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Interaction of Ribulosebisphosphate Carboxylase/Oxygenase with Transition-State Analogues†

John Pierce,* N. E. Tolbert, and Robert Barker‡

ABSTRACT: 2-*C*-Carboxy-D-ribitol 1,5-bisphosphate and 2-*C*-carboxy-D-arabinitol 1,5-bisphosphate have been synthesized, purified, and characterized. In the presence of Mg^{2+}, 2-*C*-carboxy-D-arabinitol 1,5-bisphosphate binds to ribulose-1,5-bisphosphate carboxylase/oxygenase by a two-step mechanism. The first, rapid step is similar to the binding of ribulose 1,5-bisphosphate or its structural analogues. The second step is a slower process ($k = 0.04\ s^{-1}$) and accounts for the tighter binding of 2-*C*-carboxy-D-arabinitol 1,5-bisphosphate ($K_d \lesssim 10^{-11}$ M) than of 2-*C*-carboxy-D-ribitol 1,5-bisphosphate ($K_d = 1.5 \times 10^{-6}$ M). Both carboxypentitol bisphosphates exhibit competitive inhibition with respect to ribulose 1,5-bisphosphate. 2-*C*-(Hydroxymethyl)-D-ribitol 1,5-bisphosphate and 2-*C*-(hydroxymethyl)-D-arabinitol 1,5-bisphosphate were also synthesized; both are competitive inhibitors with respect to ribulose 1,5-bisphosphate with $K_i = 8.0 \times 10^{-5}$ M and $K_i = 5.0 \times 10^{-6}$ M, respectively. Thus, the carboxyl group of 2-*C*-carboxy-D-arabinitol 1,5-bisphosphate is necessary for maximal interaction with the enzyme. Additionally, Mg^{2+} is essential for the tight binding of 2-*C*-carboxy-D-arabinitol 1,5-bisphosphate. A model for catalysis of ribulose 1,5-bisphosphate carboxylation is discussed which includes a functional role for Mg^{2+} in the stabilization of the intermediate 2-*C*-carboxy-3-keto-D-arabinitol 1,5-bisphosphate. Mechanistic implications that arise from the stereochemistry of this intermediate are also discussed.

An essential reaction for CO_2 fixation in all photosynthetic organisms is catalyzed by ribulose-P_2 carboxylase/oxygenase.[1] Interest in this enzyme has been stimulated by the discovery of the oxygenase reaction (Ogren & Bowes, 1971; Andrews et al., 1973) and its role in the glycolate pathway of photorespiration (Tolbert & Ryan, 1976).

An outline for the chemical mechanism of the carboxylation reaction was predicted by Calvin (1954) even before the discovery of the enzyme (Scheme I). The initial step is the enolization of ribulose-P_2 (**1**) to form **2**, which is attacked by CO_2 to form a 2-*C*-carboxy-3-ketopentitol bisphosphate, **3**. Addition of H_2O across the bond at C-2 and C-3 of **3** yields two molecules of D-glycerate-3-P (Fiedler et al., 1967; Cooper

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[1] Abbreviations used: ribulose-P_2, D-*erythro*-pentulose 1,5-bisphosphate; P, phosphate; P_2, 1,5-bisphosphate; ^{13}C NMR, ^{13}C nuclear magnetic resonance spectroscopy; carboxypentitol-P_2, an unresolved mixture of 2-*C*-(phosphohydroxymethyl)-D-ribonic acid 5-phosphate and 2-*C*-(phosphohydroxymethyl)-D-arabinonic acid 5-phosphate.

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Scheme I

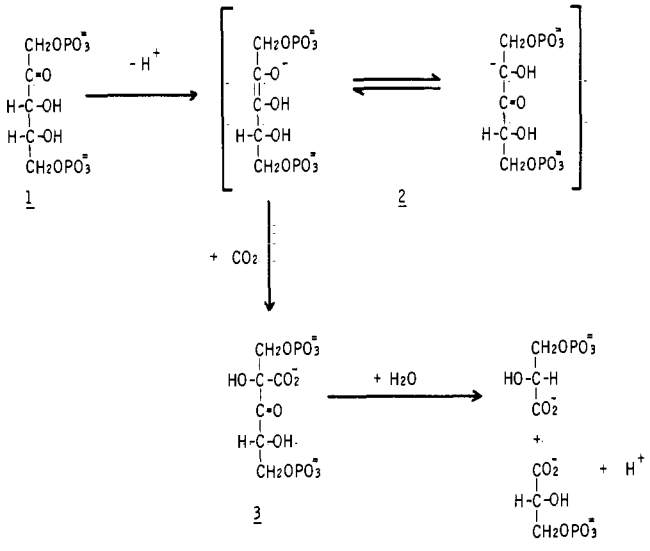
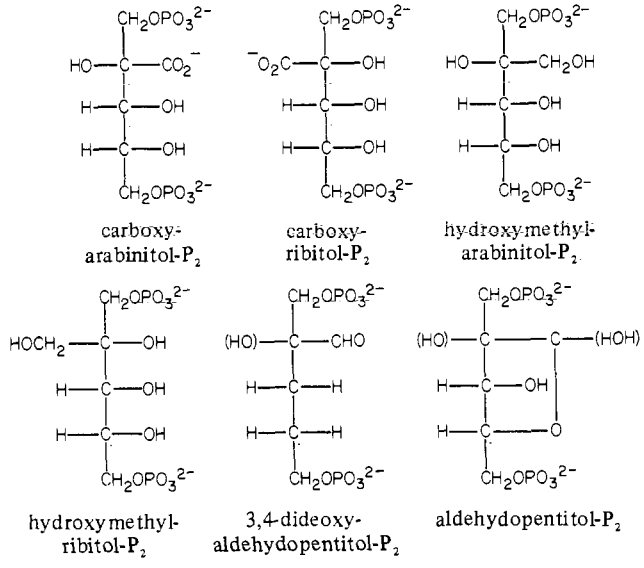


Chart I



et al., 1969; Müllhofer & Rose, 1965; Pierce et al., 1980; Weissbach et al., 1956; Jakoby et al., 1956). In addition, the oxygen atoms at C-2 and C-3 of ribulose-P_2 are retained in the products of the carboxylation reaction, ruling out the intermediacy of eneamine or dithioacetal derivatives in the reaction (Sue & Knowles, 1978; Lorimer, 1978). An analogous mechanism for the oxygenase reaction accounts for all known facts regarding the formation of D-glycerate-3-P and glycolate-2-P from ribulose-P_2 and molecular oxygen (Lorimer et al., 1973).

