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Spinach Ribulose Diphosphate Carboxylase. I. Purification and Properties of the Enzyme*

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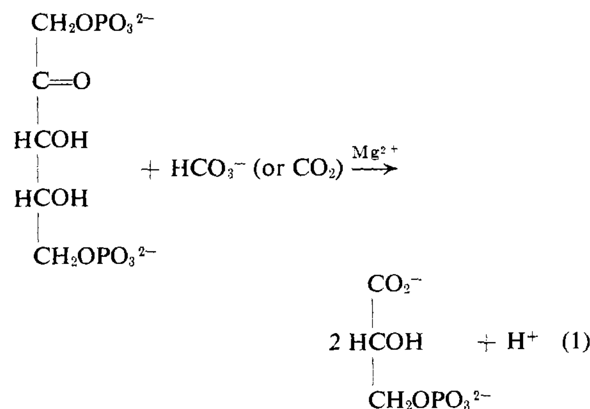
ABSTRACT: Spinach leaf ribulose diphosphate carboxylase has been purified to a homogeneous state free of 5-phosphoriboisomerase and 5-phosphoribulokinase activities.

The carboxylase has a sedimentation coefficient ($s^0_{20,w}$) of 21.0 S and molecular weight by sedimentation equilibrium of 557,000. The pure enzyme catalyzes the carboxylation of 1300 moles of D-ribulose 1,5-diphosphate/min per mole of enzyme at pH 7.9 and 30°. K_m and V values for substrates and metal activators are reported. Orthophosphate (K_i = 4.2 mM) and sulfate (K_i = 8.1 mM) inhibit the carboxylation reaction competitively with respect to ribulose diphosphate. 3-Phosphoglycerate appears to inhibit competitively with respect to HCO_3^- and noncompetitively with respect to ribulose diphosphate.

The isolation and partial characterization of ribulose diphosphate carboxylase[1] (3-phospho-D-glycerate carboxy-lyase (dimerizing) EC 4.1.1.f; Report of the Commission on Enzymes, 1961; Dixon and Webb, 1964a) from spinach leaves has been reported by a number of investigators (Weissbach *et al.*, 1956; Racker, 1957; Jakoby *et al.*, 1956; Trown, 1965). Convincing evidence has been presented by Trown (1965) and Thornber *et al.* (1965) that the principal component of leaf "fraction I protein" (Wildman and Bonner, 1947) is, in fact, RuDP carboxylase. While the stoichiometry of the reaction (reaction 1) catalyzed, as well as many properties of the carboxylase, have been known for nearly a decade, information on the carboxylation mechanism or structural characteristics of the enzyme is meager. Only recently, Müllhoffer and Rose (1965) unequivocally proved that C–C bond



cleavage occurs at the C-2–C-3 bond of RuDP during the carboxylation reaction. Carboxylation of RuDP in the presence of D_2O resulted in deuterium incorporation into 3-PGA at the carbon atom originating from the C-2 position of RuDP. Trown and Rabin (1964) have obtained evidence for an interaction between RuDP carboxylase sulfhydryl groups and RuDP. The nature of this interaction is still uncertain.

The initial objective of this investigation was to devise a procedure for the preparation of homogeneous RuDP carboxylase in sufficient quantity for structural characterization and mechanism studies, which will be reported subsequently as part of this series. In addition to describing such a purification procedure, the molec-

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[1] Abbreviations used: RuDP, D-ribulose 1,5-diphosphate; 3-PGA, 3-phospho-D-glyceric acid; R 5-P, D-ribose 5-phosphate; Ru 5-P, D-ribulose 5-phosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2(5-phenyloxazolyl)benzene; ATP, adenosine triphosphate; GSH, glutathione.

ular weight, sedimentation coefficient, and various kinetic properties of the carboxylase are reported in this paper.

Experimental Procedures

Materials. 3-PGA·Na_3 was obtained from Boehringer–Mannheim Corp., R-5-P·Na_2 from Sigma Chemical Co., ATP·Na_2 from Pabst Laboratories, and GSH from Schwarz Bioresearch, Inc. DEAE-cellulose (Type 20), hydroxylapatite (Bio-Gel HT), and Sephadex G-25, medium, were products of Carl Schleicher and Schuell Co., Bio-Rad Laboratories, and Pharmacia, respectively. [^{14}C]$NaHCO_3$ was obtained from New England Nuclear Corp.

Methods. Orthophosphate was determined by the method of Fiske and Subbarow (1925) and total phosphate as described by Umbreit *et al.* (1957). Protein was routinely determined by the method of Sutherland *et al.* (1949) using crystalline bovine serum albumin as standard. As described in a later section, the relation between protein concentration of pure RuDP carboxylase determined refractometrically and by the Sutherland method is given by: concentration (refractometric method) = 0.79 × concentration (Sutherland method). An absorbance (1-cm light path) of 1.00 at 280 mμ for the pure enzyme is equivalent to 0.602 mg of refractometrically determined protein/ml.

