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INHIBITION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE BY RIBULOSE-1,5-BISPHOSPHATE EPIMERIZATION AND DEGRADATION PRODUCTS

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Summary: Xylulose-1,5-bisphosphate in preparations of ribulose-1,5-bisphosphate (ribulose-P_2) arises from non-enzymic epimerization and inhibits the enzyme. Another inhibitor, a diketo degradation product from ribulose-P_2, is also present. Both compounds simulate the substrate inhibition of ribulose-P_2 carboxylase/oxygenase previously reported for ribulose-P_2. Freshly prepared ribulose-P_2 had little inhibitory activity. The instability of ribulose-P_2 may be one reason for a high level of ribulose-P_2 carboxylase in chloroplasts where the molarity of active sites exceeds that of ribulose-P_2. Because the K_D of the enzyme/substrate complex is ≤ 1 μM, all ribulose-P_2 generated *in situ* may be stored as this complex to prevent decomposition.

The hysteretic substrate inhibition of ribulose-P_2 carboxylase/oxygenase (EC 4.1.1.39) by ribulose-P_2 (1-4) has been attributed to an allosteric binding site (3) or competitive binding for ribulose-P_2 and CO_2 (5), either of which could produce a conformational change (6,7). However purified carboxylase cannot be fully reactivated upon addition of Mg^{2+} and bicarbonate once it has been preincubated with ribulose-P_2 (3). Partial inactivation does not occur for enzyme activity in whole chloroplasts where ribulose-P_2 may accumulate in the absence of CO_2 to a 16 fold molar excess over the carboxylase binding sites, but the carboxylase activity is fully restored within 10 min after addition of bicarbonate (8). Much of the substrate inhibitory effect and the difference between the isolated enzyme and the chloroplasts can be explained by 2 impurities which we find in ribulose-P_2 preparations used for *in vitro* assays. One is xylulose-P_2, an inhibitor of the enzyme (9), and the other apparently is a diketo compound, most likely 1-deoxy-D-*glycero*-2,3-pentodiulose-5-phosphate. Other α-dicarbonyl compounds are also inhibitors of this enzyme (10,11). The carboxylase inhibition by newly prepared ribulose-P_2 was small, as if there had been insufficient time for inhibitor formation.

Abbreviations: ribulose-P_2,ribulose-1,5-bisphosphate; deoxypentodiulose-5-P, 1-deoxy-D-*glycero*-2,3-pentodiulose-5-phosphate; xylulose-P_2, xylulose-1,5-bisphosphate.

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MATERIALS AND METHODS

Ribulose-P_2 carboxylase/oxygenase from spinach leaves was prepared as described elsewhere (12). Ribulose-P_2 was prepared enzymatically from ribose-5-P (13). Xylulose-P_2 was synthesized with rabbit muscle aldolase from glycolaldehyde-P and dihydroxyacetone-P (14). Proof of structure and purification have been described (9). (1-^{13}C) xylulose-P_2 was synthesized from (1-^{13}C) fructose-1,6-P_2, which was a gift from A. S. Serianni of Mich. State Univ., and glycolaldehyde-P by aldolase. $NaH^{14}CO_3$ and NaB^3H_4 were from Amersham-Searle; other chemicals were from Sigma Chemical Co. and were used without further purification. Ribulose-P_2 carboxylase activity was determined using a radiometric assay (15). The enzyme was incubated at 2mg/ml for at least 1 h in the assay buffer composed of 0.1 M N,N-bis(2-hydroxyethyl)glycine (Sigma, "Bicine") at pH 8.0 and 30°, 10 mM $MgCl_2$, 0.2 mM Na_2-EDTA, and then made to 10 mM $NaHCO_3$ and kept for 30 min at 30°. Aliquots from this stock solution were equilibrated in the assay buffer at 20 mM $NaH^{14}CO_3$ (0.16 Ci/mol) and a final protein concentration of 80 µg/ml (0.14µM). The reaction was initiated by addition of ribulose-P_2 to a final concentration of 0.5 mM and was run for 20 to 60 sec, stopped by acid and the fixed ^{14}C counted as described elsewhere (5). Under these conditions the specific activity of the enzyme preparations was 1.0 to 1.4 µmol CO_2 fixed per min per mg protein. Protein determinations were performed according to a modified Lowry procedure (16). Carbon-13 n.m.r. spectra were obtained with a Bruker WP-60, 15.08 MHz Fourier-transform spectrometer equipped with quadrature detection. The spectrometer was locked to the resonance of D_2O in a capillary. Chemical shifts are given relative to external tetramethylsilane (0 ppm) and are accurate to within 0.1 ppm.