Attempts have been made to synthesize the β-keto acid intermediate **3** (Siegel & Lane, 1973), and quenching from the steady state of the carboxylase reaction gives a compound with the expected properties of **3** (Sjödin & Vestermark, 1973). The finding that a stable analogue of **3**, carboxypentitol-P_2, is a competitive inhibitor with respect to ribulose-P_2 has been considered proof for the existence of intermediate **3** in the reaction (Wishnick et al., 1970; Siegel & Lane, 1972, 1973). These studies utilized a mixture of carboxyribitol-P_2 and carboxyarabinitol-P_2 that resulted from cyanide addition to ribulose-P_2. Although the studies were performed prior to 1976 when the requirement for preincubation with Mg^{2+} and CO_2 for maximal enzyme activity was clearly established (Lorimer et al., 1976; Badger & Lorimer, 1976), carboxypentitol-P_2 was shown to require Mg^{2+} for maximal inhibition. The enzyme–Mg^{2+}–carboxypentitol-P_2 complex was reported to have $K_d < 10^{-8}$ M (Siegel & Lane, 1972). These results were suggested to imply a role for Mg^{2+} in the stabilization of intermediate **3**.

In view of our interest in the structure of the active site of ribulose-P_2 carboxylase/oxygenase and in the use of the carboxypentitol-P_2 compounds by a number of workers to probe the active site of the enzyme (Miziorko & Mildvan, 1974; Ryan & Tolbert, 1975; Schloss et al., 1978; Miziorko, 1979), it was desirable to further characterize these compounds and their interaction with the enzyme. Consequently, structural analogues (see Chart I) of the reaction intermediate **3** in the carboxylation of ribulose-P_2 were synthesized and their interactions with ribulose-P_2 carboxylase/oxygenase were examined.

Materials and Methods

Compounds and Enzymes. Sodium [^{14}C]bicarbonate ($NaH^{14}CO_3$) was from Amersham/Searle, and potassium [^{14}C]cyanide ($K^{14}CN$) was from New England Nuclear. Potassium [^{13}C]cyanide ($K^{13}CN$) was supplied by the Los Alamos Scientific Laboratory, University of California, Los Alamos, NM. Other chemicals were reagent grade or better and were used without further purification. Biochemicals were purchased from Sigma Chemical Co.

Ribulose-P_2 carboxylase/oxygenase (EC 4.1.1.39) was purified from spinach (Ryan & Tolbert, 1975); the enzyme was stored as a precipitate in 50% saturated $(NH_4)_2SO_4$ at 4 °C. Ribulose-P_2 was prepared enzymatically from D-ribose-5-P (Horecker et al., 1956). Sugar phosphates with ^{13}C enrichment at various carbon atoms were prepared as described previously (Serianni et al., 1979). 1,5-Dihydroxypentan-2-one-P_2 was prepared by the procedure of Hartman & Barker (1965).

Instrumentation. ^{13}C NMR spectra were obtained at 15.08 MHz with a Bruker WP-60 Fourier-transform spectrometer equipped with quadrature detection. Spectra were obtained with 4000 spectral points. The spectrometer was locked to the resonance of [^{2}H]H_2O in a capillary insert. Chemical shifts are given relative to external tetramethylsilane and are accurate to within 0.1 ppm.

Mass spectra were obtained from a combined gas chromatograph–mass spectrometer utilizing an LKB spectrometer equipped with a computer-assisted data acquisition system. Compounds were chromatographed at 150 °C on a 2.0-m, small bore column containing 3% OV-17.

Radioactivity measurements were obtained either on a Packard Tri-Carb scintillation counter or on a LKB LS-100 scintillation counter using a toluene–Triton X-100 mixture.

Carboxyribitol-P_2 [2-C-(Phosphohydroxymethyl)-D-arabinonic Acid 5-P] and Carboxyarabinitol-P_2 [2-C-(Phosphohydroxymethyl)-D-ribonic Acid 5-P]. A 50–100 mM solution of ribulose-P_2 (with or without ^{13}C and/or ^{14}C enrichment) at pH 8.5 was added to a 0.5 M solution of KCN (with or without ^{13}C and/or ^{14}C enrichment) so that the final ratio of cyanide to ribulose-P_2 was 1.1. The resulting nitriles were allowed to hydrolyze to the acid salts at 22 °C for 48 h. The solution was treated with excess Dowex 50(H^+), filtered, concentrated to dryness in vacuo at 30 °C, and desiccated in vacuo (≤0.1 mmHg) at room temperature over $MgClO_4$ for 24 h. The lactones were dissolved in water and quickly adjusted to pH 5.5 with 1 M NaOH. For small amounts of material (≤1 mmol of total P), the solution was added to a 42 × 2 cm Dowex 1(Cl^-) (8% cross-linked; 200–400 mesh) column and eluted with a 4-L, linear gradient of 0.0–0.4

M LiCl in 3 mM HCl at a rate of 0.5–0.8 mL/min. For larger amounts of material, the solution was added to a 49 × 3.3 cm Dowex 1(Cl^-) column and eluted with a 6-L, linear gradient of 0.0–0.4 M LiCl in 3 mM HCl at a rate of 0.6–1.0 mL/min. Fractions (15 mL) were collected and assayed for total phosphate (LeLoir & Cardini, 1957) or radioactivity. Peak fractions were pooled, neutralized to pH 8.0 with 1 M LiOH, and concentrated in vacuo to approximately 100 mL. The addition of a threefold molar excess of barium acetate, followed by the addition of ethanol to a final concentration of 50% (v/v), precipitated the bisphosphates as their barium salts. After at least 1 h at −20 °C, the precipitate was collected by centrifugation and twice washed with 95% ethanol. The products were dissolved in water by the addition of excess Dowex 50(H^+), filtered, adjusted to pH 6.5 with 1 M NaOH, and stored at −20 °C until use. Recovery of radioactivity was usually about 90% of that applied to the Dowex 1(Cl^-) column. The compounds were estimated to be at least 95% pure by ^{13}C NMR, phosphate, and gas chromatographic analyses. Prior to use with ribulose-P_2 carboxylase/oxygenase, the compounds were incubated at pH 9.0 for 24 h at room temperature to ensure that no lactone forms were present.

Hydroxymethylribitol-P_2 [2-C-(Hydroxymethyl)-D-ribitol-P_2] and Hydroxymethylarabinitol-P_2 [2-C-(Hydroxymethyl)-D-arabinitol-P_2]. The γ-lactones of [2′-^{13}C]-carboxyribitol-P_2[2] and [2′-^{13}C]carboxyarabinitol-P_2 were prepared from their salts by Dowex 50(H^+) treatment and desiccation as described above. A 20-mL aliquot of a 0.5 M solution of $NaBH_4$ in 0.4 M Na_2CO_3 was added to 50 μmol of the appropriate lactone. The reduction was terminated after 24 h at room temperature by the addition of 2 mL of glacial acetic acid. Dowex 50(H^+) treatment, evaporation to dryness in vacuo, and repeated concentration from anhydrous methanol removed excess borate. The products were purified by column chromatography on Dowex 1(Cl^-) as described above. The hydroxymethyl derivatives eluted at approximately 0.1 M LiCl, followed by the carboxy derivatives; products were collected as their barium salts, converted to the sodium salts, and stored at −20 °C as described for the carboxypentitol bisphosphates. [2′-^{13}C]Hydroxymethylribitol-P_2 and [2′-^{13}C]hydroxymethylarabinitol-P_2 were characterized by ^{13}C NMR analysis. The ^{13}C NMR chemical shifts of the enriched carbons were 62.8 ppm for the ribo derivative and 63.5 ppm for the arabino derivative. The purity of these compounds was estimated to be at least 95% by ^{13}C NMR.