RuDP was synthesized enzymatically (Horecker *et al.*, 1957), isolated as the barium salt, and converted to the sodium salt (Weissbach *et al.*, 1954). Characterization of RuDP was accomplished by inorganic and total phosphate analyses, quantitative orcinol reaction, and quantitative enzymatic conversion to 3-PGA in the presence of [^{14}C]HCO_3^- and RuDP carboxylase. The orcinol reaction (Ashwell, 1957) was employed for ribulose analysis using D-arabinose as standard. The absorbance at 670 mμ of the orcinol reaction product of ribulose is 37% that for D-arabinose (Cohen, 1953). In typical RuDP preparations, the ratio of enzymatically determined RuDP: pentose (orcinol): organic phosphate was found to be 1.0:1.2:1.9. On a dry weight basis, enzymatically synthesized RuDP contained Ba_2RuDP, 63%; $Ba_3(PO_4)_2$, 29%; and free pentose, 8%.

RuDP Carboxylase Assay. The complete reaction mixture contains the following components (in micromoles, unless otherwise indicated): Tris (Cl^-) buffer, pH 7.8, 100; RuDP, 0.35; [^{14}C]$KHCO_3$ (2 μc), 25; $MgCl_2$, 5; EDTA, 0.03; GSH, 3; and RuDP carboxylase, up to 0.015 unit in the total volume of 0.50 ml. The final pH is 7.9. Separate incubations are carried out for 3, 6, 9, and 12 min at 30° and then stopped by addition of 1 ml of 2 N HCl. A 0.5-ml aliquot from each is pipetted into a liquid scintillation counting vial and is dried in a forced-draft oven for 45 min at 95°. Water (1 ml) is added to each vial followed by 10 ml of liquid scintillator.[2] Radioactivity measurements are then made using a liquid scintillation spectrometer. The carboxylation rate is proportional to enzyme concentration up to a level of approximately 0.012 unit; proportionality to enzyme concentration is maintained until at least 36% of the added RuDP has been consumed. The carboxylation reaction follows zero-order kinetics under the conditions described following a 1-min lag period. One unit of RuDP carboxylase is defined as that amount which catalyzes the fixation of 1.0 μmole of [^{14}C]bicarbonate into an acid-stable form/min under the assay conditions. 3-Phosphoglycerate was identified as the sole radioactive reaction product by paper chromatography of the assay reaction mixture after carboxylation for 5 min by 0.25 unit of pure RuDP carboxylase in the presence of high specific activity [^{14}C]bicarbonate (0.5 μc/μmole). Descending paper chromatography (Whatman 3MM paper) using the 1-butanol–acetic acid–H_2O (74:19:50, v/v) solvent system revealed a single radioactive peak (R_F 0.28, by strip counter) which cochromatographed exactly with authentic 3-phosphoglyceric acid.

5-Phosphoriboisomerase and 5-Phosphoribulokinase Assays. Combined "isomerase–kinase" assays were conducted by coupling RuDP formation from R 5-P to the RuDP carboxylase system. The following components (in micromoles, unless otherwise indicated): Tris (Cl^-) buffer, pH 7.8, 100; R 5-P, 2; ATP, 4; $MgCl_2$, 5; [^{14}C]$KHCO_3$ (2 μc), 25; GSH, 3; EDTA, 0.03; RuDP carboxylase, 0.5 unit (excess); and enzyme fraction to be assayed in a total volume of 0.5 ml were incubated at 30° for 10 min. The reaction was stopped with 1 ml of 2 M HCl; subsequent steps were the same as those described for the RuDP carboxylase assay. The method used for detecting 5-phosphoriboisomerase activity was that described by Axelrod and Jang (1954).

5-Phosphoribulokinase assays were conducted by coupling the formation of RuDP from enzymatically synthesized Ru 5-P (mixture of Ru 5-P and R 5-P) to the RuDP carboxylase system. Ru 5-P was prepared by incubation of the following components (in μmoles unless specified): Tris (Cl^-), pH 7.0, 30; R 5-P, 10; cysteine, 1; and phosphoriboisomerase (ammonium sulfate II fraction, Weissbach *et al.*, 1956), 3 mg in a total volume of 0.60 ml for 40 min at 37°. After 10 min in a boiling water bath and centrifugation, the supernatant solution (containing Ru 5-P) was used directly as the source of Ru 5-P in the 5-phosphoribulokinase assay. Kinase assays were conducted in the same manner as RuDP carboxylase assays except that 1 μmole of Ru 5-P was substituted for RuDP and 0.5 unit of RuDP carboxylase and 4 μmoles of ATP were added. [^{14}C]Bicarbonate fixed was proportional to the quantity of kinase added.