RESULTS AND DISSCUSSION

Evidence for Inhibitors in Ribulose-P_2 Preparations- Early reports (1,2) of ribulose-P_2 inhibition of the carboxylase indicated that saturating or higher concentrations had to be used. In our experience the extent of inhibition was not reproducible. Different batches of ribulose-P_2 solutions at apparently identical concentrations, as judged by phosphate (17), carbohydrate (18), and enzymatic analyses with an excess of ribulose-P_2 carboxylase and $NaH^{14}CO_3$ of a known specific activity gave different initial rates of carboxylation (data not shown). These differences became insignificant when ribulose-P_2 concentrations below 5 times the K_m were used in the assay. At these low concentrations, short reaction times of 10-40 sec are required, and the inhibition, which is time-dependent, is not significant. Nevertheless the inhibitory effect from low concentrations of ribulose-P_2 can be demonstrated (Fig. 1). After complete utilization of a known amount of substrate, severe inhibition occurred during a second reaction period initiated by adding ribulose-P_2 to the same concentration as in the first stage. Several sources of inhibition could be ruled out. Product inhibition under these conditions was negligible; 1 mM 3-P-glycerate had no effect and 0.5 mM P-gycolate caused a 4% inhibition of the inital rate. Also mono- and divalent cations (e.g.

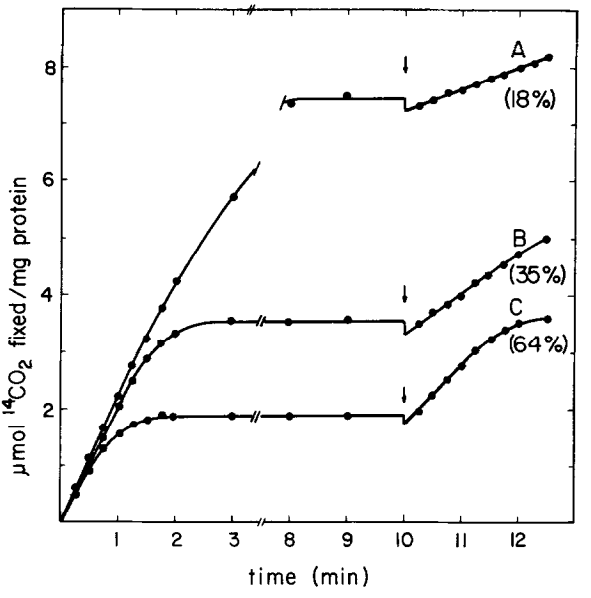


Figure 1. Progress curve of ribulose-P_2 carboxylase reactions with (A) 0.5, (B) 0.25, (C) 0.125 mM ribulose-P_2. 0.3 ml of a ribulose-P solution was added to 7.2 ml of the assay buffer containing the enzyme at 80 µg/ml and $NaH^{14}CO_3$ at 20 mM final concentration. Ten aliquots of 0.25 ml were withdrawn at indicated times and added to 0.2 ml 2 N HCl. The arrows indicate initiation of the second progress curve by addition of a similar amount of ribulose-P_2 (0.2 ml into 4.8 ml) as in the first reaction. A corresponding volume was removed prior to addition of ribulose-P_2 in the second run. Thus, the zero point is 4% lower and a maximum of 96% of the first initial rate in the second progress curve would represent no inhibition. The values in parentheses represent the observed percent inhibition of the first initial velocity.

