Ribulose 1,5-Diphosphate Carboxylase from *Hydrogenomonas eutropha* and *Hydrogenomonas facilis*. I. Purification, Metallic Ion Requirements, Inhibition, and Kinetic Constants*

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ABSTRACT: Ribulose 1,5-diphosphate carboxylase has been purified to homogeneity from two chemoautotrophic microorganisms, *Hydrogenomonas eutropha* and *Hydrogenomonas facilis*. The carboxylase from *H. eutropha* was purified 26-fold and catalyzed the carboxylation of 1.9 μmoles of D-ribulose 1,5-diphosphate per min per mg of protein at pH 8.0 and 30°.

Magnesium ion was the most effective metallic cofactor. Cobaltous ion could partially substitute (11%) for magnesium ion but manganous ion was inactive. Sulfate and orthophosphate did not inhibit the enzyme. The K_m for ribulose 1,5-diphosphate was 1.25×10^{-4} M. The carboxylase from *H. facilis* was purified 20-fold to a specific activity of 1.36 by one procedure. Magnesium ion was the most effective metallic cofactor ($K_m = 1.39 \times 10^{-3}$ M). Manganous (18%) and cobaltous (9%) ions could partially substitute for magnesium ion. Both sulfate ($K_i = 5.1 \times 10^{-3}$ M) and orthophosphate ($K_i = 10 \times 10^{-3}$ M) were competitive inhibitors with respect to ribulose 1,5-diphosphate. K_m values for ribulose diphosphate and bicarbonate were 2.35×10^{-4} and 4.16×10^{-3} M, respectively. 3-Phosphoglycerate inhibited competitively with respect to bicarbonate ($K_i = 15 \times 10^{-3}$ M) and noncompetitively with respect to ribulose diphosphate ($K_i = 14.7 \times 10^{-3}$ M). Both enzymes were free of contaminating enzyme activities often associated with the enzyme from plant sources. Homogeneous preparations could be stored for more than 4 weeks at 2° in 0.02 M Tris-sulfate, containing 0.01 M $MgCl_2$, 0.05 M $NaHCO_3$, and 1 mM EDTA with no loss in activity.

Ribulose 1,5-diphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39) catalyzes the carboxylation of ribulose 1,5-diphosphate in the reductive pentose phosphate cycle of autotrophs. The isolation and partial characterization of this enzyme from spinach leaves have been reported by numerous investigators (Weissbach *et al.*, 1956; Racker, 1957; Jakoby *et al.*, 1956; Trown, 1965; Paulsen and Lane, 1966) and in recent years the mechanism of catalysis has been partially elucidated (Müllhofer and Rose, 1965; Trown and Rabin, 1964; Fiedler *et al.*, 1967). Although this enzyme was the object of numerous investigations immediately following its detection over a decade ago, only recently have Paulsen and Lane (1966) prepared enzyme that is homogeneous by rigorous criteria. Convincing evidence for a nonidentical subunit structure has been reported by Rutner and Lane (1967).

A comparison of ribulose 1,5-diphosphate carboxylase among the photosynthetic and chemosynthetic forms of life may provide important information about the evolution of autotrophism. To date, there have been few reports on the purification of ribulose 1,5-diphosphate carboxylase from bacterial sources (Gottschalk, 1964; Anderson *et al.*, 1968; Sugiyama and Akazawa, 1968; MacElroy *et al.*, 1968) and rigorous chemical and physical studies have been lacking. The purification and partial characterization of ribulose 1,5-diphosphate carboxylase from two facultative chemosynthetic bacteria, *Hydrogenomonas facilis* and *Hydrogenomonas eutropha*, are described herein. It is significant that both enzymes have been purified from fructose-grown cells.

Materials and Methods

Materials. Barium 3-phosphoglycerate was obtained from Calbiochem Co., streptomycin sulfate from Mann Research Laboratories, $Na_2{}^{14}CO_3$ (29 mCi/mmole dissolved in 1 M NaOH) from Nuclear-Chicago Corp., dibarium ribulose 1,5-diphosphate and protamine sulfate (salmon) from Sigma Chemical Co., and DEAE-cellulose from Carl Schleicher & Schuell, Co. All other compounds were of reagent grade quality. Barium salts were converted into the sodium form prior to use by addition of a slight excess of sodium sulfate with subsequent removal of precipitated Ba_2SO_4. Prior to use in the carboxylase assay, the desired amount of $Na_2{}^{14}CO_3$ was diluted in 100 volumes of 0.15 M Tris-Cl (pH 8.0) containing 0.03 M $MgCl_2$ and 0.05 M $NaHCO_3$, unless otherwise specified.

Culture Methods. The culture of *H. facilis* was a subculture of the original isolate of Schatz and Bovell (1952) and has been maintained autotrophically since 1953 with transfers at least once per month. The culture of *H. eutropha* was kindly provided by R. Repaske. Both cultures were maintained on autotrophic medium (McFadden and Homann, 1965) supplemented with trace minerals (Repaske, 1962) and grown in

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large cultures of fructose-containing medium at 30° as described previously (Kuehn and McFadden, 1968). Large 50-l. batch cultures of *H. facilis* were grown on fructose at 30° in an American Sterilizer Biogen apparatus at an aeration rate of 3 ft^3/min and agitation of 100 cycles/min. *H. eutropha* and, in some cases, *H. facilis* were cultured in 6-l. erlenmeyer flasks containing 5 l. of fructose medium. The growth rate was controlled by agitation in the range of 120–140 cycles/min. All fructose-grown cultures were harvested near midexponential phase of growth and the cells were collected by continuous-flow centrifugation of the culture suspension. Because of the marked instability of ribulose 1,5-diphosphate carboxylase in the absence of substrate *in vivo* and *in vitro* (Kuehn and McFadden, 1968), the presence of bicarbonate was essential during all cell washings and subsequent treatments of cell-free extracts. Thus the collected cell paste was washed once at 2° in five volumes of 0.02 M Tris-SO_4 (pH 8.0, 25°), containing 1 mM EDTA, 0.01 M $MgCl_2 \cdot 6H_2O$, and 0.05 M $NaHCO_3$ (henceforth referred to as TEMB buffer), followed by centrifugation at 9000*g*. The resulting cell paste could be stored indefinitely at −20° with no loss in ribulose 1,5-diphosphate carboxylase activity.