Aldehydopentitol-P_2 [Equimolar Mixture of 2-C-(Phosphohydroxymethyl)-D-ribose-5-P and 2-C-(Phosphohydroxymethyl)-D-arabinose-5-P]. An equimolar solution of $K^{13}CN$ (containing $K^{14}CN$) and ribulose-P_2 was kept at pH 7.5 and 4 °C for 45 min. The resulting cyanohydrins were reduced on palladium–barium sulfate (5%) (Serianni et al., 1979). Analysis by ^{13}C NMR revealed the presence of glycosylamine derivatives after the reduction. The mixture of glycosylamines was converted to the aldoses by incubation at pH 8.4 for 12 h at room temperature. Aldehydopentitol-P_2 was purified on a 51 × 2.2 cm Dowex 1(formate) column by using a 4-L, linear sodium formate gradient (0.2–1.3 M, pH 6.2). The aldoses eluted at approximately 0.5 M sodium formate. After treatment of the pooled fractions with excess Dowex 50(H^+), formic acid was removed by continuous ether extraction for 24 h at 4 °C, and the compounds were stored as their sodium salts at −20 °C until use. Analysis by ^{13}C NMR revealed that aldehydopentitol-P_2 exists in solution as the cyclic, anomeric furanoses (chemical shifts: 102.9 ppm, 43%; 102.2 ppm, 8%; 98.3 ppm, 49%) with no detectable (<4%) *gem*-diol or free aldehydo forms. The purity of these compounds was estimated to be at least 95% by ^{13}C NMR.

Aldehydo-3,4-dideoxypentitol-P_2 [(RS)-2-C-(Phosphohydroxymethyl)-3,4-dideoxypentose-5-P]. An equimolar solution of $K^{13}CN$ (containing $K^{14}CN$) and 1,5-dihydroxypentan-2-one-P_2 was kept at pH 8.0 and 22 °C for 30 min. The resulting cyanohydrins were converted to the aldoses by catalytic reduction on palladium–barium sulfate (5%) (Serianni et al., 1979). Aldehydo-3,4-dideoxypentitol-P_2 was purified by Dowex 1(Cl^-) chromatography (Byrne & Lardy, 1954); it was isolated as its barium salt and stored at −20 °C in the manner described for the carboxypentitol bisphosphates. Analysis by ^{13}C NMR revealed the presence of 70% *gem*-diol (93.2 ppm) and 30% free aldehyde (207.6 ppm) forms at 30 °C and pH 7.0. The purity of these compounds was estimated to be at least 90% by ^{13}C NMR.

Characterization of Carboxyribitol-P_2 and Carboxyarabinitol-P_2. Either carboxyribitol-P_2 γ-lactone or carboxyarabinitol-P_2 γ-lactone was treated with acid phosphatase (potato) at pH 4.5, deproteinized (Somogyi, 1945), applied to a 20-mL column of Dowex 1(Cl^-), and eluted with water. The dephosphorylated γ-lactones (carboxypentitol γ-lactones) were concentrated to dryness and desiccated in vacuo for 48 h. The compounds were derivatized in pyridine with *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane for gas chromatography–mass spectrometric analysis. The carboxypentitol γ-lactones were further characterized by ^{13}C NMR analyses and by oxidation with periodate (Ferrier, 1962). Rates of periodate reduction were followed spectrophotometrically (Dixon & Lipkin, 1954). The infrared spectra of the lithium salts of the carboxypentitol bisphosphates (acid and lactone forms) were obtained in KBr pellets.

General Assay Procedures for Ribulose-P_2 Carboxylase/Oxygenase. Enzyme at a concentration of 0.4–2 mg/mL was activated with CO_2 (Lorimer et al., 1976) at 30 °C for at least 30 min in assay buffer [0.1 M *N,N*-bis(2-hydroxyethyl)glycine (Bicine), pH 8.1, 20 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM Na_2EDTA] containing 10 mM $NaHCO_3$. Ribulose-P_2 carboxylase activity was determined by the radiometric assay (Paulsen & Lane, 1966). Activated enzyme was added to the assay buffer which contained 10 mM $NaH^{14}CO_3$ (0.14–1.4 Ci/mol). The reaction was initiated with ribulose-P_2. Under these conditions the specific activity of different enzyme preparations varied between 1.5 and 2.2 μmol of CO_2 fixed per min per mg of protein. Kinetic parameters V_{max} and K_m were calculated with a computer program using a nonlinear regression analysis (Wilkinson, 1961). For the determination of inhibition constants (K_i), 0.46 mL of an enzyme solution was mixed with 40 μL of a solution containing ribulose-P_2 and inhibitor at appropriate concentrations so that the desired final concentrations were achieved. Averages of duplicate measurements are reported. The error in the assay was less than 5%. For experiments with carboxyarabinitol-P_2, the assay time was 15 s. Assay times for other inhibitors were 60 s. Linearity of the assay was confirmed for all assay conditions and assay times reported. Protein concentrations were determined by the method of Bensadoun & Weinstein (1976) or by absorbance at 280 nm with $\epsilon_{280}^{1\%}$ = 16.4 (Paulsen & Lane, 1966).

[2] The branched-chain compounds used in this report are named as derivatives of the D-pentitol-P_2 compounds and numbered so as to stress their structural relationship to ribulose-P_2. The tertiary carbon is designated C-2. The carbon derived from cyanide (i.e., carboxyl, hydroxymethyl, or aldehydo carbon) is designated C-2′.