Ba^{2+}, Ca^{2+}, Sr^{2+}) as well as $NaVO_3$ (19) were not inhibitory up to 10 mM.

Stability of Ribulose-P_2 - Treatment of ribulose-P_2 solutions at 30° and pH 11 increased the inhibition to a maximum of about 70%, presumably from reaching an inhibitor/substrate equilibrium. Prolonged base treatment or more elevated temperature caused more rapid substrate loss and appearance of orthophosphate, but no further inhibition (Fig. 2). Loss of ribulose-P_2 during alkaline treatment correlated with the formation of inorganic phosphate (20) (Fig. 3). When ribulose-P_2 was no longer detectable enzymatically, approximately 50% of its total phosphate had been liberated. No ribulose-P_2 was regenerated by incubating samples, taken during the time course of alkaline treatment, with a mixture of phosphoriboisomerase, phosphoribulokinase, and ATP; therefore neither ribulose-5-P nor ribose-5-P were formed during the alkaline treatment.

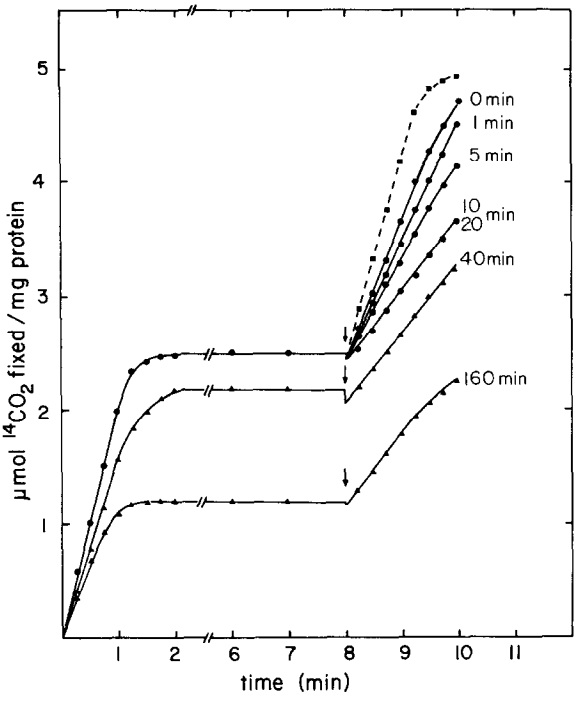


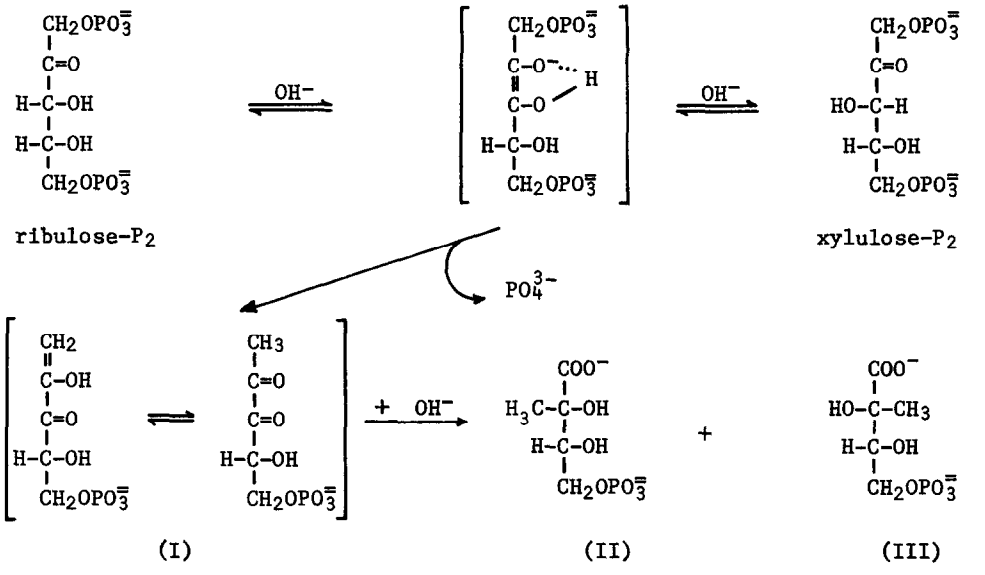
Figure 2. Time course of the ribulose-P_2 carboxylase reaction with 0.2 mM ribulose-P_2. Ribulose-P_2, 5 mM, was incubated at pH 11 and 30° for 1, 5, 10, and 20 min as indicated. The pH was then adjusted to 8.0 and an aliquot was used to run two consecutive progress curves (●——●) as described in the legend to Fig. 1. Another sample was incubated for 40 and 160 min at pH 11 and 40° and used for the same experiment (▲——▲). When ribulose-P_2 was generated from ribose-5-P enzymatically, and used directly the initial rate in the second progress curve reached 88% of the initial rate, which because of the dilution was over 90% of the maximum expected (■---■).

