which can dissociate at low enzyme concentration is being investigated. Accurate molecular weight determinations will be required, however, for elucidation of the nature of the monomer–oligomer transition.

Binding studies are currently being carried out and the preliminary results are consistent with the scheme in Figure 10; the presence of ATP or ornithine greatly decreases the apparent affinity of the enzyme for UMP, and the presence of UMP decreases the apparent affinity of the enzyme for ornithine (P. M. Anderson and J. Demetriou, 1969, unpublished data).

It is apparent from these studies that the observed properties of this enzyme are very dependent on the temperature, enzyme concentration, buffer used, and the presence of substrates or allosteric effectors, and that results obtained under different conditions should be compared with appropriate caution.

Acknowledgments

We thank Mrs. Patricia Wasmund for her skillful assistance in preparing the purified enzyme used in this work.

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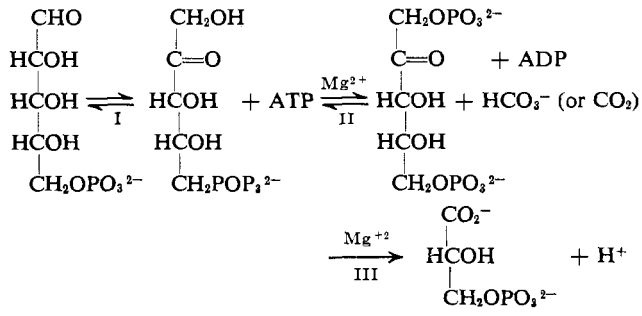
Spinach 5-Phosphoribose Isomerase. Purification and Properties of the Enzyme*

Alan C. Rutner

ABSTRACT: Spinach leaf 5-phosphoribose isomerase has been purified 2800-fold to a homogeneous state free of 5-phosphoribulokinase and ribulose diphosphate carboxylase. The isomerase has a sedimentation coefficient ($s_{20,w}$) of 4.10 S and a molecular weight by sedimentation equilibrium of 53,200 ($\bar{v}$ = 0.749). The pure enzyme catalyzes the isomerization of 1.21×10^5 moles of D-ribose 5-phosphate to D-ribulose 5-phosphate/min per mole of enzyme at pH 7.1 and 37° and the K_m for ribose 5-phosphate is 4.6×10^{-4} M. The enzyme is weakly inhibited by high concentrations of inorganic phosphorus, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, and *p*-mercuribenzoate. No evidence has been found for the existence of more than one form of 5-phosphoribose isomerase in spinach leaves.

The isolation and partial characterization of 5-phosphoribose isomerase (D-ribose 5-phosphate ketol-isomerase EC 5.3.1.6) from photosynthetic organisms has been reported by a number of investigators (Axelrod and Jang, 1954; Hurwitz *et al.*, 1956; Anderson *et al.*, 1968; Pon and Knowles, 1968). This enzyme catalyzes the first reaction (reaction I) of the carboxylative phase of the photosynthetic carbon reduction cycle. Reaction II is catalyzed by 5-phosphoribulokinase and reaction III by ribulose diphosphate carboxylase. Although the enzyme has been known for many years and the mechanism of the reaction has been studied (McDonough and Wood, 1961; Rose, 1962) little is known concerning the physical properties of the isomerase.

The initial objective of this investigation was to devise a



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mild procedure for the preparation of homogeneous spinach leaf 5-phosphoribose isomerase in sufficient quantity for physical characterization of the enzyme. In the present communication the isolation, physiochemical properties, and some kinetic parameters of the homogeneous enzyme are reported.

Experimental Procedure

Materials. R-5-P·Na_2[1] and crystalline bovine serum albumin were obtained from Sigma Chemical Co. and GSH from Schwarz BioResearch, Inc. DEAE-cellulose (Type 20) and hydroxylapatite (Bio-Gel HT) were products of Carl Schleicher & Schuell Co., and Bio-Rad Laboratories, respectively. DE-52 cellulose was obtained from Reeve Angel; Polyclar AT (insoluble polyvinylpyrolidone) was a gift from General Aniline and Film Corp. [^{14}C]$NaHCO_3$, hexokinase, and ATP were obtained from New England Nuclear Corp., Boehringer-Mannheim Corp., and P-L Inc., respectively.

Methods. Protein was routinely determined by the biuret reaction (Gornall *et al.*, 1949) using crystalline bovine serum albumin as standard. The reaction mixture contained 0.8 ml of biuret reagent and 0.1–1.5 mg of protein, in a total volume of 1.0 ml. After 30 min at room temperature the absorbance at 540 mμ was measured. As described in a later section, the relationship between protein concentration of pure R-5-P isomerase determined refractometrically and by the biuret method is given by concentration (refractometric method) = 0.907 × concentration (biuret method). An absorbance of 1.00 at 280 mμ (1-cm light path) for the pure enzyme is equivalent to 2.30 mg of refractometrically determined protein/ml.

