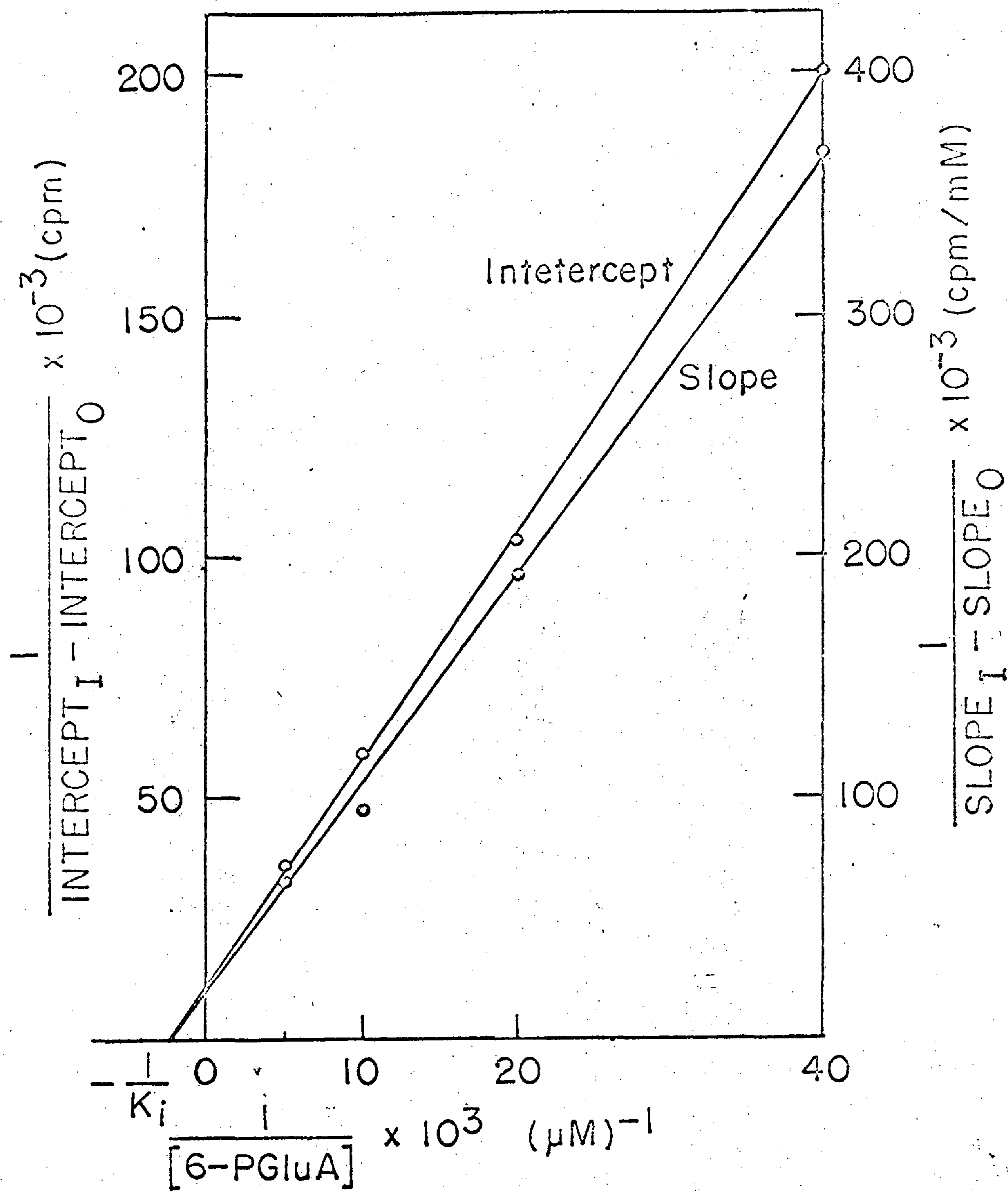

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Fig. 5

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