Even at pH 8.3 and 30° (the enzyme assay conditions) loss of ribulose-P_2 amounted to 1.25% per hour (data not shown).

Ribulose-P_2 solutions essentially free of inhibitor were obtained in a coupled assay in which ribulose-P_2 was generated from ribose-5-P or ribulose-5-P with phosphoriboisomerase, phosphoribulokinase, ATP and used immediately (■----■ in Fig. 2). In this case there was little effect from inhibitor accumulation during the second cycle of the repeat assay.

Mechanism of Degradation and Identification of Inhibitors - Ribulose-P_2 is usually prepared enzymatically from ribose-5-P and stored as the barium salt which is unstable even at 4° (13). The usual way to prepare the potassium or sodium salt is by mixing the barium salt with Dowex 50/H^+, filtering, and neutralizing with 6 N KOH. During neutralization, epimerization at C-3 induced by hydroxyl ions would form xylulose-P_2, which is a potent inhibitor

of ribulose-P_2 carboxylase (9). Alkaline treatment may also lead to phosphate elimination at C_1 from the enediol intermediate formed from either ribulose-P_2 or xylulose-P_2, to yield deoxypentodiulose-5-P. This unstable compound (I) should undergo a rearrangement to form (II) and (III).



(I) 1-deoxy-D-*glycero*-2,3-pentodiulose-5-phosphate
(II) 2-C-methyl-D-*erythro*-tetronic acid 4-phosphate
(III) 2-C-methyl-D-*threo*-tetronic acid 4-phosphate

Evidence for a phosphate elimination mechanism was the appearance of ortho phosphate (Fig. 3) and a time course of degradation of (1-^{13}C) xylulose-P_2 at pH 11 and 40° (Fig. 4). The starting compound was 91% ^{13}C enriched at C-1. As the reaction proceeded, the area under the doublet, centered about 69.4 ppm, decreased as methyl resonances at 24.0 and 23.4 ppm increased, reaching their maximum values when no further xylulose-P_2 remained. The methyl resonances are assumed to arise from the two major degradation products of the proposed diketo compound via the described mechanism. Similar mechanisms for non-phosphorylated sugars have been proposed (21).

To identify the inhibitors, samples of ribulose-P_2 were reduced with $NaBH_4$, pH 7, and treated with acid phosphatase. The products were converted to the pentaacetyl pentitols and subjected to gas liquid chromatography (22). The major peaks were identified as ribitol and arabinitol, present in approximately the same quantities. These peaks were followed by a small xylitol peak. After treatment with NaB^3H_4 and phosphate ester cleavage, the tritiated reduction products were separated by thin layer chromatography on