Purification of RuDP Carboxylase. The initial steps in the purification, *i.e.*, through the ammonium sulfate II fractionation, are based on the procedure described by Weissbach *et al.* (1956). Since some modifications have been introduced the complete procedure will be described. All operations were carried out at approximately 4°. Unless otherwise specified, all buffers used

[2] PPO (15 g), 0.375 g of POPOP, and 150 g of naphthalene in 1.5 l. of 1,4-dioxane.

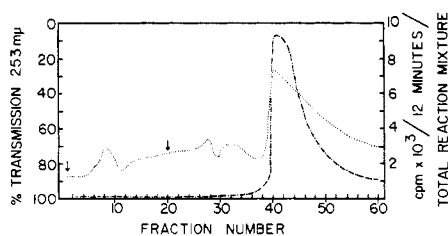

FIGURE 1: Chromatography of RuDP carboxylase on DEAE-cellulose. The per cent transmission at 253 mμ (......) of the column effluent was recorded continuously using an LKB Uvicord absorptiometer. Fractions (10 ml) were collected and aliquots assayed for RuDP carboxylase activity. Carboxylase activity is expressed (-·-·-·) as counts per minute of [^{14}C]HCO_3^- fixed × 10^3 during 12 min of incubation/0.002-ml aliquot. Vertical arrows (from left to right) indicate points of initiation of the gradient (0.1 M phosphate added to reservoir) and addition of 0.2 M phosphate to the reservoir, respectively. Fractions 39–45 were pooled and protein precipitated as described in the text.

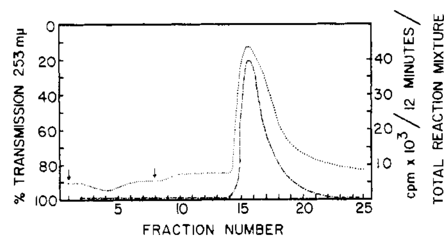

FIGURE 2: Chromatography of RuDP carboxylase on hydroxylapatite. The per cent transmission at 253 mμ (......) of the column effluent was recorded continuously using an LKB Uvicord absorptiometer. Fractions (7 ml) were collected and aliquots assayed for RuDP carboxylase activity. Carboxylase activity is expressed (-·-·-·) as counts per minute of [^{14}C]HCO_3^- fixed × 10^3 during 12 min of incubation/0.005-ml aliquot. Vertical arrows (from left to right) indicate points of addition to the column of 0.005 and 0.025 M phosphate, respectively. Fractions 15–17 were pooled and protein was precipitated as described in the text.

in the purification contained 0.1 mM EDTA and 5 mM mercaptoethanol. The results of the purification procedure are summarized in Table I.

TABLE I: Purification of RuDP Carboxylase.

Stage of Purification	Total Protein[a] (g)	Total Act. (Units)	Sp Act. (Units/mg of Protein)	Yield %
Initial extract[b]	6.10	1400	0.23	100
Ammonium sulfate I	1.97	1230	0.62	88
Ammonium sulfate II	0.644	605	0.94	43
DEAE-cellulose chromatographic fraction	0.182	246	1.35	18
Hydroxylapatite chromatographic fraction	0.074	105	1.42[c]	8

[a] Protein determined by the method of Sutherland *et al.* (1949). [b] Extract of 300 g of spinach leaves. [c] Specific activity, expressed in terms of refractometrically determined protein, is 1.80 units/mg of protein.

INITIAL EXTRACT. Fresh spinach, purchased locally, is washed with cold tap water and destemmed. Leaves (600 g) are homogenized with 2 l. of 0.01 M phosphate buffer, pH 7.5 (mercaptoethanol omitted), in a 4-l. capacity Waring Blendor for 3 min. The resulting suspension is filtered through S. and S. No. 588 fluted filter paper. The filtrate, referred to as the initial extract, is adjusted to pH 7 with 2 M NH_4OH.