All succeeding purification procedures were carried out near 2° and the pH of Tris buffers employed for all operations was adjusted with H_2SO_4 and measured at 25°.

Purification of Ribulose 1,5-Diphosphate Carboxylase from H. facilis. Frozen cells of *H. facilis* were suspended in an equal mass of TEMB buffer containing 5 mM 2-mercaptoethanol (henceforth referred to as TEMMB buffer). The cell suspension was disrupted by a French press at 15,000 psi, the extruded mixture was clarified by centrifugation at 41,190*g* for 15 min, and the supernatant was decanted and recentrifuged at 105,000*g* for 1 hr. The resulting red-brown supernatant was drawn off the gelatinous pellet with a pipet in order to ensure exclusion of particulate material. The protein concentration was generally 25–30 mg/ml. This 105,000*g* supernatant preparation, labeled fraction S-105, was dialyzed 12–15 hr against two changes of 60 volumes of TEMMB buffer. After dialysis, the protein concentration was adjusted to 13 mg/ml with TEMMB to yield fraction $S\text{-}105_D$.

Absolute ethanol, chilled to −77° in an acetone–Dry-Ice mixture, was added dropwise with gentle stirring to fraction $S\text{-}105_D$ to a final concentration of 10% (v/v). Stirring was discontinued, the mixture was incubated at 2° for 15 min, and the precipitated protein was collected by centrifugation at 30,900*g* for 10 min, and immediately dissolved in cold 0.01 M Tris-SO_4 (pH 7.0) containing 0.01 M $MgCl_2 \cdot 6H_2O$ and 5 mM 2-mercaptoethanol (TMM buffer). The buffer volume added was approximately one-sixth that of fraction S-105 and yielded a protein concentration of 5–8 mg/ml. Most of the ethanol-precipitated material dissolved after 60–90 min with intermittent stirring at 2°. A small amount of insoluble residue remaining was pelleted by centrifugation at 105,000*g* for 1 hr yielding a pale yellow supernatant fraction, E-10.

In several experiments, ribulose 1,5-diphosphate carboxylase failed to precipitate in 10% ethanol, in which case it was recovered in the protein precipitated between 25 and 30% ethanol concentration. In those cases the protein was collected and treated as just described. Purification attempts upon the precipitate obtained at 25–30% ethanol always gave lower final yields of the enzyme.

Nucleic acid was partially removed by addition of either 2% (w/v) protamine sulfate or 10% (w/v) streptomycin sulfate to fraction E-10. The 2% protamine sulfate solution was prepared in cold TMM buffer and was adjusted to pH 7.0 by addition of 0.5 M NaOH. A small amount of precipitate that formed on adjustment to pH 7.0 was pelleted by centrifugation and discarded. The 10% streptomycin sulfate solution was similarly prepared and adjusted to pH 7.0. A systematic investigation was carried out to determine the optimal conditions for precipitation of nucleic acid. Based upon these studies the following protocols were developed.

Protamine sulfate solution was slowly added to fraction E-10 with gentle stirring in 0.025-ml increments to a final ratio of 0.10 mg of protamine sulfate/mg of protein. Stirring was discontinued and the insoluble complex was allowed to precipitate over a 15-min interval. The complex was removed by centrifugation at 30,900*g* for 10 min, the supernatant was immediately recentrifuged at 105,000*g* for 30 min, and the resulting supernatant, fraction PS, was stored overnight at 2°.

A similar procedure was followed for addition of 10% streptomycin sulfate to a ratio of 0.75 mg of streptomycin/mg of protein. The resultant supernatant fraction was labeled fraction SM.

Either fraction PS or fraction SM could be stored for weeks at 2° with little loss in ribulose 1,5-diphosphate carboxylase activity and were further purified on DEAE-cellulose using elution rates of 30–40 ml/hr.

Fraction PS was adjusted to pH 8.0 by addition of 1.0 M Tris-SO_4 (pH 8.0) and was immediately applied to a 2.3 × 35 cm DEAE-cellulose column previously equilibrated with 0.02 M Tris-SO_4 (pH 8.0) containing 0.01 M $MgCl_2 \cdot 6H_2O$ (TM buffer). Stepwise elution employing increasing sodium chloride prepared in TM buffer in steps of 0.1 M salt (from 0 to 0.3 M) was conducted. Each succeeding buffer–salt solution was applied to the column only after ultraviolet-absorbing fractions had been eluted.

After loading fraction SM as described for PS, gradient elution was accomplished by using two 600-ml beakers as a mixing chamber and reservoir. Both beakers were mounted at the same level and connected by tubing so as to maintain hydrostatic equilibrium; 400 ml of TM buffer was placed in the mixing chamber and 400 ml of 0.3 M NaCl buffered in TM was added to the reservoir.

All fractions absorbing at 254 mμ were further analyzed by measuring the absorbancy at 280 mμ and the ribulose 1,5-diphosphate carboxylase activity.

Various attempts to concentrate pooled fractions containing ribulose 1,5-diphosphate carboxylase from *H. facilis* failed to yield enzymatically active preparations. Hence the enzyme was stored at 2° at concentrations of less than 1.5 mg of protein/ml after dialysis of pooled fractions against TM buffer containing 0.05 M $NaHCO_3$ plus 1 mM EDTA. Under these conditions it was stable for at least 4 weeks.