R-5-P Isomerase Assay. The assay procedure was modified from that described by Hurwitz *et al.* (1956). The complete reaction mixture contained the following components (in micromoles): imidazole (Cl^-) buffer (pH 7.10), 32; R-5-P, 2.0; GSH, 4; EDTA, 4; and R-5-P isomerase, up to 1.5 units in a total volume of 0.8 ml. Isomerase was diluted as necessary immediately before assay with 0.05 M Tris (Cl^-) (pH 7.5), 1 mM EDTA, 10 mM GSH; 0.01–0.05-ml aliquots of enzyme were added to each tube. The presence of EDTA and a thiol were essential to preserve active isomerase in dilute solution. After incubation for 10 min at 37°, the reaction was stopped by addition of 5 ml of 70% (v/v) H_2SO_4 followed by 0.2 ml of 1.2% cysteine–HCl and 0.2 ml of 0.1% carbazole (recrystallized from absolute ethanol). The mixture was thoroughly mixed by inversion, incubated at 50° for 30 min (Lim and Cohen, 1966), and then read immediately in a Klett photoelectric colorimeter with a No. 540 filter. The isomerization rate was linear up to a level of approximately 1.2–1.5 units of isomerase. One unit of R-5-P isomerase activity is defined as that amount of enzyme which catalyzes an increase of 100 Klett scale divisions in 10 min under the assay conditions described. A factor for converting units into μmoles of Ru-5-P formed in in 10 min was obtained as follows. Several concentrations of R-5-P were incubated with an excess of isomerase to give the equilibrium mixture of aldose and ketose (24.4% conversion into Ru-5-P at 37°, Axelrod and Jang, 1954) and the cysteine–carbazole color yield was measured as described above. These values were corrected for blanks due to the calculated residual concentration of aldose to yield the relationship, units × 0.0489 = μmoles of Ru-5-P formed/10 min.

Ribulose Diphosphate Carboxylase and 5-Phosphoribulokinase Assays. RuDP carboxylase was prepared and assayed as described by Paulsen and Lane (1966). The limit of detection under present conditions was approximately 1×10^{-3} unit. Ru-5-P kinase assays were conducted by coupling RuDP formation from enzymatically synthesized Ru-5-P to the RuDP carboxylase assay system (reactions II and III). Kinase contamination of the carboxylase preparation was determined as follows. The following components (in micromoles unless specified) were incubated in 0.43 ml: Tris (Cl^-) buffer (pH 7.8), 100; $MgCl_2$, 5; EDTA, 0.03; GSH, 3; R-5-P, 2; R-5-P isomerase, 10 μg (~13 μmole/min). After incubation for 15 min at 37°, to reach equilibrium in the isomerization reaction, the reaction mixture was heated for 5 min at 90°. The solution was cooled to 37° and the following components added: ATP, 2 μmoles; [^{14}C]$KHCO_3$ (2 μCi), 25 μmoles; RuDP carboxylase, 0.5 unit, to produce a final volume of 0.5 ml. After a 15-min incubation, 0.2 ml of 6 N HCl was added to stop the reaction and the number of micromoles of [^{14}C]-3-PGA formed was determined. Kinase activity associated with these samples of RuDP carboxylase was less than 1 mμmole of 3-PGA formed/min per 0.5 unit of carboxylase. Kinase activity present in the isomerase preparation was estimated by two procedures. First, the complete reaction mixture (containing R-5-P, isomerase, [^{14}C]$KHCO_3$, and carboxylase) was incubated for 15 min at 37°. The reaction was stopped with HCl, and the acid-stable radioactivity determined. Radioactivity in excess of that found with carboxylase alone (or in the two-step assay system described above) was due to kinase present in the isomerase sample. In the second procedure, isomerase was incubated with the complete reaction mixture, minus carboxylase, for 15 min at 37°, then 5 μmoles of glucose and 3 units of hexokinase were added, incubated for an additional 15 min to remove ATP. Finally, carboxylase was added and $H^{14}CO_3^-$ incorporation into acid-stable form determined. In this assay, RuDP synthesis occurred prior to the removal of ATP, and was, therefore, independent of the subsequent carboxylase addition.

Purification of R-5-P Isomerase. All operations were carried out at approximately 4°. Unless otherwise indicated, all buffers contained 1 mM EDTA and 10 mM 2-mercaptoethanol. The results of the purification procedure are summarized in Table I.

Initial Extract. Fresh spinach, purchased locally, was destemmed, washed with cold running tap water, and drained. Leaves (2400 g) were homogenized in four batches with 8 l. of 0.01 M potassium phosphate (pH 7.2) (mercaptoethanol omitted) in a 4-l. capacity Waring Blendor for 1.5 min. The brei was filtered through S. & S. No. 588 fluted filter paper.

Ammonium Sulfate Fractionation. The initial extract was brought to 35% saturation with solid ammonium sulfate (209 g/l. of extract) and, after stirring for 30 min, was centrifuged at 13,000*g* for 30 min. The dark green precipitate was discarded and the supernatant solution brought to 65% saturation with solid ammonium sulfate (200 g/l.). After centrifugation as described above, the precipitate was dissolved in a minimal volume (*ca.* 50 ml/600 g of leaves) of 5 mM phosphate buffer (pH 7.2) (mercaptoethanol omitted) and dialyzed overnight against 20 l. of the same buffer. Following dialysis, the

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: R-5-P, ribose 5-phosphate; Ru-5-P, ribulose 5-phosphate; RuDP, ribulose 1,5-diphosphate; 3-PGA, 3-phosphoglyceric acid; PVP, polyvinylpyrrolidone.