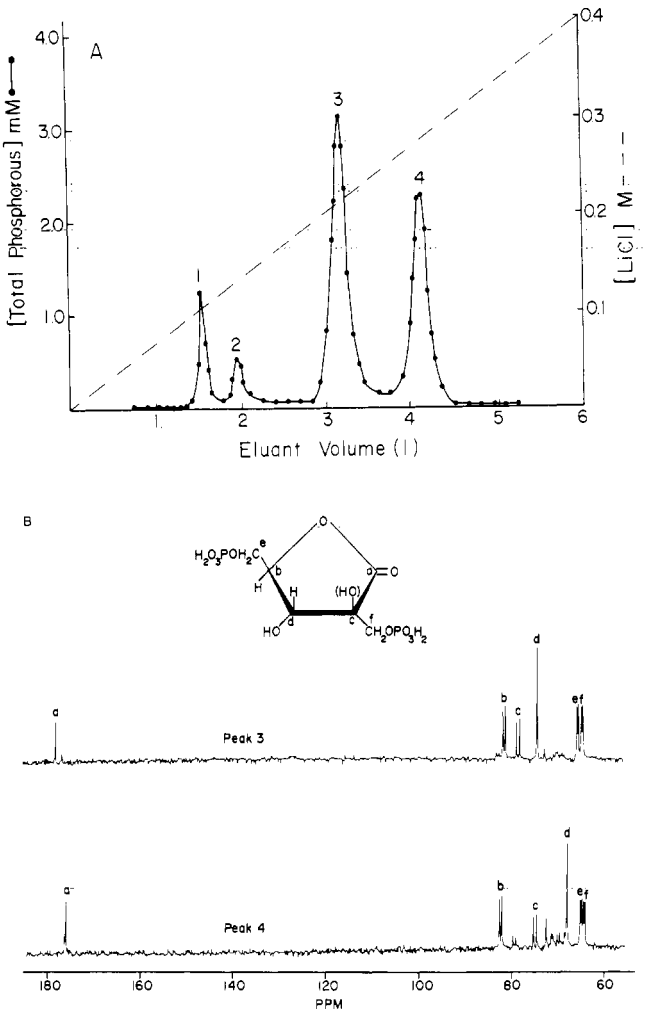


FIGURE 1: Separation of carboxyribitol-P_2 and carboxyarabinitol-P_2 and ^{13}C NMR analysis of the separated compounds. (A) Chromatography of the reaction products of cyanide addition to 1.2 mmol of ribulose-P_2 on a 49 × 3.3 cm Dowex 1-X8 column in the chloride form with a linear gradient of LiCl (6 L, 0.0–0.4 M) in 3 mM HCl. Peaks 3 and 4 contain, respectively, 40 and 32% of the material applied to the column. (B) The ^1H-decoupled, ^{13}C NMR spectra of the compounds from peaks 3 and 4. Spectra were obtained at 25 °C, pH 2.5, with a sweep width of 3000 Hz. Unassigned resonances arise from hydrolysis of the compounds during the NMR analysis.

Results and Discussion

Purification and Characterization of the Carboxypentitol Bisphosphates. The chromatographic separation of the products of cyanide addition to ribulose-P_2 is presented in Figure 1. The compound from peak 1 and the compound from peak 2 appear to be the free acid forms of the compound from peak 4 and the compound from peak 3, respectively. This was demonstrated by the observation that rechromatography of the peak 1 compound gave peaks 1 and 4, while rechromatography of the peak 2 compound gave peaks 2 and 3. Peaks 1 and 2 were not observed if very careful lactonization was performed prior to column chromatography. Alternatively, if the products of cyanide addition to ribulose-P_2 were chromatographed without prior acid treatment and desiccation, partial lactonization occurred on the column, peaks 1 and 2 predominated, and a smeared elution profile resulted. When $K^{14}CN$ was used, all peaks contained compounds that had a ratio of total phosphate/^{14}C of 2:1.

The peak areas in the elution profile indicate that the cyanohydrin synthesis, as applied to ribulose-P_2 here, gives two epimeric products in approximately a 1:1 ratio. In addition,

Table I: Properties of the γ-Lactones

		peak 3 compd	peak 4 compd	γ-arabinonolactone	γ-ribonolactone
periodate redn, $t_{1/2}$ (min)[a,f]		330	15	750[b]	23[b]
^{13}C-^1H coupling constant, $^3J_{(H_3-C_3-C_2-C_1)}$ (Hz)[c,f]		0.5	2.6		
^{13}C NMR chemical shifts (ppm)[d]	C-2	78.7	75.7	73.2[e]	70.4[e]
	C-3	74.7	68.7	73.8[e]	70.8[e]

[a] Time required to reduce 0.5 molar equiv of periodate at 25 °C; $[IO_4^-] = [\gamma\text{-lactone}] = 5.4 \times 10^{-5}$ M. [b] From Ferrier (1962). [c] Determined at 25 °C and pH 7.0 with a sweep width of 1500 Hz. The coupling constants are accurate to within 0.7 Hz. [d] Conditions are given in Figure 1. [e] From H. A. Nunez (unpublished observations). [f] Measurements were made with the dephosphorylated γ-lactones.

^{13}C NMR resonances of equal intensity at 178.1 and 176.7 ppm were observed after lactonization when $K^{13}CN$ was used, confirming the equal distribution of products.

The infrared spectra of the pentalithium salts of the compounds from peaks 3 and 4 gave absorptions typical of carboxylic acids at 1620 cm^{-1}. After treatment with Dowex 50(H^+) and desiccation, infrared absorptions typical of γ-lactones at 1780 cm^{-1} were observed (Barker et al., 1958). Mass spectrometric analyses showed that these compounds were epimers since similar fragmentation patterns were obtained, the only differences being in the relative abundances of the mass fragments (Petersson, 1970). The ^{13}C NMR spectra of the compounds from peaks 3 and 4 show resonances of six carbon atoms (Figure 1B). Assignments of the resonances were made by using acids prepared from ribulose-P_2 selectively enriched with ^{13}C at C-1 and the patterns of carbon–phosphorus coupling (Lapper et al., 1973; Lapper & Smith, 1973). In addition, by use of $K^{13}CN$ to enrich the carbonyl carbons, it was shown that the epimeric carbon atoms with resonances at approximately 76 and 79 ppm were coupled to the carbonyl atoms by approximately 56 Hz, indicating direct bonding of these atoms. Unlabeled resonances in Figure 1 arise from the free acid forms due to hydrolysis of the γ-lactones during the NMR analysis.

The only structures consistent with the above data are those for carboxyribitol-P_2 and carboxyarabinitol-P_2. The absolute stereochemistry about C-2 of these compounds was determined by comparing the rates of periodate oxidation of their dephosphorylated γ-lactones. Carboxyribitol-P_2 forms a γ-lactone with the C-2 and C-3 hydroxyl groups in a *trans* configuration, while carboxyarabinitol-P_2 forms a γ-lactone with the C-2 and C-3 hydroxyl groups in a *cis* configuration. The rate of periodate oxidation of adjacent hydroxyl groups depends on the stereochemistry of the glycol group being oxidized, *cis*-glycols being oxidized much faster than *trans*-glycols (Ferrier, 1962). The dephospholactone from peak 4 is oxidized much more rapidly than that from peak 3 (Table I). Production of formaldehyde from the oxidation of the 1,2-glycol accounted for less than 5% of the oxidation products after 0.6 molar equiv of periodate had been reduced. Thus, periodate cleaved predominately at the 2,3-glycol, and the differences in the rates of oxidation reflect differences in the stereochemistry at this site (Ferrier, 1962). On this basis, the peak 4 compound is tentatively identified as carboxyarabinitol-P_2 γ-lactone.