Purification of Ribulose 1,5-Diphosphate Carboxylase from H. Eutropha. To $S\text{-}105_D$, prepared as described for *H. facilis*, 10% streptomycin sulfate solution (see previous section) was added in 0.025-ml increments to a final ratio of 0.75 mg of streptomycin sulfate per mg of protein; 15 min after the final addition, the insoluble precipitate was removed by centrifugation at 29,000*g* for 15 min. The resulting supernatant, fraction SM, was slowly brought to 0.40 ammonium sulfate saturation by addition of 0.67 volume of alkaline ammonium sulfate solution that had been saturated at 0° and the pH of which

had been adjusted to 7.8 with concentrated ammonium hydroxide. Precipitated protein was collected after 40-min centrifugation at 29,000*g* for 15 min, and the pellet was redissolved in 16 ml of TEMB buffer yielding fraction AAS.

Each of four 4.0-ml aliquots of fraction AAS was applied to the top of four 2.3 × 35 cm Sephadex G-50 gel columns equilibrated with TEMB buffer. Elution of each column was achieved with the same buffer at a flow rate of 30–40 ml/hr and 5-ml fractions were collected. The first four to five fractions after the void volume effluent containing 100% of the ribulose 1,5-diphosphate carboxylase activity were pooled, stored at 2° overnight, and then applied to a 2.3 × 35 cm DEAE-cellulose column. Elution was initially carried out with TEMB buffer containing 0.1 M NaCl until an ultraviolet-absorbing peak had been eluted. A 1-l. gradient from 0.1 to 0.3 M NaCl buffered in TEMB was then constructed as described earlier from 500-ml portions of salt–TEMB solutions. The flow rate was maintained at 30–40 ml/hr and 10-ml fractions were collected. Peak fractions which displayed ribulose 1,5-diphosphate carboxylase activity, 280 mμ/260 mμ ratios greater than 1.8, and contained more than 1.0 mg of protein/ml were pooled. The resulting solution was brought to 0.45 saturation with the saturated alkaline ammonium sulfate, and the precipitate was collected by centrifugation and dissolved in 3.3 ml of TEMB buffer. This protein solution was then subjected to descending Sephadex G-200 chromatography on a 2.3 × 50 cm column. Flow rates of 10–15 ml/hr were achieved by positioning the reservoir 15–20 cm above the effluent orifice. Elution was achieved with TEMB buffer and peak fractions containing ribulose 1,5-diphosphate carboxylase activity were again concentrated by alkaline ammonium sulfate precipitation. The protein was collected, dissolved in TEMB buffer to yield a concentration of 5–7 mg of protein/ml, and finally dialyzed against 1 l. (three changes) of the same buffer. This enzyme solution was stable for weeks when stored at 2°.

Assay. The general procedure of McFadden and Tu (1967) including preincubation was followed for the estimation of enzyme activity at 30°, pH 8.0. It was established that substrates and Mg^{2+} in 0.25 ml of assay mixture were saturating at the following levels (in micromoles): Tris-Cl, 16; $MgCl_2$, 5; $NaH^{14}CO_3$, 5; and RuDP, 0.4. Accordingly that was the composition of all assay mixtures unless otherwise specified. One enzyme unit is the amount of enzyme catalyzing the carboxylation of 1 μmole of ribulose 1,5-diphosphate in 1 min at 30°. Specific activity is expressed as enzyme units per milligram of protein and was calculated from rates that were first order with respect to enzyme concentration. Protein was estimated by the method of Lowry *et al.* (1951) with dried homogeneous ribulose 1,5-diphosphate carboxylase as the standard.

Prior to studies of the specificity of metallic ion requirements, ribulose 1,5-diphosphate carboxylase was dialyzed 24 hr in 0.01 M Tris-Cl (pH 8.0) containing 1 mM EDTA (1000 volumes, two changes). Suitable aliquots were then added to the preincubation mixture in which various metal ions had been substituted for Mg^{2+} and the normal assay was conducted.

In studies of kinetic dependence of the reaction at pH 8 the concentration of one component was varied while all other components were used at saturating concentrations. The pH optimum was defined within the limits of 4.7 and 9.55 by the use of 60 mM citrate, histidine, imidazole, and Tris buffers with assurance obtained that in no case did Mg^{2+} become limiting through coordination with the buffers.

Assays designed to demonstrate enzyme inhibition or activation were conducted in the usual manner with the exception that the compound in question was present during the preincubation and the reaction (McFadden and Tu, 1967).

Ribose 5-phosphate isomerase activity was measured by chemical detection of ribulose 5-phosphate generated from ribose 5-phosphate. Ribulose 5-phosphate was determined by the procedure of Hurwitz *et al.* (1956).

Provision of ribulose 5-phosphate for ribulose-5-phosphate kinase assays was achieved by incubation of ribose 5-phosphate with commercial phosphoriboisomerase (Sigma Chemical Co.). The ribulose 1,5-diphosphate carboxylase preparation to be tested was then added to the resultant incubation mixture and the final mixture was exposed to $H^{14}CO_3^-$. The ribulose 1,5-diphosphate carboxylase used in the assay was a highly purified preparation containing 1.35 units of carboxylase activity with no isomerase activity.

3-Phosphoglycerate kinase and 3-phosphoglyceraldehyde dehydrogenase activities were assayed by the methods of Bücher (1955) and Beisenherz *et al.* (1953), respectively.

Carbohydrate analyses were conducted by the cystein–carbazole test described by Ridley *et al.* (1967).

Phospholipid analyses were carried out on 5 mg of protein using methanolic phosphomolybdic acid (1 g/ml) reagent (Mangold, 1965).