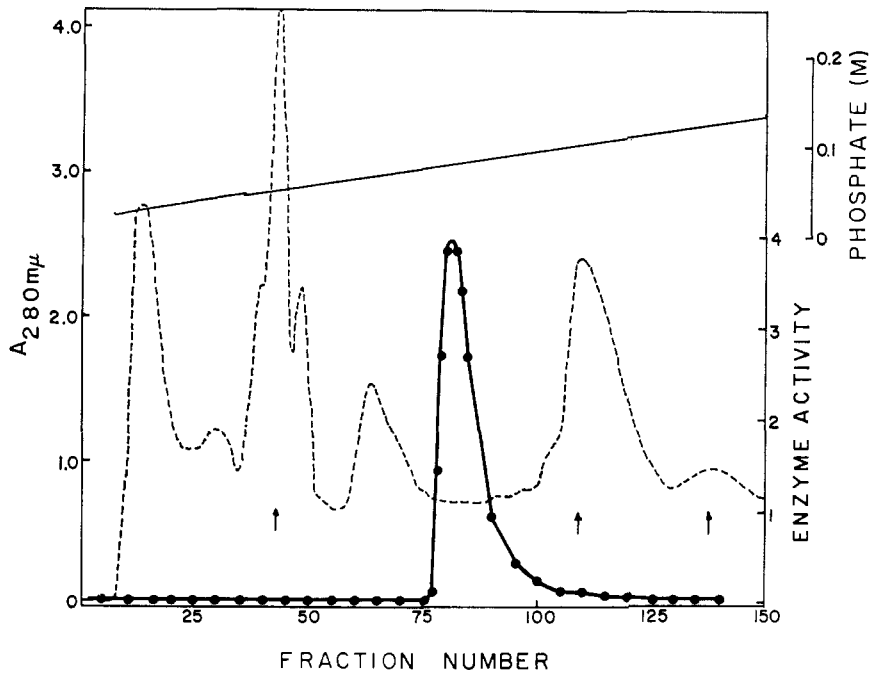

FIGURE 1: DEAE-cellulose chromatography of R-5-P isomerase. The 280-mμ absorbance (----) of each fraction of the column effluent was measured using a Zeiss PMQ II spectrophotometer. An aliquot from each fraction was assayed for R-5-P isomerase activity. Isomerase activity is plotted (-○-) as enzyme units per 0.1-μl aliquot (see Methods). The calculated phosphate buffer gradient is shown (—). The vertical arrows (from left to right) indicate the elution positions of the 280-mμ absorbing material removed by Polyclar AT, RuDP carboxylase, and Ru-5-P kinase, respectively. Fractions 79–90 were pooled and protein was collected as described in the text.

TABLE I: Purification of 5-Phosphoribose Isomerase.

Step	Protein (mg)[a]	Units	Specific Activity[b]	Purification	Yield (%)
Extract[c]	136,248	2.18×10^7	1.60×10^2	1	100
Ammonium sulfate I	17,840	2.00×10^7	1.12×10^3	7	92
Ammonium sulfate II	1,660	1.28×10^7	7.68×10^3	48	59
DEAE-cellulose	63.5	0.63×10^7	9.93×10^4	622	29
Hydroxylapatite	13.9	0.39×10^7	2.80×10^5	1753	18
DE-52-cellulose	5.77	0.26×10^7	4.44×10^5	2775	12

[a] Protein determined by the Biuret method (Gornall *et al.*, 1949). [b] Specific activity is units per milligram. Expressed in terms of micromoles per milligram per minute, the specific activity of the pure enzyme is 2171. [c] Extract of 2400 g of spinach leaves.

suspension was centrifuged at 13,000*g* for 30 min and the green precipitate discarded. The supernatant solution referred to as ammonium sulfate I was brought to 30% saturation with solid ammonium sulfate (176 g/l.), and the dark green precipitate removed by centrifugation. The supernatant solution was then brought to 65% saturation with solid ammonium sulfate (235 g/l.) and centrifuged as before. The precipitate recovered after centrifugation was suspended in 2.36 M buffered ammonium sulfate [313 g of salt brought to a volume of 1 l. at 25° with 0.1 M potassium phosphate buffer (pH 7.0); mercaptoethanol omitted], vigorously stirred for 30 min, and centrifuged. The supernatant solution was discarded and the precipitate resuspended in 1.75 M ammonium sulfate [231 g of salt brought to a volume of 1 l. at 25° with 0.1 M of potassium phosphate buffer (pH 7.0), without mercaptoethanol]. After extracting and centrifuging as above, the supernatant (1.75–2.36 M ammonium sulfate soluble fraction) solution was passed through a 4 × 6 cm column of PVP equilibrated with buffered 1.75 M ammonium sulfate. While treatment with PVP is not essential for the ultimate purification of R-5-P isomerase, this step removes approximately one-half of the 280-mμ absorbance (Loomis and Battaile, 1966) and improves the resolution obtained in subsequent chromatographic steps. The eluate was concentrated by adding 200 g of ammonium sulfate (65% saturation) per liter of solution and centrifuging. The precipitate was dissolved in a minimal volume of 0.025 M phosphate buffer (pH 7.2) (about 50 ml), dialyzed overnight against 4 l. of the same buffer, and then clarified by centrifugation (27,000*g* for 15 min). The dialyzed protein was designated as the ammonium sulfate II fraction.