This assignment is supported by analysis of ^{13}C NMR

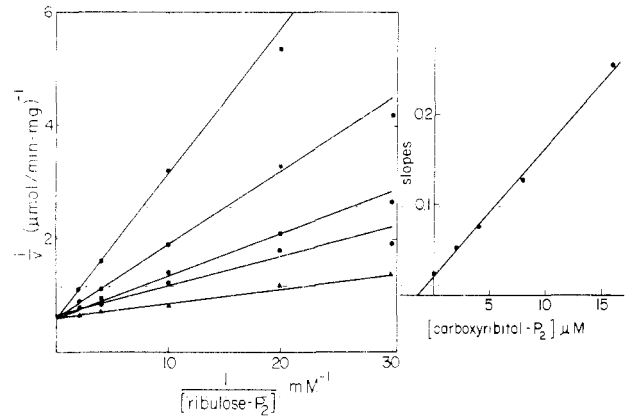


FIGURE 2: (Left) Lineweaver–Burk plot of the inhibition of ribulose-P_2 carboxylase activity by carboxyribitol-P_2. The general assay was used with no inhibitor (▲) or with 2, 4, 8, or 16 μM concentrations of carboxyribitol-P_2 (●). K_m(ribulose-P_2) = 36 μM. (Right) A plot of the slopes of the Lineweaver–Burk plot vs. the concentration of carboxyribitol-P_2. K_i = 1.5 μM.

spectra of the lactones synthesized from [1-^{13}C]ribulose-P_2. The angle between H-3 and C-1 is different in the two compounds, being close to 0° for carboxyarabinitol γ-lactone and approximately 110° for the ribo epimer. The coupling constants for ^{13}C and ^{1}H nuclei separated by three bonds, in this case $^3J_{(H_3\text{-}C_3\text{-}C_2\text{-}C_1)}$, depends on the angle subtended by the bonds (Schwarz & Perlin, 1972; Perlin et al., 1974), being maximal at 0 and 180° and minimal at 90°. The three-bond coupling constant obtained from the ^{1}H-coupled, ^{13}C NMR spectrum of the dephospholactone from peak 4 was larger than that observed in the peak 3 dephospholactone (Table I), indicating that peak 4 contains the arabino isomer, in agreement with the conclusion drawn from the periodate oxidation experiments.

In addition, the correctness of the above assignment is supported by the ^{13}C NMR chemical shifts of the ring carbons of the epimeric lactones. The dependence of chemical shifts on stereochemistry has been demonstrated for the furanose ring system of pentose 5-phosphates (Serianni et al., 1979) and for the γ-lactone ring system of pentono-γ-lactones (H. A. Nunez, unpublished observations). When hydroxyls at C-2 and C-3 are *cis*, the chemical shifts of these carbon atoms are at a higher field than when the C-2 and C-3 hydroxyls are *trans*. The chemical shifts of C-2 and C-3 of the peak 4 compound occur at higher field (smaller chemical shift values) than those for C-2 and C-3 of the peak 3 compound (Figure 1; Table I) in keeping with the stereochemical assignment made above.

Although none of the above criteria constitutes proof of the stereochemical assignment, the agreement between all of the chemical and physical analyses supports the identification of the peak 3 compound as carboxyribitol-P_2 γ-lactone and of the peak 4 compound as carboxyarabinitol-P_2 γ-lactone. Since the 2-*C*-hydroxymethyl derivatives were prepared from the separated lactones, their stereochemistry is also established.

Interaction of the Carboxypentitol Bisphosphates with Ribulose-P_2 Carboxylase/Oxygenase. The inhibition of ribulose-P_2 carboxylase/oxygenase by carboxyribitol-P_2 (Figure 2) is strictly competitive with respect to ribulose-P_2 with K_i = 1.5 μM (assuming rapid equilibrium between enzyme and carboxyribitol-P_2). The rapid equilibrium assumption was verified by preincubating the activated enzyme with inhibitor for 5 s to 20 min prior to the addition of ribulose-P_2. The preincubation was without effect on the subsequent assay. Therefore, the equilibrium is achieved rapidly (within 5 s) with respect to the assay time (1 min). Equilibrium dialysis studies which gave K_d = 1.5 μM for the enzyme–carboxyribitol-P_2 complex indicate that there are eight binding sites per 560 000 daltons (data not shown). The binding is not cooperative.

The inhibition of ribulose-P_2 carboxylase/oxygenase (E) by carboxyarabinitol-P_2 (I) is time dependent, as reported by Siegel & Lane (1972) for the epimeric mixture. They proposed a mechanism (eq 1) based on the observation that en-

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \qquad (1)$$

zyme at 4 °C bound carboxypentitol-P_2 tightly but still retained full activity in the standard carboxylase assay. Incubation at 30 °C was reported to be required for enzyme inhibition. In addition, they observed that the irreversible inhibition of the enzyme by carboxypentitol-P_2 was second order. None of these results could be obtained with carboxyarabinitol-P_2. These findings, together with the known flexibility of the enzyme and its conformational sensitivity to temperature (Chollet & Anderson, 1976, 1977; Wildner & Henkel, 1977), prompted experiments designed to test the proposed mechanism of inhibition directly.

The mechanism is conceptually identical with the Michaelis–Menten mechanism for enzymatic catalysis. Therefore, the rate of inhibition by carboxyarabinitol-P_2 should demonstrate saturation kinetics. Thus, if k_4 is much smaller than the other three rate constants, then, when [carboxyarabinitol-P_2] > [enzyme active sites], the improved steady-state approximation of McDaniel & Smoot (1956) can be applied to yield the integrated rate equation

$$[E]_t = \frac{[E]_0}{1 + ([I]/K_s)} \exp(-k_{obsd}t) \qquad (2)$$

where

$$k_{obsd} = \frac{k_3[I]}{K_s + [I]} \qquad (3)$$

and

$$K_s = \frac{k_2 + k_3}{k_1} \qquad (4)$$

The rate of inhibition of the enzyme by carboxyarabinitol-P_2 may be determined by measuring the amount of enzyme activity remaining after preincubation of the enzyme with carboxyarabinitol-P_2 for various times and at various inhibitor concentrations. When enzyme activity (V) is proportional to free enzyme concentration, a plot of ln ($V_t/V_{control}$) vs. time of preincubation should yield straight lines with slopes (k_{obsd}) varying with inhibitor concentration according to eq 3 and with extrapolated, ordinal intercepts varying with inhibitor concentration according to the preexponential term in eq 2. The proportionality of enzyme activity with enzyme concentration will hold only if the EI complex is catalytically incompetent. In this case, eq 3 indicates a hyperbolic relationship between k_{obsd} and [I], and a plot of k_{obsd}^{-1} vs. [carboxyarabinitol-P_2]$^{-1}$ should yield a straight line with an intercept on the ordinate of $1/k_3$ and a slope of K_s/k_3. As shown in Figure 3 the data are consistent with these predictions and indicate a rapid equilibration of the enzyme with carboxyarabinitol-P_2, followed by a slow interaction of the enzyme with the inhibitor which has a rate constant k_3 = 0.04 s^{-1}.