Electrophoresis. The purity of ribulose 1,5-diphosphate carboxylases prepared from both *H. facilis* and *H. eutropha* was examined by polyacrylamide gel disc electrophoresis. Polyacrylamide gel columns were prepared with running-gel, stacking-gel, and sample-gel sections according to the general procedure of Davis (1964); 7 or 10% cross-linked cylindrical gels were used as supporting media. All running-gel stacks were prepared at pH 8.9 with runs at pH 9.5 (see instruction bulletin for the Model 6 system of Canalco, Canal Industrial Corp., Rockville, Md.). A current of less than 2 mA/gel was applied at room temperature until a tracking dye (bromophenol blue) was approximately 0.2 cm from the bottom of the gel. Gels were stained in aniline black (1 g in 200 ml of 7% acetic acid) for 1 hr and then destained electrophoretically at a current of 3 mA/gel (Schwabe, 1966).

Results

Ribulose 1,5-Diphosphate Carboxylase from H. facilis. Preliminary experiments revealed that ribulose 1,5-diphosphate carboxylase in extracts from *H. facilis* could not be submitted to standard salt fractionation with retention of activity. Hence the organic solvent precipitation method was developed. Results from one of the two general purification procedures described for the isolation of ribulose 1,5-diphosphate carboxylase from 30 g (wet weight) of *H. facilis* are summarized in Table I. Enzyme activity was recovered quantitatively in S-105 from supernatants resulting from slower centrifugation (*e.g.*, 5000*g*) indicating that it was not particle bound. The protocol for the isolation procedure included removal of nucleic acid with streptomycin sulfate followed by gradient-elution chromatography on DEAE-cellulose. The elution profile is depicted in Figure 1.

Of major interest was the high yield of enzyme activity (Table I) and the unusually selective precipitation of ribulose 1,5-diphosphate carboxylase by 10% ethanol. The polyacrylamide gel electrophoretogram of fraction SM exhibited in

TABLE I: Isolation Summary of Ribulose 1,5-Diphosphate Carboxylase from *H. facilis* (30 g wet weight).

Fraction[a]	Total Protein (mg)	$A_{280\ m\mu}/A_{260\ m\mu}$ Ratio	Sp Act. (units/mg)	Total Units	Purifcn
$S\text{-}105_D$	800	0.67	0.069	55.0	(1)
E-10	77	0.63	0.18	14.0	2.6
SM	59	0.79	0.22	13.2	3.2
DEAE-cellulose chromatography	25	1.71	1.36	23.8	20

[a] Fraction abbreviations are defined in Materials and Methods.

TABLE II: Crystallization of Ribulose 1,5-Diphosphate Carboxylase from *H. facilis*.

Fraction[a]	Total Protein (mg)	$A_{280\ m\mu}/A_{260\ m\mu}$ Ratio	Sp Act. (units/mg)	Total Units	Purifcn
$S\text{-}105_D$	760	0.72	0.017	20.4	(1)
E-10	74	0.61	0.14	10.1	8
PS	42	0.90	0.16	6.9	10
Crystals	15	1.10	0.85	12.9	50
DEAE-cellulose chromatography	8	1.09	1.45	11.5	85

[a] Fraction abbreviations are defined in Materials and Methods.

Figure 2 and the data in Table I showing slight purification in going from fraction E-10 to SM clearly demonstrate that ribulose 1,5-diphosphate carboxylase is the major protein precipitated in 10% ethanol. The total enzyme activity recovered after DEAE-cellulose chromatography was 180% in excess of the activity initially applied to the column in fraction SM. This phenomenon was observed in all isolations of ribulose 1,5-diphosphate carboxylase from *H. facilis* and suggests removal of an inhibitor during ion-exchange chromatography. The peak fractions containing ribulose 1,5-diphosphate carboxylase proved to be homogeneous (Figure 2).

The experimentally generated expression for enzyme concentration was: $OD_{500\ m\mu} = 0.894C + 0.022$, where $OD_{500\ m\mu}$ is the reading of a standard Lowry protein assay and C is the

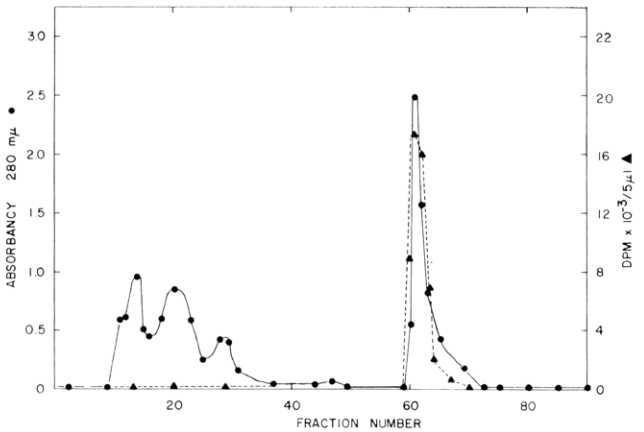

FIGURE 1: DEAE-cellulose chromatography by gradient elution of fraction SM prepared from *H. facilis* extracts. The details of chromatography are described in the text. Protein concentration is represented by closed circles. Ribulose 1,5-diphosphate carboxylase activity is expressed (▲) as disintegration (dpm) of [^{14}C]bicarbonate fixed during 5-min incubation per 5-μl aliquot from each fraction of 20 ml in a standard carboxylase assay.

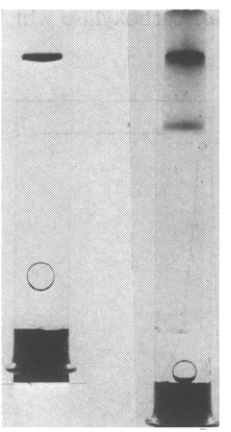

FIGURE 2: Polyacrylamide (7%) gel electrophoretograms (displayed in test tubes) of ribulose 1,5-diphosphate carboxylase from: left, *H. facilis*, carried through DEAE-cellulose chromatography (see Table I); right, *H. facilis* fraction SM. The display has been cut at a position corresponding to the origin of each running gel with the bottom of the gel resting on the stopper. Electrophoresis conditions for each gel: 60 μg of protein, 500 V, 2 mA, 80 min.