AMMONIUM SULFATE FRACTIONATION. The initial extract is brought to 37% saturation with solid ammonium sulfate (226 g/l. of extract) and after standing for 30 min is centrifuged at 13,000*g* for 30 min. The precipitate is discarded and the supernatant solution is brought to 50% saturation with solid ammonium sulfate (92.5 g/l.). After centrifugation as described above, the precipitate is dissolved in 100 ml of 0.1 M phosphate buffer, pH 7.6. This solution (37–50% saturated ammonium sulfate fraction), referred to as ammonium sulfate I, is stored at 4° overnight. It is then diluted with an equal volume of ion-free water and saturated ammonium sulfate[3] (pH 7.3, 41 ml/100 ml of diluted ammonium sulfate I) is added. After centrifugation, the precipitate is discarded and saturated ammonium sulfate[3] (pH 7.3, 8.5 ml/100 ml) is added to the supernatant solution. Following centrifugation, the precipitate is again discarded and additional saturated ammonium sulfate[3] (pH 7.3, 9.1 ml/100 ml) is added to the supernatant solution. The precipitate recovered after centrifugation is dissolved in 27 ml of 0.1 M phosphate buffer, pH 7.6, and constitutes a 39–45% saturated ammonium sulfate fraction. This solution is divided into two equal fractions (about 600 mg of protein in each) and is stored at −20°. One of these fractions is thawed and centrifuged at 27,000*g* for 30 min, and the supernatant solution applied to a Sephadex G-25 column (4.4 × 26 cm), previously equilibrated with 5 mM phosphate, pH 7.6. The enzyme is eluted from the column with the same buffer. The light brown fastest moving band, which is clearly resolved from

[3] Ammonium sulfate is saturated at room temperature and neutralized with NH_4OH so that when diluted fivefold, the pH is that indicated.

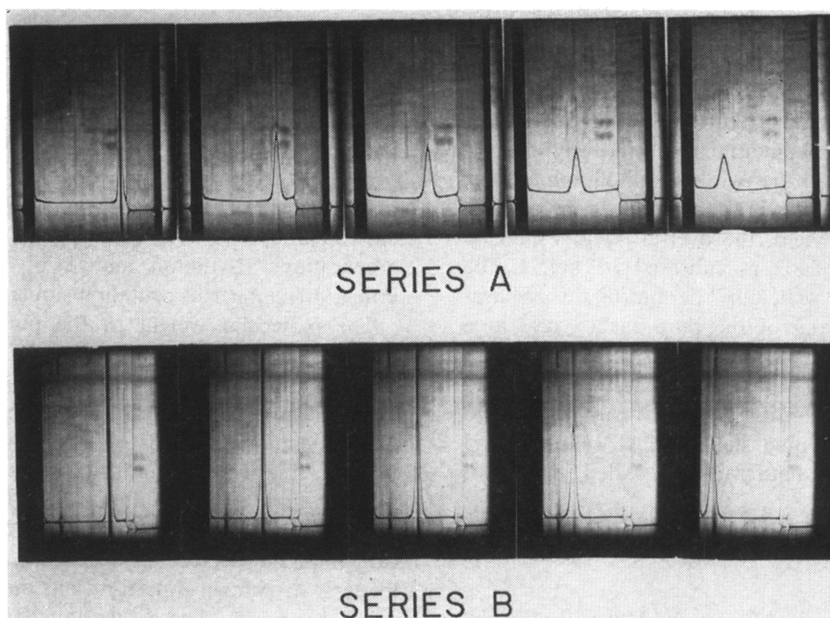

FIGURE 3: Schlieren patterns of purified RuDP carboxylase obtained in the Spinco Model E ultracentrifuge. Purified RuDP carboxylase (sp act. 1.4 units/mg of protein) was centrifuged at 37,020 rpm (series A, 5.0 mg of protein[4]/ml, single-sector cell) and 39,460 rpm (series B, 9.5 mg of protein[4]/ml, double-sector cell) at 20°. Photographs (from left to right) were taken at 8, 24, 40, 57, and 80 min in series A and at 16, 28, 36, 48, and 64 min in series B. The direction of sedimentation is from right to left. Prior to centrifugation the enzyme preparations were dialyzed *vs.* 0.01 M Tris (Cl^-) buffer, pH 8.3, containing 0.05 M NaCl, 0.1 mM EDTA, and 5 mM mercaptoethanol.

a bright yellow, slower moving band, is collected visually and contains about 90% of the protein applied to the column. This fraction, referred to as ammonium sulfate II, is immediately subjected to DEAE-cellulose chromatography as described in the next section.

DEAE-CELLULOSE CHROMATOGRAPHY. DEAE-cellulose columns (OH^- phase, 2.2 × 29 cm) are equilibrated with 0.5 M phosphate buffer, pH 7.6, and then washed with 5 mM phosphate buffer, pH 7.6. After application of the Sephadex G-25 gel-filtered enzyme solution (about 600 mg of protein) to the DEAE-cellulose column, stepwise gradient elution is accomplished by placing 200 ml of 0.005 M phosphate in a closed mixing chamber and introducing 200 ml of 0.1 M phosphate and then 400 ml of 0.2 M phosphate into the reservoir. All phosphate buffers are pH 7.6. The column effluent is continuously monitored for protein with an LKB Uvicord absorptiometer and is collected fractionally. Fractions exhibiting peak RuDP carboxylase specific activity (containing approximately 50% of the total activity applied to the column) are pooled and the enzyme is precipitated by bringing the saturation to 55% with saturated ammonium sulfate,[3] pH 6.5. Figure 1 shows a typical elution pattern of the DEAE-cellulose chromatography. In preparation for the next purification step, the enzyme suspension is centrifuged and the precipitate redissolved in 3.0 ml of 5 mM phosphate buffer, pH 7.6. This solution is subjected to Sephadex G-25 gel filtration (2.2 × 25 cm column) as described in the preceding section. The gel-filtered enzyme, referred to as the DEAE-cellulose fraction, then undergoes hydroxylapatite chromatography as described in the next section.