DEAE-Cellulose Chromatography. DEAE-cellulose was prewashed with 15 volumes of 0.5 N HCl, water, 0.5 N NaOH, and water, and titrated to pH 7.2 with KH_2PO_4. The washed cellulose was suspended in 0.25 M phosphate buffer (pH 7.2) (no mercaptan), freed of "fines" by decantation, degassed on a water pump, and poured into chromatography columns (2.2 × 80 cm). The column bed was then equilibrated with 0.025 M phosphate buffer (pH 7.2). After application of the dialyzed ammonium sulfate II enzyme solution to the column, a 3-l. linear gradient [0.025 M and 0.175 M phosphate buffers

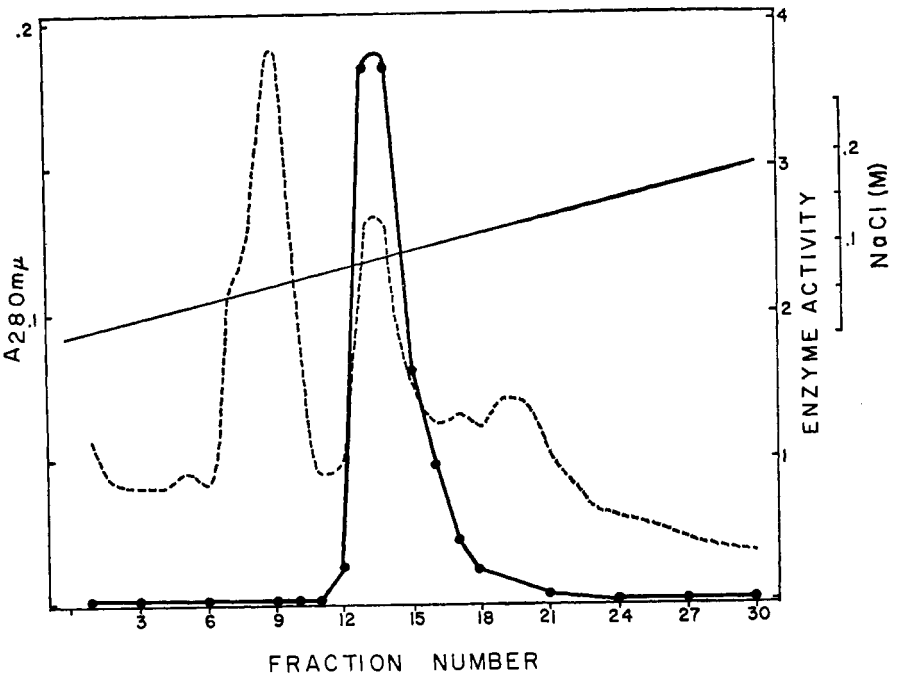

FIGURE 2: DE-52 chromatography of R-5-P isomerase. The 280-mμ absorbance (----) of each fraction of the column effluent was measured using a Zeiss PMQ II spectrophotometer. An aliquot from each fraction was assayed for R-5-P isomerase activity. Isomerase activity is plotted (-O-) as units per 0.01-ml aliquot (see Methods). The calculated NaCl gradient is shown (—). Fractions 12–15 were pooled and protein was collected as described in the text.

(pH 7.2)] was used to elute the isomerase. The column effluent was collected fractionally and was monitored for protein (by 280-mμ absorbance) and R-5-P isomerase activity. Fractions containing in excess of 10^4 units ml^{-1} were pooled (Figure 1) and dialyzed against ammonium sulfate to produce 70% saturation at equilibrium [700 ml of saturated ammonium sulfate, 100 ml of 1 M phosphate buffer (pH 7.2), dialysis bag containing pooled fractions, and H_2O to produce a total volume of 1 l.]. The enzyme suspension was collected by centrifugation, dissolved in 6 ml of 5 mM phosphate buffer (pH 7.2), and dialyzed overnight against 500 ml of the same buffer. The dialyzed protein is referred to as the DEAE-cellulose fraction.