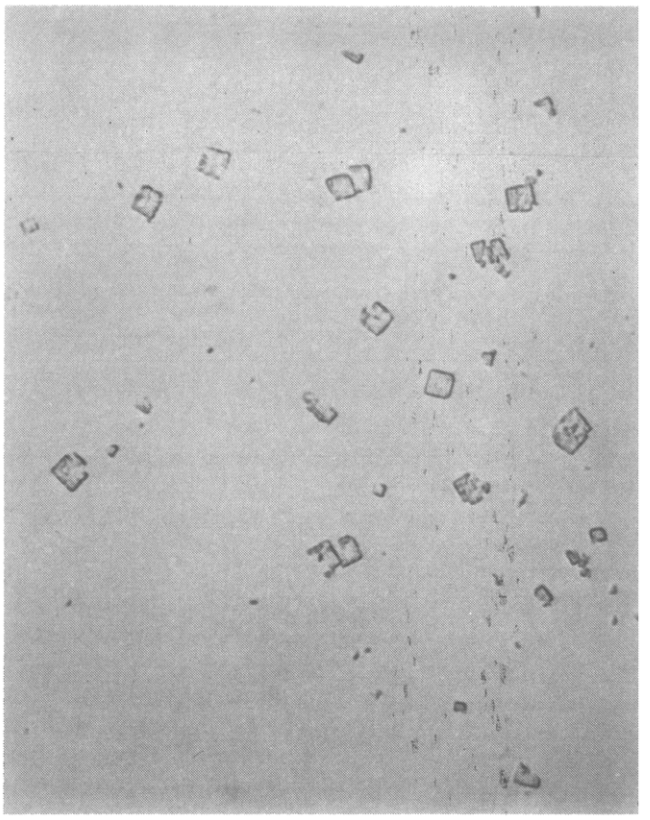

FIGURE 3: Crystalline ribulose 1,5-diphosphate carboxylase derived from fraction PS prepared from an *H. facilis* extract. Magnification: approximately 1300 ×.

concentration in milligrams per milliliter of ribulose 1,5-diphosphate carboxylase.

An alternate procedure for ribulose 1,5-diphosphate carboxylase isolation from *H. facilis* differed from the previous protocol in that nucleic acid was removed with protamine sulfate. Table II summarizes a single experiment in which ribulose 1,5-diphosphate carboxylase crystallized from fraction PS during overnight storage at 2°. Figure 3 represents a photograph of the square platelets of carboxylase which was obtained after

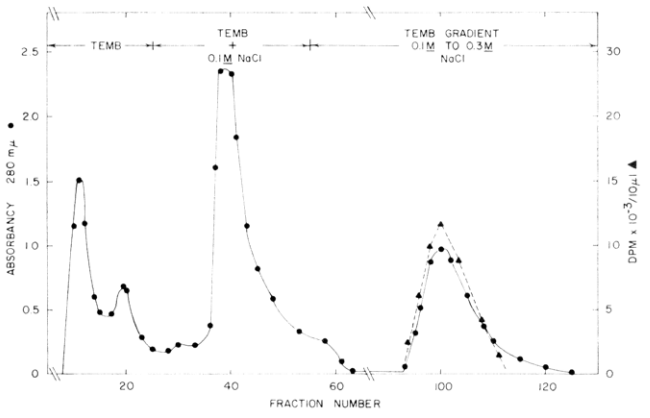

FIGURE 4: DEAE-cellulose chromatography of Sephadex G-50 fractions derived from fraction SM of *H. eutropha*. The details of chromatography are described in the text. The symbols are defined in Figure 1.

FIGURE 5: Polyacrylamide (7%) gel electrophoretogram of ribulose 1,5-diphosphate carboxylase from *H. eutropha* carried through Sephadex G-200 chromatography. Electrophoresis conditions: 60 μg of protein, 500 V, 1.4 mA, 95 min. This display has been cut at the gel bottom.

considerable difficulty due to the fragility of the crystals. After removal of the crystals by centrifugation, the mother liquor was chromatographed by stepwise elution from DEAE-cellulose as described in the preceding section and additional enzyme was recovered during elution with 0.1 M NaCl. Again, as evident from Table II, recovery of enzyme in the latter stages of purification increased markedly both with crystallization and chromatography of the mother liquor. We have no explanation for the unusually low starting specific activity (Table II).

The specific activity of the crystalline material was considerably lower than that of ribulose 1,5-diphosphate carboxylase obtained from column chromatography of the mother liquor (Table II). Both preparations, however, had lower 280 mμ: 260 mμ ratios than that of the enzyme obtained by the method described earlier (Table I) yet gave a single band at the same position upon disc gel electrophoresis as observed for the product obtained by the fractionation procedure described in Table I. In all studies of the enzyme from *H. facilis*, the product obtained by the first procedure (Table I) was used.

Ribulose 1,5-Diphosphate Carboxylase from H. eutropha. In contrast to the carboxylase from *H. facilis*, the enzyme in extracts of *H. eutropha* could be fractionated with alkaline ammonium sulfate precipitation after treatment of fraction $S\text{-}105_D$ with streptomycin sulfate. Table III summarizes data obtained for a typical isolation from 62 g (wet weight) of cells. Significant purification of the carboxylase was achieved during Sephadex G-50 chromatography by selecting only the first several fractions eluted after the column void volume. These fractions contained approximately 50% of the protein applied to the column and 100% of the carb oxylase activity. These early fractions were pooled and chromatographed on a DEAE-cellulose (Figure 4). The peak fractions were pooled, concentrated with alkaline ammonium sulfate, and further purified by Sephadex G-200 chromatography to yield a homogeneous product (Figure 5) with a specific activity of 1.9. The correla-

TABLE III: Isolation Summary of Ribulose 1,5-Diphosphate Carboxylase from *H. eutropha*.