The rapid interaction of carboxyarabinitol-P_2 with the enzyme was investigated by measuring inhibition when carboxyarabinitol-P_2 and ribulose-P_2 were added simultaneously to

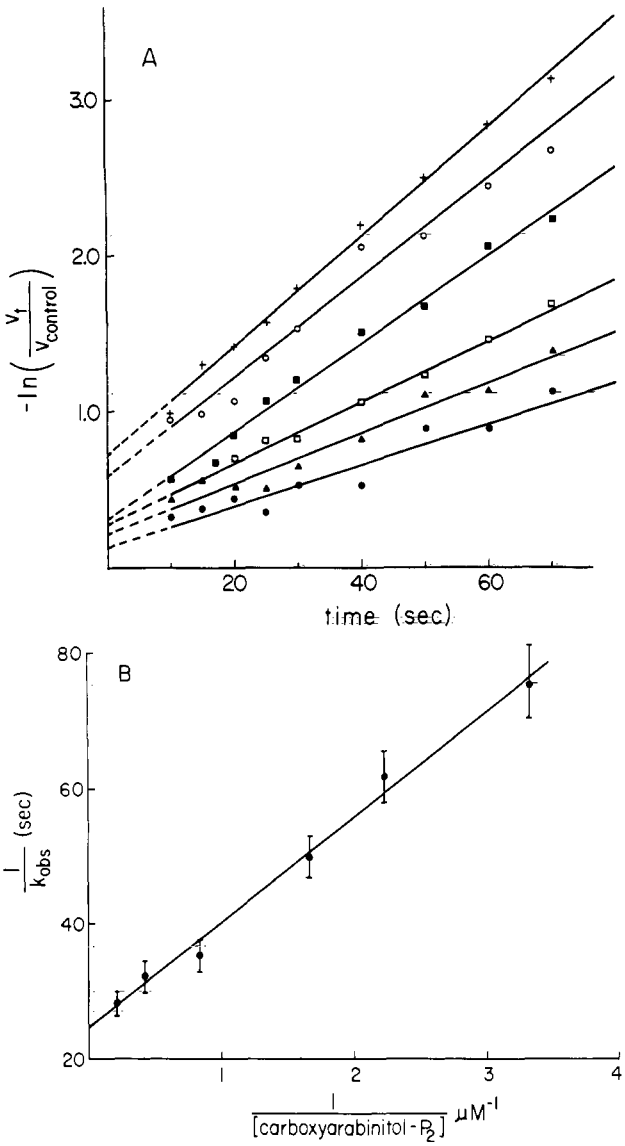


FIGURE 3: Kinetics of inhibition of ribulose-P_2 carboxylase activity by carboxyarabinitol-P_2. A 0.1-mL aliquot of a solution containing carboxyarabinitol-P_2 at various concentrations was rapidly mixed with 4.9 mL of assay buffer at 25 °C containing activated enzyme and $NaH^{14}CO_3$ (1.4 Ci/mol) so that the final concentrations were 7 μg of enzyme per mL and 10 mM $NaH^{14}CO_3$. The final concentration of carboxyarabinitol-P_2 was varied between 0.0 and 4.8 μM. At indicated times, 0.48-mL aliquots of these solutions were added to 20 μL of a 25 mM ribulose-P_2 solution, and the extent of CO_2 fixation was determined over a 15-s period. (A) Pseudo-first-order plot. See the text for details. Concentrations of carboxyarabinitol-P_2 were 0.3 (●), 0.45 (▲), 0.60 (□), 1.20 (■), 2.40 (○), and 4.80 μM (+). The lines were drawn from fitting the data by regression analysis. (B) Double-reciprocal plot of the rate constants obtained in (A) with varying concentrations of carboxyarabinitol-P_2. k_3 = 0.04 s^{-1}. K_s = 0.6 μM.

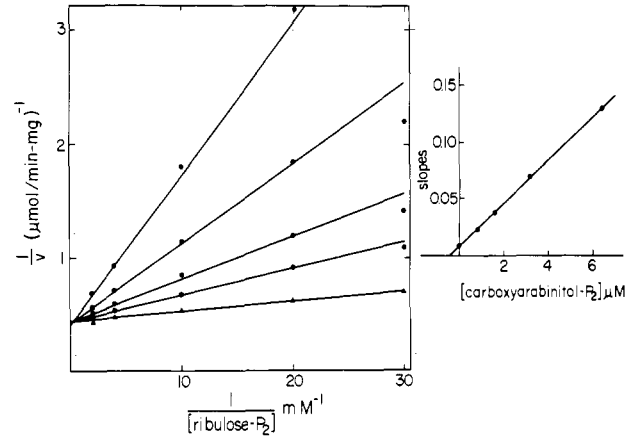


FIGURE 4: (Left) Lineweaver–Burk plot of the inhibition of ribulose-P_2 carboxylase activity by carboxyarabinitol-P_2. The general assay was used with no inhibitor (▲) or with 0.8, 1.6, 3.2, or 6.4 μM concentrations of carboxyarabinitol-P_2 (●). K_m(ribulose-P_2) = 20 μM. (Right) A plot of the slopes of the Lineweaver–Burk plot vs. carboxyarabinitol-P_2 concentration. K_i = 0.4 μM.

Table II: Dissociation of Carboxyarabinitol-P_2 from Ribulose-P_2 Carboxylase/Oxygenase[a]

conditions	time (h)	mol of [2'-^{14}C]-carboxyarabinitol-P_2 per mol of enzyme
+Mg^{2+}	0	6.3
	24	6.2
	48	6.0
	96	6.0
	120	5.8
+NaDodSO$_4$	120	0.0
−Mg^{2+}	0	6.2
	1	0.8

[a] Rates of dissociation of the enzyme–carboxyarabinitol-P_2 complex were determined by following the time course of release of radioactive carboxyarabinitol-P_2. Ribulose-P_2 carboxylase/oxygenase (20 mg, 36 nmol) was incubated for 1 h at 25 °C with 0.9 μmol of [2'-^{14}C]carboxyarabinitol-P_2 (0.81 Ci/mol) in a final volume of 1 mL of assay buffer containing 10 mM $NaHCO_3$ with or without 20 mM $MgCl_2$. This solution was applied to a 60 × 1 cm Sephadex G-25 column previously equilibrated with the same buffer solution. Elution with buffer solution separated unbound carboxyarabinitol-P_2 from the enzyme. Fractions containing protein were pooled, 29 μmol of nonradioactive carboxyarabinitol-P_2 was added, and the solution was adjusted so that its final composition was 2 mg/mL enzyme, 50 mM Bicine (pH 8.1), 2 mM dithiothreitol, 10 mM $NaHCO_3$, and 2.9 mM carboxyarabinitol-P_2 (with or without 20 mM $MgCl_2$). The rate of exchange of radioactive carboxyarabinitol-P_2 with unlabeled carboxyarabinitol-P_2 at 25 °C was determined over a 5-day period by passing 1-mL aliquouts of the incubation solution over the gel filtration column, pooling the protein-containing fractions, and assaying for radioactivity and protein (see Materials and Methods). After 5 days, the solution was made 1% in sodium dodecyl sulfate and a 1-mL aliquot was passed over the gel filtration column as before.

the enzyme. Short assay times (15 s) were used to minimize the effects of the slow, second phase of inhibition (Figure 4). The first phase of inhibition by carboxyarabinitol-P_2 is purely competititve with ribulose-P_2 with K_i = 0.4 μM. This value is in excellent agreement with the value of K_s = 0.6 μM obtained by using the steady-state assumption (Figure 3; eq 4) and validates the assumption that the EI complex is catalytically incompetent.