Hydroxylapatite Chromatography. Hydroxylapatite gel was partially freed of fines by suspending in 5 mM phosphate buffer (pH 7.2) and decanting the supernatant solution after settling for 15 min. A gel column (2 × 12.5 cm) was washed with 5 mM phosphate buffer (pH 7.2), and after the DEAE-cellulose fraction was applied, batchwise elution was carried out with successive 150-ml volumes of 0.005 M, 0.035 M, and 0.070 M phosphate buffer (pH 7.2). The column effluent was monitored for protein ($A_{253\ m\mu}$) and R-5-P isomerase activity; isomerase activity was eluted as a discrete peak with 0.070 M phosphate. Enzymically active fractions were pooled and precipitated with 70% saturated ammonium sulfate (by dialysis) as described above. The precipitated enzyme was dissolved in 3 ml of 0.01 M Tris (Cl^-) (pH 7.2) and dialyzed overnight against two 500-ml changes of the same buffer. The recovered protein is designated as the hydroxylapatite chromatography fraction.

DE-52 Cellulose Chromatography. Microangular DE-52 (DEAE-cellulose) was pretreated with 0.5 N HCl and 0.5 N NaOH as recommended in the Whatman Technical Bulletin IE2. The dialyzed hydroxylapatite fractionated enzyme was applied to a DE-52 column (1.6 × 17 cm) equilibrated with 0.01 M Tris (Cl^-) (pH 7.2). Elution was accomplished with a 900-ml linear gradient from 0 to 0.25 M NaCl; all buffers contained 0.1 M Tris (Cl^-) (pH 7.2). Fractions were collected and assayed for protein and isomerase activity as previously described. As illustrated in Figure 2, the enzyme activity was eluted as a single symmetrical peak coincident with an $A_{280\ m\mu}$ peak. Fractions containing in excess of 3 × 10^4 units/ml were pooled and precipitated by dialysis against ammonium sulfate as before. R-5-P isomerase suspensions were stored in this manner at 0–2° and were stable for at least 2 months. Table I summarizes the results obtained from the purification of R-5-P isomerase from 2.4 kg of fresh spinach leaves. More than ten preparations of homogeneous enzyme have been obtained with similar yields using this procedure.

Results

Purity, Sedimentation Velocity, and Molecular Weight. Two preparations of R-5-P isomerase purified according to the procedures outlined in the previous section (*i.e.*, through the DE-52 chromatography step, Table I) have been examined for purity using the analytical ultracentrifuge. In both cases, sedimentation patterns revealed a single symmetrical sedimentation boundary. A typical sedimentation pattern is shown in Figure 3. Sedimentation velocity experiments were conducted at 20° after overnight dialysis of the enzyme against either 0.01 M Tris (Cl^-) buffer (pH 7.5), 0.05 M NaCl, 1 mM EDTA, and 0.01 M mercaptoethanol, or 0.01 M phosphate buffer (pH 7.23), 1 mM EDTA, and 0.01 M mercaptoethanol. The sedimentation coefficient ($s_{20,w}$), was 4.10 ± 0.02 S.

More convincing evidence for the homogeneity of the enzyme was obtained by acrylamide gel electrophoresis. Samples at several stages of purification were diluted 1:1 with 50% sucrose–0.2 M mercaptoethanol and applied to gel columns having a separating gel composed of 7.0% acrylamide, with a 0.2% cross linkage, in Tris (Cl^-) (pH 8.9) (Davis, 1964). Between 10 and 200 μg of protein was applied per column and a current of 5 mA/gel was applied for 2 hr at 4°. The electro-

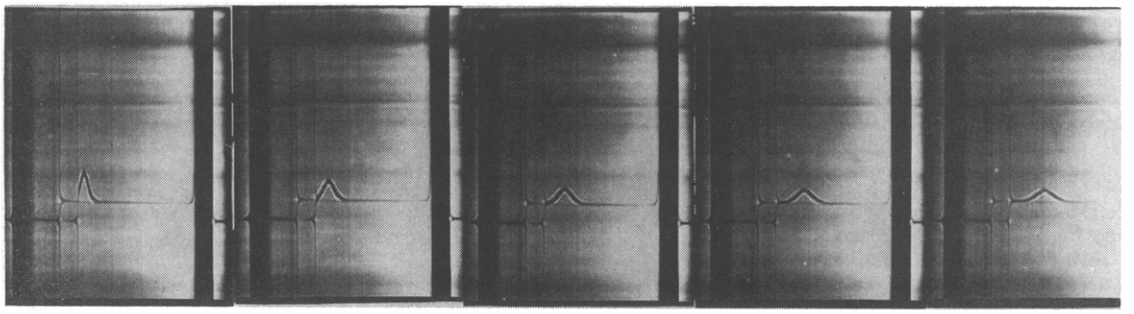

FIGURE 3: Schlieren patterns of purified R-5-P isomerase obtained in the Spinco Model E ultracentrifuge. Purified isomerase (specific activity~2000 μmole/min per mg of protein) was centrifuged at 56,100 rpm (3.0 mg of protein/ml, double sector cell) at 5°. Photographs (from left to right) were taken at 8, 16, 32, 48, and 64 min. The direction of sedimentation is from left to right. Prior to centrifugation the enzyme preparation was dialyzed to equilibrium against 0.01 M phosphate buffer (pH 7.23), 1 mM EDTA, and 0.01 M mercaptoethanol.

phoretic patterns obtained, including a representative gel of the homogeneous enzyme (gel D), are shown in Figure 4. Zones of enzymic activity were located by assaying disks cut from a gel following electrophoretic separation. Ten to forty units of isomerase, in 5 mM Tris (Cl^-) (pH 7.5) and 20% (v/v) glycerol, were applied to gel columns and subjected to electrophoretic separation as above. Immediately after shutting off the current, the gel was removed from the tube, 2-mm sequential sections were cut with a razor blade, and the disks dropped into standard assay mix. After 5 min at 37° the reaction was started with the addition of R-5-P. Figure 5 shows that the single zone of isomerase activity in the crude extract is coincident with the protein band of homogeneous enzyme.