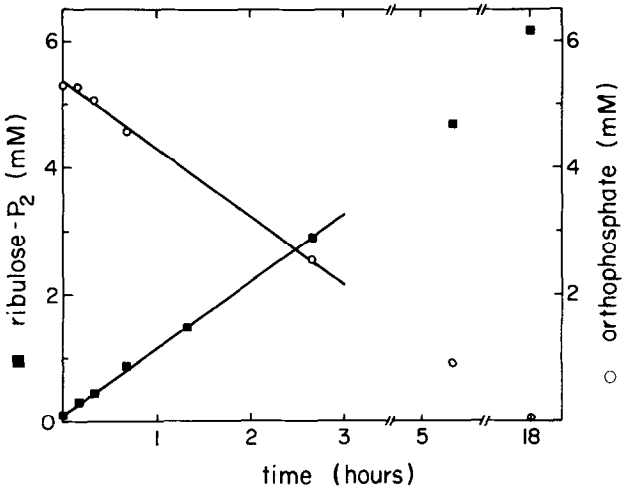


Figure 3. Loss of ribulose-P_2 at pH 11 and 40°, and formation of inorganic phosphate as a function of time. The ribulose-P_2 content was measured enzymatically with a pure preparation of ribulose-P carboxylase and $NaH^{14}CO_3$ of a known specific activity.

borate-impregnated silica gel plates. The two major labeled spots corresponded to arabinitol and ribitol, and a minor spot corresponded to xylitol. These compounds were recrystallized with the corresponding unlabeled alcohols to constant specific activity. The presence of approximately 1% xylulose-P_2 in preparations of ribulose-P_2 could account for the inhibitory phenomenon of ribulose-P_2 on carboxylase activity. That xylulose-P_2 solutions may also epimerize to ribulose-P_2 was demonstrated by assaying them with the carboxylase. 3-P-Glycerate was formed, but the reaction reached completion with most of the xylulose-P_2 remaining. Thus it is unlikely that xylulose-P_2 was acting as a substrate for ribulose-P_2 carboxylase.

The β-elimination product from ribulose-P_2 has not been characterized unequivocally but is proposed to be 1-deoxy-D- *glycero*- 2,3-pentodiulose-5-phosphate for the following reasons: (a) β-elimination is kinetically favored over hydrolysis under alkaline conditions. The deoxypentodiulose-5-P would not accumulate and its further degradation to the end product shown in the scheme would explain why the inhibitory effect from alkaline treatment did not further increase. (b) Incubation of ribulose-P_2 preparations with o-penylenediamine to complex the dicarbonyl compound reduced inhibition of the enzyme by the ribulose-P_2 preparation. (c) Dicarbonyl derivatives of carbohydrates are sensitive to oxygen under alkaline conditions and undergo rapid degradation and rearrangement (23). (d) Deoxypentodiulose-5-P belongs to a class of reagents specific for arginyl residues in enzymes (24), and it has been shown that