Dissociation of Carboxyarabinitol-P_2 from Ribulose-P_2 Carboxylase/Oxygenase. The rate of exchange of enzyme-bound, radioactive carboxyarabinitol-P_2 with added, nonradioactive carboxyarabinitol-P_2 was measured (Table II) to evaluate k_4. In the presence of Mg^{2+}, only 6–7% of the labeled carboxyarabinitol-P_2 dissociated from the enzyme in 5 days. Complete dissociation is effected by protein denaturation with sodium dodecyl sulfate. The close agreement between the values of the thermodynamic constant, $K_i = k_2/k_1$, and the kinetic constant, K_s (eq 4), indicates that k_2 is greater than k_3. Since the values of k_2 and k_3 are much greater than the rate of dissociation of carboxyarabinitol-P_2 from the enzyme, the rate-limiting process in this dissociation is that process related to k_4. When the observed rate of exchange is equated with k_4, an upper estimate of k_4 from these data is 5×10^{-7} s^{-1}.[3] The equilibrium constant for the second phase of in-

hibition depicted in eq 1 is therefore $K_2 = [EI]/[EI]^* = k_4/k_3 \lesssim 1.2 \times 10^{-5}$. The overall binding constant for the interaction of carboxyarabinitol-P_2 with ribulose-P_2 carboxylase/oxygenase is then $K = K_i K_2 \lesssim 10^{-11}$ M. This value is 10^5 times less than the K_i of 1.5 μM determined for carboxyribitol-P_2 and also 10^5 times smaller than the dissociation constant for ribulose-P_2 (Wishnick et al., 1970).

Mg^{2+} Effects on the Dissociation of Carboxyarabinitol-P_2 from Ribulose-P_2 Carboxylase/Oxygenase. Siegel & Lane (1972) demonstrated that Mg^{2+} is essential for maximal inhibition of ribulose-P_2 carboxylase/oxygenase by carboxypentitol-P_2. This result has since been confirmed and clarified in studies which indicate that enzyme active sites, Mg^{2+}, CO_2, and carboxypentitol-P_2 form a quaternary complex in a 1:1:1:1 stoichiometry (Miziorko & Mildvan, 1974; Miziorko, 1979). Since the published data are most consistent with the data given above for carboxyarabinitol-P_2, the effect of Mg^{2+} on the binding of carboxyarabinitol-P_2 to the enzyme was investigated.

It has been shown that carboxyarabinitol-P_2 is bound very tightly to the enzyme ($K \lesssim 10^{-11}$ M) in the presence of Mg^{2+}. In the absence of Mg^{2+}, exchange between bound and unbound carboxyarabinitol-P_2 occurs relatively rapidly (Table II) although the compound binds tightly enough to remain with the protein during a gel filtration. Thus, only the second phase of inhibition by carboxyarabinitol-P_2 exhibits a requirement for Mg^{2+}. It is not known whether carboxyarabinitol-P_2 binds to the enzyme in a two-step fashion in the absence of Mg^{2+}, nor can this question be addressed by following enzyme activity since Mg^{2+} is required for activation of the enzyme.

Wishnick et al. (1970) proposed that the carboxyl group of carboxypentitol-P_2 is involved in its interaction with metal ions such as Mg^{2+}. This proposal is supported by the observation that the ^{13}C NMR resonance of the carboxyl group of carboxyarabinitol-P_2 is strongly affected by low concentrations of paramagnetic ions such as Mn^{2+} which do not affect resonances of the other carbons (data not shown). To examine the importance of the C-2′ carboxyl group on the interaction of carboxyarabinitol-P_2 with the enzyme, we synthesized the hydroxymethyl and aldehyde analogues of the carboxypentitol bisphosphates and examined their interactions with the enzyme.

Interaction of Ribulose-P_2 Carboxylase/Oxygenase with Structural Analogues of the Carboxypentitol Bisphosphates. The hydroxymethyl derivatives of the carboxypentitol bisphosphates are simple competitive inhibitors with respect to ribulose-P_2. The K_i values are 80 μM for hydroxymethylribitol-P_2 and 5 μM for hydroxymethylarabinitol-P_2. Neither compound exhibited a large time dependence in their inhibition of the enzyme.

The enhanced binding of carboxyarabinitol-P_2 ($K_i \lesssim 10^{-11}$ M) as compared to that of hydroxymethylarabinitol-P_2 ($K_i = 5 \times 10^{-6}$ M) might be due to an interaction of the negatively charged carboxyl group with either protein-bound Mg^{2+} or a positively charged amino acid group that is exposed when Mg^{2+} binds to the enzyme. A number of investigators have reported the presence of several lysine groups at the active site of ribulose-P_2 carboxylase/oxygenase (Norton et al., 1975; Schloss & Hartman, 1977; Paech et al., 1977; Whitman & Tabita, 1978a,b; Paech & Tolbert, 1978). Since the branched-chain inhibitors mentioned are tightly bound to the enzyme, it appeared that aldehydo analogues of the carboxypentitol bisphosphates might form covalent adducts in a very specific fashion with these active-site lysine groups.

Although aldehydopentitol-P_2 and 3,4-dideoxyaldehydopentitol-P_2 inhibited the enzyme at low concentrations, attempts to detect Schiff base formation between the enzyme and the ^{14}C-labeled inhibitors by reduction with sodium borohydride or sodium cyanoborohydride at pH values from 6.5 to 8.5 in the presence or absence of CO_2 and Mg^{2+} were unsuccessful. The inhibition of the enzyme by these reagents was presumably due to binding via their negatively charged phosphate groups.

Active-Site Geometry. From the studies presented here, it is clear that the "carboxyribitol-P_2" used by earlier workers is a mixture of ribo and arabino isomers. The two isomers are similar to ribulose-P_2 in their initial binding to the enzyme, but only the arabino isomer exhibits the second phase of tight binding. Therefore, the potent inhibition of ribulose-P_2 carboxylase/oxygenase by "carboxyribitol-P_2" cited in earlier literature is due to carboxyarabinitol-P_2.[4]

The slow, second phase of binding of carboxyarabinitol-P_2 accounts for the 10^5-fold greater affinity of this ligand for the enzyme relative to ribulose-P_2. It is possible that carboxyarabinitol-P_2 mimics the transition state of the substrates and that the slow, tight binding of the analogue models a similar, but more rapid process that occurs with the transition state of the substrates. In the latter case, the rate of the binding process must equal or exceed the turnover rate of the enzyme. The difference between the rate of formation of the second binding state with carboxyarabinitol-P_2 (0.04 s^{-1}) and the turnover rate of the enzyme active sites ($\simeq$2.5 s^{-1}) may reflect the differences in structure between carboxyarabinitol-P_2 and the transition state of the substrates or the carboxylated intermediate in the reaction.