HYDROXYLAPATITE CHROMATOGRAPHY. A hydroxylapatite column (approximately 2 × 7 cm) is equilibrated with 5 mM phosphate buffer, pH 7.6. After application of the DEAE-cellulose fraction (about 180 mg of protein) to the column, batchwise elution is carried out with 50 ml of 0.005 M followed by 100 ml of 0.025 M phosphate, pH 7.6. The column effluent is monitored for protein and RuDP carboxylase activity. As illustrated in Figure 2, the carboxylase appears in the 0.025 M phosphate eluate as a discrete protein peak. Fractions containing RuDP carboxylase with maximal specific activity (approximately 1.4 units/mg of protein) and comprising about 50% of the carboxylase activity applied to the column are pooled. These fractions are brought to 55% saturation with saturated ammonium sulfate,[3] pH 6.5; EDTA and mercaptoethanol are added to produce final concentrations of 0.1 and 5 mM, respectively. RuDP carboxylase suspensions, stored in this manner at 0–2°, retain nearly full activity for at least 1 month. Table I summarizes the results of the purification of RuDP carboxylase from 300 g of spinach

[4] Refractometrically determined protein concentration in milligrams per milliliter is $0.602(A_{280m\mu}^{1cm})$ or 0.79 (protein concentration in milligrams per milliliter according to the method of Sutherland *et al.*, 1949).

leaves. The specific activity of the purified carboxylase, shown subsequently to be homogeneous, is 1.4–1.5 units/mg. Since the objective of this purification procedure was to obtain RuDP carboxylase essentially free of even minor contaminants, relatively narrow cuts were employed in the two chromatographic steps, hence yield was sacrificed. If the latter steps are adjusted to maximize yield, the over-all yield would be at least 25 instead of 8% as indicated in Table I. The procedure has been "scaled up" permitting the preparation of up to 400 mg of the pure carboxylase in a single isolation. Large quantities (20 g of protein) of ammonium sulfate II fraction are accumulated and stored frozen; 3-g quantities are then carried through the two chromatographic steps (DEAE-cellulose and hydroxylapatite chromatography) "scaled up" proportionally.

Results

Purity, Sedimentation Velocity, and Molecular Weight. Eleven preparations of RuDP carboxylase, purified according to the procedures described (*i.e.*, through the hydroxylapatite chromatographic step; Table I) have been examined for purity in the analytical ultracentrifuge. In each case, sedimentation patterns revealed a single symmetrical component with no evidence of inhomogeneity. Two typical sedimentation patterns are shown in Figure 3. Sedimentation velocity experiments were conducted at 20° after overnight dialysis of the enzyme *vs.* 0.01 M Tris (Cl^-) buffer, pH 8.3, 0.05 M NaCl, 0.1 mM EDTA, and 5 mM mercaptoethanol. The sedimentation coefficient, based on results from four experiments at varying protein concentrations[4] (2.38–9.54 mg/ml) and involving three enzyme preparations, was found to be $s^0_{20,w}$ = 21.0 S (extrapolated to zero protein concentration). The equation, $s_{20,w} = s^0_{20,w} - kc$, in which c represents the protein concentration[4] in milligrams per milliliter and k = 0.298 S mg^{-1} ml, fits the results of these experiments. In order to calculate the protein concentration refractometrically, total peak areas on several schlieren patterns were determined from comparator readings corrected for radial dilution. Protein concentration was then calculated using 1.862×10^{-4} as the specific refractive index increment/mg per ml. This figure is the average for several proteins studied by Perlmann and Longsworth (1948) corrected to 20° and a wavelength of 546 mμ. The relation between absorbancy at 280 mμ and the refractometrically determined protein concentration is given by the equation, $c = 0.61(A^{1cm}_{280m\mu})$, where c is protein concentration in milligrams per milliliter and A is absorbancy at 280 mμ (1-cm light path). The absorbancy ratio $A_{280m\mu}/A_{260m\mu}$ of the pure enzyme is 1.85. Ultraviolet and visible absorption spectra reveal no characteristic chromophoric groups which would distinguish this enzyme from most other proteins. To convert protein concentration determined by the method of Sutherland *et al.* (1949) to refractometrically determined protein concentration, the former should be multiplied by a factor of 0.79. Sedimentation patterns of carboxylase preparations carried only part way through the purification procedure, *i.e.*, through DEAE-cellulose chromatography, reveal a slow moving, 6S, component in addition to RuDP carboxylase. This contaminant is completely removed by hydroxylapatite chromatography. The RuDP carboxylase preparation of Weissbach *et al.* (1956) and chloroplast fraction I protein preparations (Lyttleton and Ts'o, 1958; Park and Pon, 1961) appear to contain a similar contaminant.