Fractions[a]	Total Protein (mg)	$A_{280\,m\mu}/A_{260\,m\mu}$ Ratio	Sp Act. (units/mg)	Total Units	Purifcn
$S\text{-}105_D$	2695	0.53	0.081	218	(1)
SM	2690	0.89	0.080	216	1.1
AAS	1078	1.00	0.14	148	1.8
Sephadex G-50	564	1.21	0.28	155	3.7
DEAE-cellulose chromatography	88	1.87	1.62	143	22
Sephadex G-200	47	1.90	1.94	91	26

[a] Fraction abbreviations are defined in Materials and Methods.

tion between colorimetric Lowry assay and enzyme concentration (C in milligrams per milliliter) was: $OD_{500\,m\mu} = 0.830 \cdot C + 0.030$.

Stability of Ribulose 1,5-Diphosphate Carboxylase. One of the unique properties of the carboxylases is their instability in the absence of substrate, bicarbonate, both in intact cell suspensions and in extracts (Kuehn and McFadden, 1968). Hence, washing and cell rupture were routinely conducted in the presence of 0.05 M sodium bicarbonate. At any stage of the purification procedure the enzyme was safely stored at 2° in the presence of bicarbonate. Fraction E-10 and succeeding fractions prepared from *H. facilis* were also stable at 2° and pH 8.0 in the absence of bicarbonate. Similarly, fraction AAS and succeeding fractions from *H. eutropha* were stable for several weeks in the absence of bicarbonate. Although 2-mercaptoethanol, dithiothreitol, or glutathione was occasionally included in some buffers, they were not essential for retention of carboxylase activity either during isolation or storage. Thiol reagents were not required for catalytic activity. The presence of sulfate ion was important for stability of the enzyme from *H. facilis* and sulfuric acid was therefore employed in the adjustment of the pH of Tris solutions used in isolation procedures.

In the presence of 1 mM EDTA and 2–5 mM thiol, dilute enzyme solutions (0.05 mg/ml) of both proteins lost 90% of their activity at pH 8.0 in 12 hr at 2°. Activity was only partially restored (to about 75%) by addition back of 20 mM $MgCl_2$. Freezing or storage of enzyme in glycerol or sucrose at −20° resulted in loss of activity. The preferred method of storage was in TEMB buffer (pH 8.0) at 2° and a concentration of 5 mg/ml for enzyme from *H. eutropha* and at a concentration of less than 1.5 mg/ml for enzyme from *H. facilis*.

Purity. Assays for ribose 5-phosphate isomerase, ribulose 5-phosphate kinase, 3-phosphoglycerate kinase, and 3-phosphoglyceraldehyde dehydrogenase conducted on ribulose 1,5-diphosphate carboxylase from both *H. eutropha* and *H. facilis* were negative. Tests for carbohydrate and phospholipid were also negative.

Electrophoretograms of purified preparations of both enzymes yielded a single protein zone in both 7 and 10% cross-linked gels thus reducing the likelihood of a contaminant with similar electrophoretic properties but a different molecular weight (Hedrick and Smith, 1968).

The absorption spectra of the carboxylases were similar and typical of proteins. The absorbancies at 280 mμ and 25° for the enzymes from *H. eutropha* and *H. facilis* were 1.551 and 1.228, respectively, in 0.02 M Tris-sulfate containing 0.1 M $MgCl_2$ (pH 8.0, 25°) at a protein concentration of 1.0 mg/ml and a path length of 1.0 cm.

Effect of Preincubation of Assay Components upon Ribulose 1,5-Diphosphate Carboxylase Activity. The effects of preincubation of ribulose 1,5-diphosphate carboxylase with various combinations of its substrates and Mg^{2+} have been thoroughly described for the spinach enzyme (Pon *et al.*, 1963). For comparative purposes, analogous experiments were conducted with pure enzyme from *H. eutropha*. Although data are not shown in Table IV, fixation required the addition of ribulose 1,5-diphosphate. As evident (Table IV), Mg^{2+} was required for enzyme activity (expt 9). Moreover, simultaneous preincubation of dialyzed enzyme with Mg^{2+} plus $H^{14}CO_3^-$ prior to addition of ribulose 1,5-diphosphate afforded the highest rate of 3-phosphoglycerate synthesis.

Metal Ion Dependence. The metal-ion dependence of ribulose 1,5-diphosphate carboxylase has been investigated in extracts prepared from *H. facilis* (McFadden and Tu, 1967) and spinach (Pon *et al.*, 1963). After prolonged dialysis at 2° of pure carboxylase against 0.01 M Tris-sulfate (pH 8.0) containing 1 mM EDTA, 97% of the initial activity was abolished. Various metallic ions were subsequently examined for their capacity to restore ribulose 1,5-diphosphate carboxylase activity to these dialyzed samples. Mg^{2+} at 20 mM restored full activity following 25-min preincubation at 2° with buffered enzyme solution (pH 8.0) and bicarbonate. Restoration of activity by other metal ions at 20 mM compared with Mg^{2+} (100%) was as follows for enzyme from *H. facilis* and *H. eutropha*, respectively: Co^{2+}, 8 and 8; Mn^{2+}, 17 and 0; Ca^{2+}, 0 and 3. The cations Zn^{2+}, Ni^{2+}, and Cd^{2+} did not restore activity to either dialyzed enzyme.