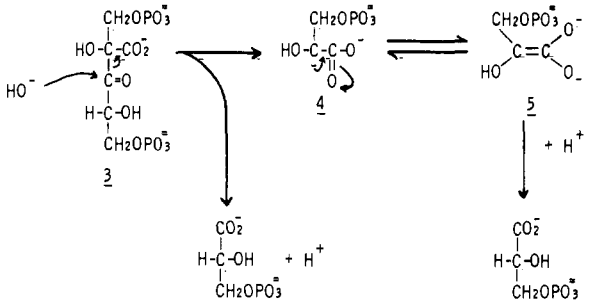
Exceedingly tight binding of an inhibitor to an enzyme has been used to infer a structural similarity to the transition state of the substrates in enzyme-catalyzed reactions (Wolfenden, 1972). However, the nature of the transition state in the carboxylation reaction is quite uncertain. Kinetic isotope experiments have been interpreted to imply that proton abstraction from C-3 of ribulose-P_2 and proton addition to the (formal) carbanion of P-glycerate are slow relative to other catalytic events (Simon et al., 1964; Hurwitz et al., 1956; Fiedler et al., 1967). Additionally, nonlinear Arrhenius plots for the carboxylation reaction may reflect the existence of different rate-limiting steps at different temperatures (Björkman & Pearcy, 1971). Nevertheless, the 10^5-fold tighter binding of carboxyarabinitol-P_2 relative to ribulose-P_2 or carboxyribitol-P_2 is consistent with the proposal that carboxyarabinitol-P_2 more closely resembles the transition state or the carboxylated intermediate of the reaction than does carboxyribitol-P_2. Accordingly, the intermediate in the enzyme-catalyzed carboxylation of ribulose-P_2 is most likely 2-*C*-carboxy-3-ketoarabinitol-P_2 (**3**).

This conclusion has interesting mechanistic implications. If, in the cleavage of intermediate **3** by H_2O, C-2 achieves a

[3] This value is considered an upper estimate. Although the apparent rate of dissociation is first order in [EI*], the enzyme may denature during the time that would be required to determine the rate constant k_4 over a greater percentage of the exchange reaction.

[4] In the original experiments with carboxypentitol-P_2 (Siegel & Lane, 1973), a purification procedure involving ion-exchange chromatography gave an asymmetric peak containing the carboxypentitol-P_2 compounds. Only fractions around the peak's maximum were pooled and used for the inhibition studies. Our results indicate that these fractions probably contained an approximately 3:1 mixture of the two epimers, the larger fraction corresponding to carboxyarabinitol-P_2. Preparation of the carboxypentitol-P_2 compounds by other workers may have resulted in mixtures containing different proportions of the epimers.

Scheme II



(formal) carbanion character, attack of hydroxide ion at C-3 and addition of a proton at C-2 would result in the formation of an equimolar mixture of D- and L-glycerate-3-P. In fact, solution studies revealed that the glycerate-3-P formed by nonenzymatic hydrolysis of **3** was a mixture of D- and L-glycerate-3-P and that the L-glycerate-3-P was derived from C-1, C-2, and C-2′ of **3** (Siegel & Lane, 1973). In contrast, two molecules of D-glycerate-3-P are produced in the enzymatic reaction (Weissbach et al., 1956; Jakoby et al., 1956). This difference in the enzymatic and nonenzymatic reactions might be explained by postulating a glycerate-3-P epimerase activity in ribulose-P_2 carboxylase/oxygenase, a carbanion inversion mechanism, or other, less likely mechanisms involving an electron-deficient carbon at C-2 of **3**. We have shown that the enzyme doos not epimerize DL-glycerate-3-P (data not shown). Therefore, a mechanism involving carbanion inversion seems to be most probable.

Since the formation and inversion of the C-2 carbanion of glycerate-3-P would be expected to be slow and thermodynamically unfavorable, a mechanism for its stabilization in the active site should exist. This stabilization may occur via the mechanism shown in Scheme II. Addition of a proton to the front face of the double bond in **5** yields D-glycerate-3-P. According to this mechanism, the efficient conversion of **3** to two molecules of D-glycerate-3-P requires the stabilization of **5** relative to **4**. This stabilization might be conveniently accomplished by complextion of **5** with the Mg^{2+} required for enzymatic activity or with a positively charged amino acid side chain in the active site.

Studies with hydroxymethylarabinitol-P_2 indicate that the carboxyl group of carboxyarabinitol-P_2 is required for the second phase of inhibition. This second phase of binding which accounts for the 10^5-fold difference in binding between the hydroxymethyl and carboxy derivatives also requires Mg^{2+}, in agreement with reports that the enzyme active site, CO_2, Mg^{2+}, and carboxypentitol-P_2 form a very slowly dissociating, quaternary complex (Miziorko, 1979). To the extent that this quaternary complex represents the transition state or intermediate in the enzyme reaction, these results indicate a specific role for Mg^{2+} in the stabilization of the transition state of the substrates or of intermediate **3**. In this regard, the recent findings (O'Leary et al., 1979) of a protein-bound carbamate resonance in the ^{13}C NMR spectrum of *Rhodospirillum rubrum* enzyme incubated with $KH^{13}CO_3$ and the existence of a slowly exchanging species of CO_2 in the presence (Miziorko, 1979) and absence (Lorimer, 1979) of carboxypentitol-P_2 support the model of enzyme activation (Lorimer et al., 1976) in which the negative charge of an enzyme-bound carbamate promotes the binding of Mg^{2+} to form the active enzyme–carbamate–Mg^{2+} species. When the Mg^{2+} in this complex is replaced by Mn^{2+}, a large effect on the longitudinal relaxation rate of a rapidly exchanging species of $^{13}CO_2$ is noted, and a distance of 5.4 Å between the Mn^{2+} and the rapidly exchanging CO_2 species was calculated (Miziorko & Mildvan, 1974). Therefore, the close proximity of Mg^{2+} to both a slowly and rapidly exchanging species of CO_2, together with the requirement of Mg^{2+} and a negatively charged carboxyl group for the tight binding of carboxyarabinitol-P_2 to the enzyme, indicates a role for Mg^{2+} in both activation of the enzyme and catalysis. Earlier reports indicating that Mg^{2+} is not required beyond the amount required for enzyme activation (Laing & Christeller, 1976) may be rationalized by concluding that the "activator" Mg^{2+} and the "catalytic" Mg^{2+} are one and the same.

The observation that Mn^{2+} interacts preferentially with the carboxyl group of carboxypentitol-P_2 in solution indicates that a similar interaction may occur at the active site. Indeed, Miziorko (1978) has presented preliminary evidence that the environment of Mn^{2+} in the quaternary complex is distorted from the octahedral environment of Mn^{2+} in solution. This distortion may be explained by insertion of a new ligand into the inner coordination sphere of Mn^{2+}. Whether the new ligand is from carboxypentitol-P_2, enzyme, or both is unresolved, though the preferential binding of Mn^{2+} to the carboxyl group of carboxyarabinitol-P_2 in solution supports the possibility that this ligand is one of the carboxyl oxygens.

An alternate explanation for the Mg^{2+}-dependent, tight binding of carboxyarabinitol-P_2 is that a positively charged amino acid residue is exposed by a Mg^{2+}-induced conformational change in enzyme structure. In this regard, experiments with active site directed reagents indicate that Mg^{2+} enhances the reactivity of a particular lysine group with *N*-(bromoacetyl)ethanolamine-P. However, a reagent more similar to ribulose-P_2, 1,4-dihydroxy-3-bromobutanone 1,4-bisphosphate, modified the same lysine residue in the absence of Mg^{2+} (Schloss et al., 1978; Norton et al., 1975). Since experiments with the aldehyde analogues of carboxyarabinitol-P_2 were inconclusive, any role of lysine groups in the binding of carboxyarabinitol-P_2 and enzymatic catalysis must be resolved by further experimentation.

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