The molecular weight of the purified carboxylase was determined by sedimentation equilibrium according to the method of Yphantis (1964). Six experiments were conducted in which enzyme from two preparations (hydroxylapatite chromatographically purified; Table I) was subjected to equilibrium centrifugation at 20° following dialysis to equilibrium *vs.* 0.01 M Tris (Cl^-), pH 8.3, 0.05 M NaCl, 0.1 mM EDTA, and 5 mM mercaptoethanol. Molecular weight determinations were made at six protein concentrations ranging from 0.0954 to 0.954 mg/ml. These determinations resulted in a molecular weight of 557,000 ± 16,000;[5] a partial specific volume of 0.74 (Thornber *et al.*, 1965) was used in this calculation. In all cases linear plots of natural logarithm of Rayleigh fringe displacement *vs.* position variable (comparator x coordinate) were obtained. This is additional evidence of the homogeneity of RuDP carboxylase purified according to the procedure outlined in the preceding section. The frictional coefficient ratio (f/f_0) of the enzyme calculated from molecular weight (5.57×10^5), $s^0_{20,w}$, and partial specific volume (0.74; Thornber *et al.*, 1965) data is 1.11. This ratio is in the usual range for a protein molecule of spherical shape having typical hydration characteristics (*i.e.*, about 0.2 g of water/g of protein). The molecular weight of 557,000 obtained for spinach leaf RuDP carboxylase in this investigation agrees closely with the average value of 565,000 for the carboxylase component of fraction I protein reported by Thornber *et al.* (1956), but is considerably higher than that (515,000) reported by Trown (1965).

RuDP carboxylase preparations at different stages of the purification procedure were tested for 5-phosphoriboisomerase and 5-phosphoribulokinase activity using the assay methods described in "Experimental Procedure." Combined isomerase–kinase assays conducted on DEAE-cellulose chromatographic fractions indicated peak activity just following, but partially overlapping (fraction 44–55; Figure 1), the carboxylase peak. Therefore, carboxylase preparations carried through the DEAE-cellulose chromatographic step still contained isomerase–kinase activity; however, this was completely removed in the subsequent purification step. Even when high levels of the hydroxylapatite-purified carboxylase (1 mg of protein, 1.4 units of carboxylase) were tested, no 5-phosphoriboisomerase or 5-phosphoribulokinase activity could be detected. The assay methods for each of the latter two enzymatic

[5] Standard deviation.

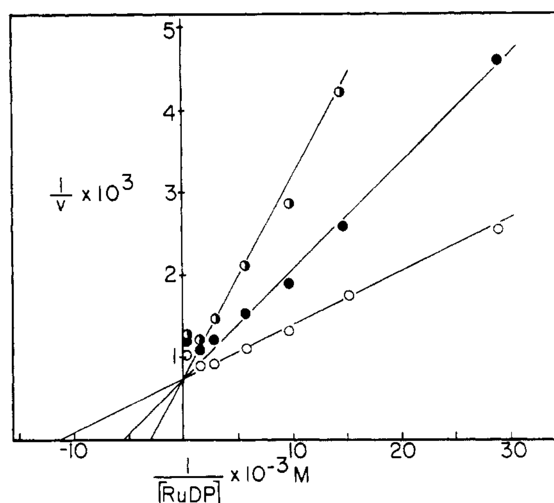

FIGURE 4: Inhibition of the RuDP carboxylase catalyzed reaction by orthophosphate. Standard RuDP carboxylase assays (5 min of incubation, 8.6 μg of carboxylase) were conducted in the absence of (O-O-O) and in the presence of 5 (●-●-●) and 10 mM (◑-◑-◑) of potassium phosphate. v is expressed as counts per minute of [^{14}C]bicarbonate (1.21 × 10^5 cpm/μmole) fixed per minute and RuDP concentration is expressed in molarity.