Substrate Dependence and pH Optimum. The following proved to be saturating concentration ranges for reaction components at pH 8.0: 20–50 mM HCO_3^-, 8–50 mM Mg^{+2}, and 0.6–1.2 mM ribulose 1,5-diphosphate. In studies of kinetic dependence, rectangular hyperbolic plots were obtained for each reaction component and there was no evidence of inhibition at the higher concentrations tested. The pH–activity curves were closely similar for both ribulose 1,5-diphosphate carboxylases and showed a pH optimum of 8.0. Both curves were bell shaped and revealed no activity at a pH lower than 6

TABLE IV: Effect of Preincubation[a] with Various Combinations of Substrates and Mg^{2+} upon Ribulose 1,5-Diphosphate Carboxylase from *H. eutropha*.

Expt	Components in First Incubn at 2° (25 min)	Components in 2nd Incubn at 2° (15 min)	Zero-Time Addition	μmoles of $^{14}CO_2$ Fixed/min per mg of Enzyme
1	Enzyme, Mg^{2+}, $H^{14}CO_3^-$		Ribulose 1,5-diphosphate	1.41
2	Enzyme, ribulose 1,5-diphosphate		Mg^{2+}, $H^{14}CO_3^-$	0.61
3	Dialyzed enzyme,[b] Mg^{2+}, $H^{14}CO_3^-$		Ribulose 1,5-diphosphate	1.40
	Components in 1st Incubn at 2° (15 min)	Components in 2nd Incubn at 2° (15 min)		
4	Dialyzed enzyme,[b] Mg^{2+}	$H^{14}CO_3^-$	Ribulose 1,5-diphosphate	0.51
5	Dialyzed enzyme,[b] $H^{14}CO_3^-$	Mg^{2+}	Ribulose 1,5-diphosphate	0.39
6	Dialyzed enzyme,[b] Mg^{2+}	Ribulose 1,5-diphosphate	$H^{14}CO_3^-$	0.16
7	Dialyzed enzyme,[b] ribulose 1,5-diphosphate	Mg^{2+}	$H^{14}CO_3^-$	0.13
8	Dialyzed enzyme,[b] $H^{14}CO_3^-$	Ribulose 1,5-diphosphate	Mg^{2+}	0.15
9	Dialyzed enzyme,[b] $H^{14}CO_3^-$	No 2nd incubation	Ribulose 1,5-diphosphate	0.02

[a] Components were incubated in the first incubation at 2° in the presence of Tris-Cl (pH 8.0). After the first or first plus second incubation (as indicated), the temperature was increased to 30° over a 5-min interval and the reaction was initiated as shown. Micromoles of all components including Tris are defined in Materials and Methods (see Assay). Components were provided in the following volumes of Tris buffer (in microliters): enzyme (10), Mg^{2+} (100), $H^{14}CO_3^-$ (100), and ribulose 1,5-diphosphate (50). Buffered Mg^{2+} and $H^{14}CO_3^-$ where provided together were added in a single 100-μl addition. The final reaction volume was brought to 0.26 ml with addition of 100 μl of Tris buffer. [b] Stock enzyme that had been stored at 2° in 0.01 M Tris-sulfate (pH 8.0, 25°) containing 2 mM 2-mercaptoethanol plus 0.01 M $MgCl_2$ was dialyzed exhaustively against the same storage buffer lacking Mg^{2+} and containing 1 mM EDTA.

and higher than 9.5. Some of the kinetic constants for reaction components for the enzyme from *H. facilis* are summarized in Table V.

Effect of Sulfate and Orthophosphate. Purification trials for enzyme from *H. facilis* suggested that concentrations of sulfate or orthophosphate lower than 0.02 M protected the enzyme against inactivation during isolation. Although ammonium sulfate and sodium sulfate were found to competitively inhibit the carboxylase from *H. facilis* with respect to ribulose 1,5-diphosphate (Table V), in routine assays sulfate ion in the enzyme preparation was diluted to a noninhibitory level. Potassium phosphate inhibited analogously (Table V).

In contrast, with ribulose 1,5-diphosphate carboxylase from *H. eutropha* sulfate at concentrations up to 20 mM has little or no effect and orthophosphate at 5–10 mM stimulated the carboxylation reaction twofold.

Product Inhibition of Ribulose 1,5-Diphosphate Carboxylase from H. facilis. Since 3-phosphoglycerate contains a phosphate moiety, it was thought that it might also inhibit competitively with respect to ribulose 1,5-diphosphate. Instead, 3-phosphoglycerate inhibition appeared to be competitive with respect to bicarbonate suggesting that the carboxylate group of 3-phosphoglycerate binds to ribulose 1,5-diphosphate carboxylase at the bicarbonate binding site (Table V).

TABLE V: Summary of Kinetic Constants for the Carboxylation Reaction Catalyzed by Ribulose 1,5-Diphosphate Carboxylase from *H. facilis*.

Substrate or Inhibitor	K_m (M)[a]	K_i (M)[a]
Ribulose 1,5-diphosphate	2.35×10^{-4}	
HCO_3^-	4.16×10^{-3}	
Mg^{2+} (as $MgCl_2 \cdot 6H_2O$)	1.39×10^{-3}	
K_2HPO_4		10.0×10^{-3}
$(NH_4)_2SO_4$[b]		5.1×10^{-3}
Na_2SO_4[b]		5.1×10^{-3}
3-Phosphoglycerate[c]		15.0×10^{-3}
3-Phosphoglycerate[d]		14.7×10^{-3}

[a] K_m and K_i were determined as described by Dixon and Webb (1964). [b] Competitive inhibitor with respect to ribulose 1,5-diphosphate. [c] Competitive inhibitor with respect to HCO_3^-. [d] Noncompetitive inhibitor with respect to ribulose 1,5-diphosphate.