Protein concentration was determined refractometrically from the Rayleigh fringe displacement (J_0) of the pure isomerase in a synthetic boundary cell after centrifugation (40 min at 4500 rpm and 20°) in the analytical ultracentrifuge. Protein concentration was calculated using the equation $c = \lambda J_0/a\Delta h$, where c = mg of protein/ml, λ = wavelength of light in Ångströms, J_0 = fringe displacement, a = light path through the centrifuge cell (in Ångströms), and $\Delta h = 1.873 \times 10^{-4}$ ml mg^{-1}, the specific refractometric index for an "average" protein (Perlmann and Longsworth, 1948) corrected to 20° and a wavelength of 546 mμ (Cannata *et al.*, 1965). The relationship between absorbancy at 280 mμ and the refractometrically determined protein is given by the equation $c = 2.30(A_{280\ m\mu}^{1\ cm})$; c is protein concentration in milligrams per milliliter, A is absorbancy at 280 mμ (1-cm light path). Protein concentration determined by the biuret method is converted into refractometrically determined protein by the relationship $c = 0.907\ (C_{biuret})$. The absorbancy ratio $A_{280\ m\mu}/A_{260\ m\mu}$ of the pure enzyme is 1.55. The low molar absorbance at 280 mμ suggests an unusually low aromatic amino acid content.

The molecular weight of R-5-P isomerase was determined by sedimentation equilibrium according to the method of Yphantis (1964). Enzyme from two preparations (DE-52 purified; Table I) was subjected to equilibrium centrifugation at 20° following dialysis to equilibrium against 0.01 M Tris (Cl^-) (pH 7.40), 1 mM EDTA, 10 mM mercaptoethanol, and either 0.05 M or 0.2 M NaCl. Molecular weight determinations were made at six protein concentrations between 0.25 and 1.13 mg/ml, in nine experiments at rotor speeds of 26,000 and 28,000 rpm. The partial specific volume of the isomerase was determined by the "falling-drop" technique of Linderstrom-Lang and Lanz (1938), as described by Schachman (1957). A bromobenzene–kerosene density gradient and aqueous KCl standards were employed in this procedure, yielding a partial specific volume of 0.749 for the protein in 0.01 M Tris (Cl^-) (pH 7.4), 0.2 M NaCl. These determinations resulted in a molecular weight of 53,203 ± 1,712. This value is somewhat lower than the approximation of the mol wt of 57,000 estimated from sucrose density gradient sedimentation and Sephadex chromatography data, reported for the isomerase from *Rhodospirillum rubrum* (Anderson *et al.*, 1968).

Isomerase purified through the step of hydroxylapatite chromatography was tested for the presence of RuDP carboxylase and 5-phosphoribulokinase activities using the assay methods described in Experimental Procedures. RuDP carboxylase activity was completely absent from isomerase carried through the hydroxylapatite column step. However, because of the low specificity activity of the carboxylase (1.8 units/mg; Paulsen and Lane, 1966), less than a 3% weight contaminant would not have been detectable. That this was not the case was evident from the absence of any sign of protein in polyacrylamide gels in the region where RuDP carboxylase would normally have been found (Ridley *et al.*, 1967; Rutner and Lane, 1967).

Negligible Ru-5-P kinase activity was found in the hydroxylapatite-purified isomerase; less than 0.01 unit of kinase activity/mg of isomerase protein was observed. By the two methods described (Experimental Procedure) for detecting kinase activity, it was possible to attribute the very low but measurable $H^{14}CO_3^-$ fixation to kinase contamination of the carboxylase, rather than of the isomerase.

Kinetic Properties. K_m and V_{max} values for R-5-P were determined by Lineweaver–Burk analysis (Lineweaver and Burk, 1934; Dixon and Webb, 1964). The K_m value found for R-5-P was 4.6×10^{-4} M under our assay conditions. This value is considerably lower than the K_m of 4×10^{-3} M reported for the isomerase from *R. rubrum* (Anderson *et al.*, 1968). Based on maximum velocity data, the pure R-5-P isomerase catalyzes the formation of 1.21×10^5 moles of Ru-5-P/min per mole of enzyme at pH 7.1 and 37°. If the turnover number of 2.4×10^5 moles/min per 10^5 g of protein of the alfalfa isomerase (Axelrod and Jang, 1954) and the specific activity of 5000