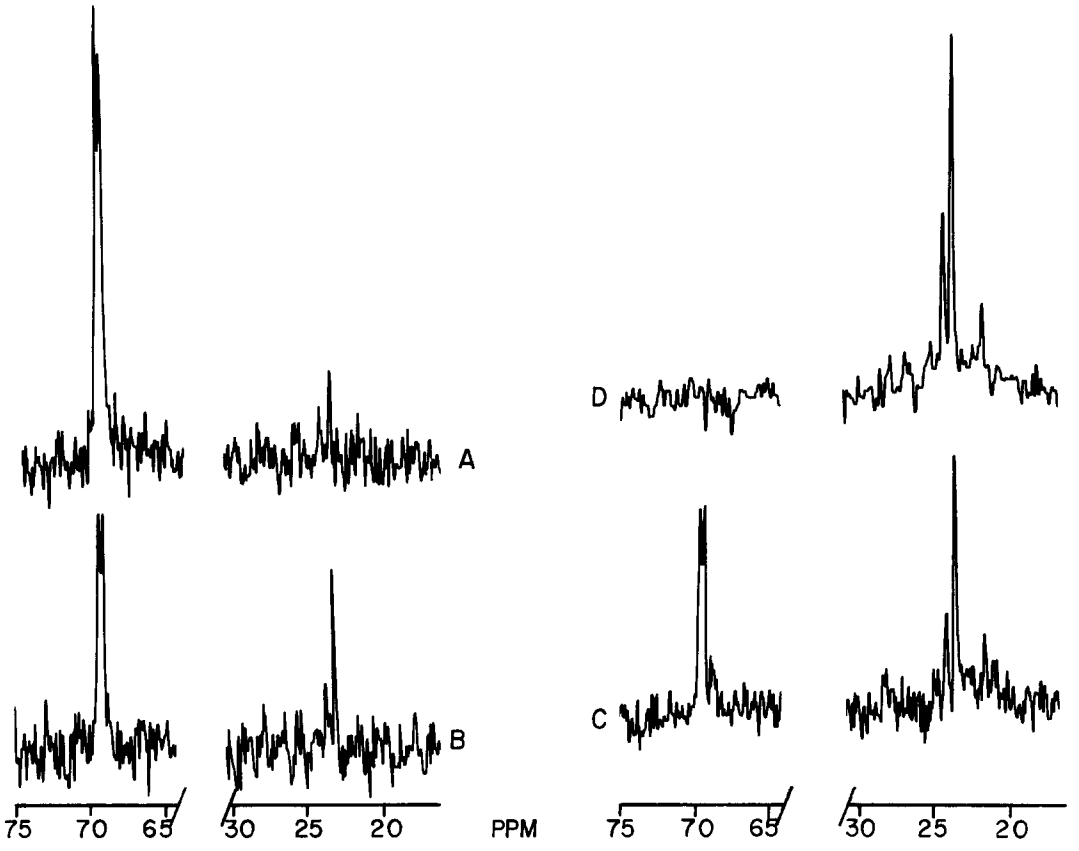


Figure 4. Time course of degradation of (1-^{13}C) xylulose-P_2 at pH 11 and 40°. (A) 4-16 min, (B) 47-58 min, (C) 125-160 min, (D) 245-295 min. The doublet centered about 69.4 ppm is due to coupling of the ^{31}P nucleus with the ^{13}C nucleus at C-1 of (1-^{13}C) xylulose-P_2 (J_{C-P} = 4.4 Hz).

ribulose-P_2 carboxylase is inhibited by such compounds, e.g. phenylglyoxal and 2,3-butanedione (10,11). (e) Dicarbonyl compounds have characteristic absorption spectra which are pH dependent and include peaks in the region 400-460 nm (25). Such spectral properties have been observed with ribulose-P_2 solutions (data not shown).

Physiological Implications - The rapid formation of inhibitors in solutions of ribulose-P_2 is important with respect to kinetic studies and the carboxylase assay. Recognition of these inhibitors may also be the key to some yet unsolved questions concerning the enzyme *in vivo*. The average concentration of ribulose-P_2 in chloroplasts is less than the total concentration of binding sites of ribulose-P_2 carboxylase (26). Therefore, ribulose-P_2 probably never occurs in free solution in the chloroplasts (R.G. Jensen, personal

communication). Conditions for chemical epimerization or β-elimination of ribulose-P_2 could occur in the chloroplasts, for example during the day when temperatures may reach 40° and the pH over 8. The presence of a large excess of protein (ribulose-P_2 carboxylase) would account for the binding of almost all the ribulose-P_2 as soon as it is formed by phosphoribulokinase, as the dissociation constant for the enzyme/ribulose-P_2 complex is ≤ 1 μM (5,27).

If xylulose-P_2 and deoxypentodiulose-5-P were formed *in situ* there would be sufficient ribulose-P_2 carboxylase to bind these inhibitors without any loss in rate of the whole photosynthetic carbon cycle. Bahr and Jensen (28) have estimated that in the chloroplast only 40 to 60% of the carboxylase is functioning at any given time; the rest may be a storage reserve or trap for ribulose-P_2 or these inhibitory products, respectively. The deoxypentodiulose-5-P seems too unstable to accumulate in chloroplasts, so that the removal of xylulose-P_2 would be of more serious concern. A phosphatase catalyzing the conversion of xylulose-P_2 to xylulose-5-P has not been reported, but such an enzyme would effectively recycle the inhibitor through the photosynthetic carbon cylce.

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