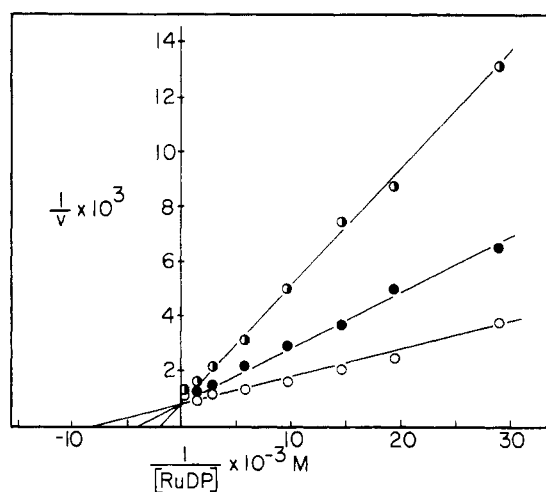

FIGURE 5: Inhibition of the RuDP carboxylase catalyzed reaction by ammonium sulfate. Standard RuDP carboxylase assays (6 min of incubation, 6.7 μg of carboxylase) were conducted in the absence of (O-O-O) and in the presence of 10 (●-●-●) and 20 mM (◑-◑-◑) ammonium sulfate. v is expressed as counts per minute of [^{14}C]bicarbonate (1.12 × 10^5 cpm/μmole) fixed per minute and RuDP concentration is expressed in molarity.

TABLE II: Summary of Kinetic Constants for the RuDP Carboxylase Catalyzed Carboxylation Reaction.

Substrate, Cofactor, or Inhibitor	K_m[a] (M)	K_i[a] (M)	V[a] (μmoles of HCO_3^- Fixed/Min μmole of Enzyme × 10^{-3})
RuDP	1.2 × 10^{-4}	...	1.27
HCO_3^-	2.2 × 10^{-2}	...	1.34
Mg^{2+}	1.1 × 10^{-3}	...	1.06
Mn^{2+}	3.9 × 10^{-5}	...	0.59
$P_i(K^+)$[b]	...	4.2 × 10^{-3}	...
$(NH_4)_2SO_4$[b]	...	8.1 × 10^{-3}	...
3-PGA[c]	...	8.3 × 10^{-3}	...
3-PGA[d]	...	9.5 × 10^{-3}	...

[a] K_m, V, and K_i were determined as described by Lineweaver and Burk (1934) and Dixon and Webb (1964b). [b] Competitive inhibitor with respect to RuDP. [c] Noncompetitive inhibitor with respect to RuDP. [d] Competitive inhibitor with respect to HCO_3^-.

activities were sufficiently sensitive to have detected 10^{-3} unit of either enzyme. Since both the isomerase and kinase have higher specific activities (Hurwitz *et al.*, 1956) than RuDP carboxylase, contamination of purified RuDP carboxylase by these enzymes is also negligible on a weight basis.

Kinetic Studies. K_m and V values for RuDP, HCO_3^-, Mg^{2+}, and Mn^{2+} were determined by Lineweaver–Burk analysis (Lineweaver and Burk, 1934; Dixon and Webb, 1964b). These kinetic constants are summarized in Table II. The K_m values found for HCO_3^- and RuDP are 2.2 × 10^{-2} and 1.2 × 10^{-4} M, respectively. These constants are in good agreement with those reported by Weissbach *et al.* (1956) and Racker (1957). RuDP becomes inhibitory at >7 × 10^{-4} M under standard assay conditions. At saturating concentrations, activation by Mn^{2+} is 56% that by Mg^{2+}. Based on maximum velocity data from Table II, pure RuDP carboxylase catalyzes the fixation of 1340 moles of HCO_3^-/min per mole of enzyme or the formation of 2600 moles of 3-PGA/min per mole of enzyme under standard assay conditions.

Orthophosphate was observed by Weissbach *et al.* (1956) to inhibit the carboxylation reaction. Since substrate itself (RuDP), which bears two phosphomonoester groups, is inhibitory in low concentration (1 mM), the nature of the inhibition by orthophosphate was investigated. It is apparent from the Lineweaver–Burk plot shown in Figure 4 that 5–10 mM orthophosphate inhibits competitively (K_i = 4.2 mM) with respect to RuDP. Furthermore, $(NH_4)_2SO_4$ also inhibits competi-

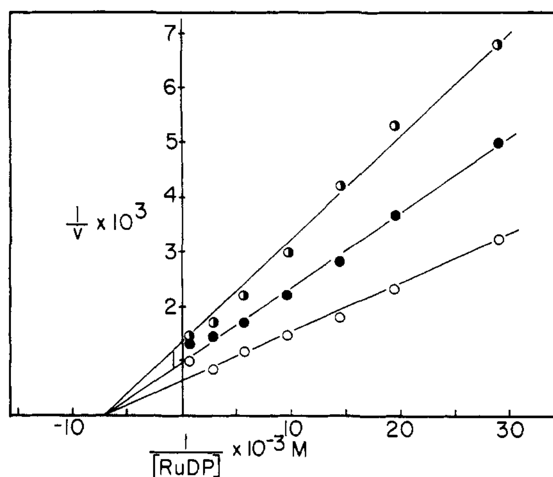

FIGURE 6: Noncompetitive inhibition of the RuDP carboxylase catalyzed reaction by 3-PGA. Standard carboxylase assays (6 min of incubation, 7.4 μg of carboxylase) were conducted in the absence of (○-○-○) and in the presence of 5 (●-●-●) and 10 mM (◐-◐-◐) 3-PGA. v is expressed as counts per minute of [^{14}C]bicarbonate (1.12 cpm $\times$ $10^5/\mu$mole) fixed per minute and RuDP concentration is expressed in molarity.