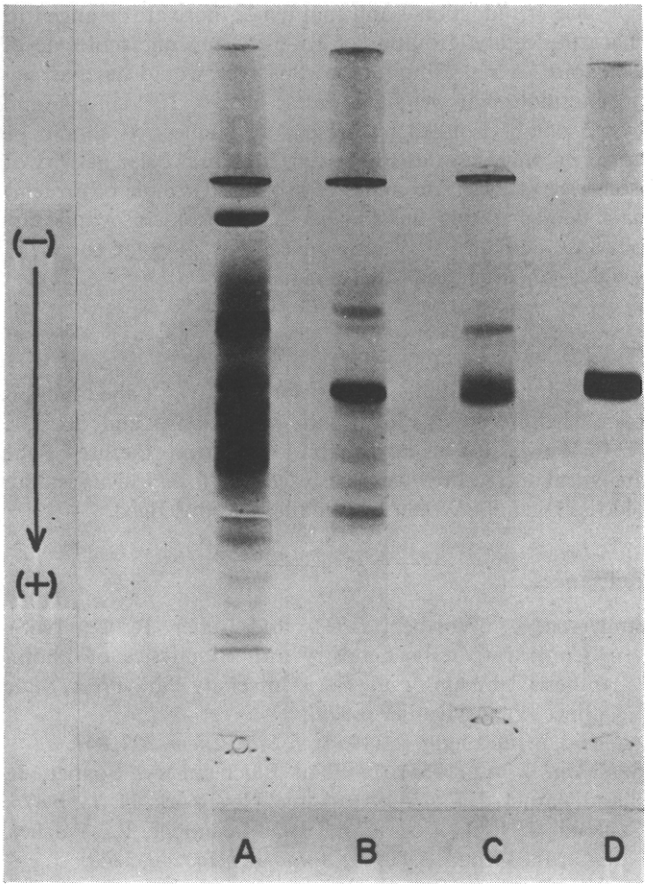

FIGURE 4: Polyacrylamide gel electrophoresis of R-5-P isomerase at four different stages of the purification. Gel A: second ammonium sulfate fraction (190 μg of protein); gel B: the enzyme after DEAE-cellulose chromatography (34 μg of protein); gel C: following hydroxylapatite chromatography (56 μg of protein); and gel D: the enzyme after DE-52 chromatography (52 μg of protein). Electrophoresis was carried out as described in the Methods section. The anode is at bottom. The gels were stained overnight with 1% Amido Black, in 50% methanol–10% acetic acid. Excess stain was removed by washing with 10% acetic acid.

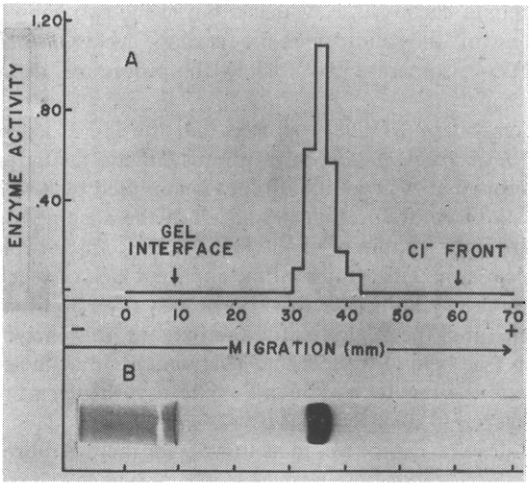

FIGURE 5: Coelectrophoretic migration of R-5-P isomerase enzymic activity and protein. Gel A: electrophoretic migration of enzyme activity. A fresh spinach extract was prepared (see Methods section) and a 5-ml aliquot dialyzed overnight against 1 l. of 5 mM potassium phosphate buffer (pH 7.2). The sample was clarified by centrifugation and 11.6 units (~70 μg of protein) of enzyme applied to the gel. For electrophoresis and assay conditions, see text. Gel B: electrophoretic migration of 5-phosphoribose isomerase. Enzyme (48 μg) purified through the DE-52 step was applied to the gel and migration effected as for gel A. Protein was visualized by overnight staining in 1% Amido Black–10% acetic acid, followed by destaining in 10% acetic acid.

Discussion

R-5-P isomerase purified using the procedure described in this paper appears to be homogeneous. Sedimentation velocity patterns (see Figure 3) show no indication of heterogeneity. As mentioned above, the linear plots of ln Rayleigh fringe displacement *vs.* radial distance are also indicative of homogeneity. Finally, only a single stained band was obtained after acrylamide gel electrophoresis of the purified enzyme. Two enzymic activities, 5-phosphoribulokinase and ribulose

units/mg (based on 1.0 $A_{280\ m\mu}$ = 1 mg of protein; Pon and Knowles, 1968) are converted into moles/min per mole of enzyme (refractometric protein, mol wt 53,200), the results of the three laboratories are in very close agreement: 1.21×10^5, 1.25×10^5, 1.13×10^5 moles/min per mole of enzyme, respectively.