Discussion

The present studies represent the first isolation of pure ribulose 1,5-diphosphate carboxylase from chemosynthetic bacteria. It is a major protein that comprises 3.8–5% of the soluble protein in derepressed *Hydrogenomonas*. This is not surprising when one considers that in higher autotrophic species fraction I protein, consisting largely of ribulose 1,5-diphosphate carboxylase, comprises up to 55% of the leaf cytoplasmic proteins (Dorner *et al.*, 1957). In the present work advantage has

been taken of the fact that culturing of *Hydrogenomonas* on fructose with slow shaking conserves synthesis of the carboxylase (Kuehn and McFadden, 1968). It is of deep interest that the enzyme can be isolated after heterotrophic culture since it has no known function in heterotrophic metabolism. It is of practical significance because of the extreme difficulty in mass culturing the hydrogen bacteria autotrophically.

The isolation and partial characterization of ribulose 1,5-diphosphate carboxylase from *H. facilis* and *H. eutropha* provides the first opportunity for comparison of these catalysts with those from photosynthetic species. To date, only partially purified carboxylases from a few autotrophic bacteria have been investigated (Gottschalk, 1964; Anderson *et al.*, 1968; Sugiyama and Akazawa, 1968; MacElroy *et al.*, 1968) and there exists a paucity of information about the bacterial enzyme. Hence, comparisons discussed below are largely restricted to those between the present enzymes and the spinach enzyme.

The results described establish the absence in either ribulose 1,5-diphosphate carboxylase of contaminating enzymes that are frequently associated with the plant enzyme (Weissbach *et al.*, 1956; Lyttleton and T'so, 1958; Park and Pon, 1961) and assays failed to detect significant activities of ribose 5-phosphate isomerase, 3-phosphoglycerate kinase, phosphoglyceraldehyde dehydrogenase, or ribulose 5-phosphate kinase. The latter enzyme cofractionates with ribulose 1,5-diphosphate carboxylase from the thiobacilli (MacElroy *et al.*, 1968). The ultraviolet absorption spectra of both carboxylases from *Hydrogenomonas* show absorbancy maxima at 280 mμ and 280 mμ/260 mμ ratios greater than 1.7, indicating that there is little or no associated nucleic acid. Similarly, the enzymes are apparently free of carbohydrate and phospholipid which also frequently accompany ribulose 1,5-diphosphate carboxylase preparations from spinach (Ridley *et al.*, 1967). These observations coupled with more convincing results with disc electrophoresis in gels of different concentrations and with results from sedimentation equilibrium and velocity studies (Kuehn and McFadden, 1969) constitute excellent evidence that the procedures described here yield a single, homogeneous protein. The apparent differences between the plant and hydrogenomonad enzymes may only be due to differences in the state of purity. There are numerous similarities. For example, in accord with observations for the spinach enzyme (Pon *et al.*, 1963) ribulose 1,5-diphosphate carboxylase from *H. eutropha* catalyzes phosphoglycerate formation at a maximal rate from ribulose 1,5-diphosphate plus a preformed magnesium ion–bicarbonate complex.

Differences in modes of inhibition of both carboxylases indicate subtle differences in the structures of the two proteins. Sulfate ion competitively inhibits the *H. facilis* carboxylase at fairly low concentrations but protects it against inactivation during isolation procedures. Orthophosphate at pH 8.0 and at low concentrations (<2 mM) has a similar effect. However, at higher concentrations such as are required for salt fractionation both potassium phosphate and ammonium sulfate irreversibly inactivate ribulose 1,5-diphosphate carboxylase from *H. facilis*. In direct contrast, the enzyme from *H. eutropha* retains complete activity after exposure to high concentrations of ammonium sulfate. Stimulation of activity was observed by 5 and 10 mM orthophosphate at pH 8.0 and 20 mM sulfate was noninhibitory. These observations suggest different environments at or near the active site of these two carboxylases.

In comparative terms, the enzyme from *H. facilis* much resembles the spinach enzyme. Thus, Weissbach *et al.* (1956) reported that phosphate inhibited spinach carboxylase and Paulsen and Lane (1966) subsequently established that phosphate and sulfate competitively inhibited with respect to ribulose 1,5-diphosphate.

Differences in metal ion requirements and specific activities provide additional evidence for subtle dissimilarities between the two hydrogenomonad carboxylases. Both enzymes exhibit absolute divalent metal ion requirements and Mg^{2+} is the most effective metallic ion of those examined. However, Mn^{2+} will not replace Mg^{2+} with the enzyme from *H. eutropha*, but partially substitutes with the carboxylase from *H. facilis*. Calcium ion slightly stimulates the former but is inactive with enzyme from *H. facilis*. Zn^{2+}, Cd^{2+}, and Ni^{2+} failed to replace Mg^{2+} with either enzyme. In comparison, Mg^{2+} stimulates activity with the spinach enzyme, Ni^{2+} may stimulate slightly, and Mn^{2+} and Co^{2+} are inactive (Sugiyama *et al.*, 1968). A report by Anderson *et al.* (1968) that the carboxylase from *R. rubrum* does not require a metal ion for activation has recently been questioned (Sugiyama and Akazawa, 1968).

The present investigations of the kinetic dependence upon HCO_3^- and ribulose 1,5-diphosphate were only conducted at saturating concentrations of Mg^{2+} and at a pH of 8.0, the pH optimum under conditions of saturation with respect to all reaction components. It will be interesting to see whether the hydrogenomonad enzymes show the homotropic interactions for HCO_3^- particularly apparent at certain Mg^{2+} concentrations and pH values with the spinach enzyme (Sugiyama *et al.*, 1968). Mg^{2+}- and pH-dependent homotropism for ribulose 1,5-diphosphate has also been observed with the partially purified enzyme from one of the photosynthetic bacteria, *Rhodopseudomonas spheroides* (Sugiyama and Akazawa, 1968). A cataloging of ligand–ligand interactions may prove of deep interest in probing the evolution of these catalysts and their regulation.

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