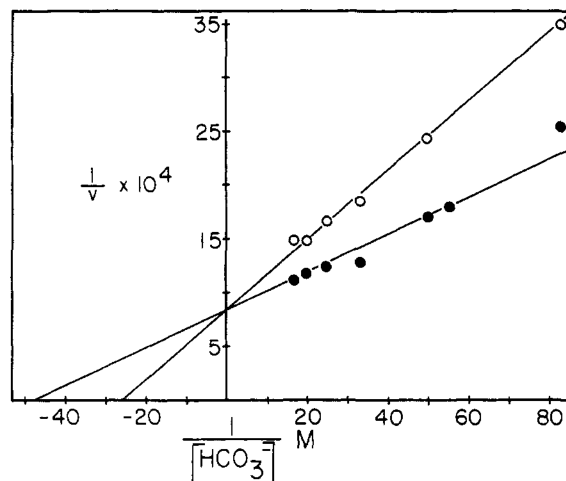

FIGURE 7: Competitive inhibition of the RuDP carboxylase catalyzed reaction by 3-PGA. Standard RuDP carboxylase assays (6 min of incubation, 7.6 μg of carboxylase) were conducted in the absence (○-○-○) and presence (●-●-●) of 8 mM 3-PGA. Final pH of reaction mixtures varied from 7.7 to 7.8. v is expressed as counts per minute of [^{14}C]bicarbonate (1.12 $\times$ 10^5 cpm/μmole) fixed per minute and HCO_3^- concentration is expressed in molarity.

tively (K_i = 8.1 mM) with respect to RuDP (see Figure 5). It seems likely, therefore, that one or both phosphate groups of RuDP are involved in binding to the enzyme. While it was thought that 3-PGA might also inhibit competitively with respect to RuDP, Figure 6 indicates that the inhibition is probably noncompetitive. Furthermore, inhibition by 3-PGA appears (Figure 7) to be competitive with respect to HCO_3^-. It is possible that the carboxylate group of 3-PGA binds to carboxylase at the bicarbonate binding site.

Discussion

RuDP carboxylase purified using the procedure described in this report appears to be homogeneous. Sedimentation velocity analysis at relatively high protein concentration gives schlieren patterns (see Figure 3) with no indication of heterogeneity. To our knowledge, schlieren patterns indicative of RuDP carboxylase preparations of comparable purity have not appeared in the literature. As pointed out in the section on molecular weight determination by sedimentation equilibrium, the linear plots obtained for log Rayleigh fringe displacement *vs.* x coordinate variable are also indicative of homogeneity. Two enzymatic activities, 5-phosphoriboisomerase and 5-phosphoribulokinase, which frequently contaminate RuDP carboxylase or leaf "fraction I protein" preparations (Wildman *et al.*, 1961; Mendiola and Akazawa, 1964), were completely absent from RuDP carboxylase purified by the procedure described in this paper. Carbohydrate has been detected (Akazawa *et al.*, 1965, Thornber *et al.*, 1965) in purified leaf "fraction I protein." Akazawa *et al.* (1965) have presented evidence suggesting that fraction I protein isolated from rice leaves is a glycoprotein. Carbohydrate analyses conducted by Dr. Alan Rutner in our laboratory on purified RuDP carboxylase revealed only traces of carbohydrate which could conceivably have been due to carbohydrate carry-over from preceding steps involving Sephadex and DEAE-cellulose. Dialyzed 10-mg samples of carboxylase analyzed by the quantitative phenol–sulfuric test (Montgomery, 1961) were found to contain no greater than 2 moles of monosaccharide equivalent/mole of carboxylase. We conclude, therefore, that RuDP carboxylase does not contain an oligosaccharide component.

The pure carboxylase has a relatively low molecular activity (1300 moles of RuDP carboxylated/minute per mole of enzyme at 30°) even at saturating bicarbonate concentrations (*i.e.*, 50 mM). At physiological bicarbonate concentrations, which must be considerably <50 mM, the catalytic activity would still be lower. It has been pointed out by Weissbach *et al.* (1956) that this may be compensated for by the high concentration of RuDP carboxylase in leaf extracts. From the purification data summarized in Table I, it can be calculated that approximately 16% of the protein in the initial spinach leaf homogenate can be accounted for as RuDP carboxylase.

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