The inhibitory effects of P_i, AMP, ADP, ATP, NAD, and NADP on R-5-P isomerase (Axelrod and Jang, 1954) have been reexamined and these results are summarized in Table II. It is apparent that P_i is a poor inhibitor, being without effect at a concentration of 10 mM and exhibiting approximately 50% inhibition at 50 mM. In the case of the nucleotides, all of which contain a C-1-substituted D-ribosyl 5-phosphate moiety, the inhibitory effects are essentially identical: little or no inhibition was observed at 1 mM and 30–50% inhibition at 10–15 mM. These results are in accord with the data obtained earlier for the alfalfa enzyme (Axelrod and Jang, 1954). In contrast to the report of Anderson *et al.* (1968) for the *Rhodospirillum* enzyme, evidence for an unusual inhibitory effect of either AMP or NAD was not obtained.

TABLE II: Effect of Inhibitors on Spinach 5-Phosphoribose Isomerase Activity.[a]

	% Inhibition			
Inhibitor	0.1 mM	1 mM	10 mM	50 mM
P_i			0	55
AMP		0	41	
ADP		2	47	
ATP		7	32	
NAD		0	33	
NADP		0	49	
*p*CMB	60			

[a] Each tube contained the standard assay mixture (see Methods), inhibitor, and 1.5 units of DE-52 purified isomerase added to start the reaction. Appropriate blanks (inhibitor minus enzyme) were obtained for each nucleotide. Enzyme preincubated for 10 min at 37° in the presence and absence of *p*CMB, and the reaction started by adding substrate.

diphosphate carboxylase, which catalyze the two subsequent reactions of the photosynthetic carbon cycle, are absent from R-5-P isomerase prepared by the procedures described in this paper.

Anderson *et al.* (1968) reported that the R-5-P isomerase from *Rhodospirillum rubrum* (a photosynthetic bacterium) is not inhibited at ATP and ADP, but is inhibited by AMP and NAD. We have found that P_i and all of the adenine nucleotides are poor inhibitors of the spinach isomerase (Table II). For this reason and because of the apparent lack of specificity of nucleotide inhibition (Table II), the easy reversibility of the reaction, and the high relative activity of this enzyme in spinach (see below) we feel that it is unlikely that the R-5-P isomerase is a regulatory site in the photosynthetic carbon reduction cycle in spinach.

The question can be raised as to whether there is more than a single 5-phosphoribose isomerase in the spinach. The purification scheme reported here could be selective for one enzyme species. We have been unable to obtain evidence to support this notion. Analytical acrylamide gel electrophoresis of the crude extract, AS-I fraction, and DE-52 purified enzyme from fresh spring spinach leaves, followed by isomerase assays of gel slices, reveals only one region of enzyme activity, coincident with the zone occupied by the homogeneous enzyme (Figure 5). Furthermore, at no point in the fractionation procedure was evidence obtained for more than a single 5-phosphoribose isomerase species.

Since there are now several well-documented examples of multienzyme complexes (*e.g.*, fatty acid synthetase, pyruvic dehydrogenase; Reed and Cox, 1966), there is a tendency to implicate them in other sequential biochemical reactions. The carboxylative phase of the CO_2 reduction cycle is no exception (Criddle, 1966; Bassham, 1965; Mendiola and Akazawa, 1964). We would like to point out several considerations which should be borne in mind when considering a model for a carboxylation complex in the chloroplast. First, based on the properties of the purified enzymes (RuDP carboxylase, Paulsen and Lane, 1966; Racker, 1957; R-5-P isomerase, this paper), the simplest model, *i.e.*, 1:1:1, would have a molecular weight in the order of 650,000 daltons (RuDP carboxylase, 557,000; Ru-5-P kinase, approximately 50,000; R-5-P isomerase, 53,000). However, such a complex would have a molecular activity ratio of approximately 1:3.5:100. In other words, there would be approximately 100 units of isomerase activity/unit of carboxylase, at saturating CO_2 tensions. We have calculated the enzyme molar concentration, of carboxylase and isomerase, in spinach leaves from the data of Paulsen and Lane (1966) (RuDP carboxylase) and this work (isomerase; from fresh spring spinach, total units in extract divided by the molecular activity) and arrive at a figure of one molecule of R-5-P isomerase for ten molecules of RuDP carboxylase. We are assuming, for this discussion, as the maximum limit that all the spinach leaf isomerase activity is associated with the photosynthetic carbon reduction cycle. If a carboxylative phase multifunctional enzyme complex does exist, we believe that the molar stoichiometry just discussed would require a model in which: (a) only a small percentage (perhaps 5–10%) of the total RuDP carboxylase molecules are significantly active *in vivo*, and that these molecules are part of the 1:1:1 multienzyme complex, or (b) all molecules of carboxylase are functional and that ten or more are arranged to share the catalytic output of Ru-5-P from one molecule of isomerase. A repeating unit of this type would have an aggregate molecular weight in excess of 5×10^6 daltons (and would not be isolated by present techniques). It should be borne in mind that due to the very high molecular activity of isomerase (1.21×10^5 moles of product per mole of protein) data demonstrating the coincidence of isomerase and carboxylase activities may only reflect a 1% molar (or 0.1% weight) contamination with isomerase.

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