Ribulose 1,5-Diphosphate Carboxylase from *Hydrogenomonas eutropha* and *Hydrogenomonas facilis*. II. Molecular Weight, Subunits, Composition, and Sulfhydryl Groups*

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ABSTRACT: Numerous chemical and physical properties of homogeneous ribulose 1,5-diphosphate carboxy-lyase isolated from *Hydrogenomonas eutropha* and *Hydrogenomonas facilis* are reported. Meniscus-depletion sedimentation equilibrium experiments yielded an average molecular weight value of 515,000 for the carboxylase from *H. eutropha*. The $s_{20,w}$ was 20×10^{-13} sec and the calculated $D_{20,w}$ was 3.7×10^{-7} cm² per sec.

The average subunit molecular weight determined in 3, 4, and 6 M guanidinium chloride or 8 M urea was 40,700. Subunits formed in the presence of 0.03% sodium dodecyl sulfate or 8 M urea were homogeneous by the criterion of gel electrophoresis at pH 9.5. Amino acid analyses yielded all the amino acids commonly found in proteins. Titration of the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) reagent in 8 M urea yielded 35 free sulfhydryl groups/mole of enzyme. A total of 97 half-cystines/mole of enzyme was inferred from amino acid analysis of performic acid treated and hydrolyzed enzyme. Analogous experiments performed on carboxylase isolated from *H. facilis* yielded an average molecular weight of 551,000 and an average subunit molecular weight of 38,000. The amino acid analyses of the two carboxylases were closely similar; 93 half-cystines were recovered per mole of enzyme for the carboxylase from *H. facilis* of which 37 apparently existed as cysteine residues. Comparisons between these two proteins and carboxylases from other sources are discussed.

The isolation of ribulose diphosphate carboxylase (3-phospho-D-glycerate carboxylyase (dimerizing), EC 4.1.1.39) in homogeneous form from both *Hydrogenomonas eutropha* and *Hydrogenomonas facilis* is described in the preceding paper (Kuehn and McFadden, 1969). The specific activities of both enzymes (1.4–1.9 units/mg of protein) are comparable with that of ribulose diphosphate carboxylase from spinach (Paulsen and Lane, 1966). Many of the kinetic constants of the hydrogenomonad enzymes are also similar to those reported for this catalyst from higher plants. However, variations in metal ion requirements and kinetic responses to orthophosphate and sulfate suggested subtle differences between the two carboxylases from *Hydrogenomonas*. In light of this, the studies of amino acid composition, subunit structure, and molecular weight described in the present report are of particular interest.

Materials and Methods

Materials. D_2O (99.89%) was obtained from Bio-Rad Laboratories, guanidine hydrochloride (Ultra Pure) and urea (Ultra Pure) from Mann Research Laboratories, 5,5′-dithiobis(2-nitrobenzoic acid) from Aldrich Chemical Co., and ethylenimine (White Label) from Eastman Organic Chemicals. All other reagents were of reagent grade quality.

Amino Acid Analyses. Ribulose diphosphate carboxylases were purified to homogeneity as outlined in the preceding paper (Kuehn and McFadden, 1969). Prior to amino acid analyses, enzyme solutions were dialyzed 24–36 hr against four changes of 1000 volumes of doubly distilled water. Duplicate aliquots of protein containing 1–3 mg of enzyme were pipetted into tared aluminum pans and subsequently dried to constant weight *in vacuo* at 70°. Dry weights were determined on an electrical Cahn microbalance. Other aliquots were transferred to hydrolysis vials containing an equal volume of 12 M HCl, degassed *in vacuo*, and then sealed under N_2. Hydrolysis was conducted at 110–113°. All hydrolysates were analyzed with a Beckman-Spinco 120C automatic amino acid analyzer.

Cysteine plus cystine was determined as cysteic acid, and methionine as methionine sulfone after performic acid oxidation of protein for 3 hr at 0° followed by acid hydrolysis (Hirs, 1956). Sulfhydryl groups were determined by titration with 5,5′dithiobis(2-nitrobenzoic acid) reagent (Ellman, 1959) in 0.1 M potassium phosphate (pH 8.0) in the presence and absence of 8 M urea. Tryptophan was measured spectrophotometrically (Goodwin and Morton, 1946).

Ultracentrifugal Analyses. Prior to all ultracentrifugal studies with native ribulose diphosphate carboxylase preparations, enzyme solutions (0.3–0.5 mg of protein/ml) were equilibrated with 0.1 M potassium phosphate at pH 7.0 by 24-hr dialysis against three changes of 300 volumes of buffer at 2°. The solution outside of the dialysis membrane after the final dialysis was used as a blank where necessary.

All measurements were conducted with a Spinco Model E

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TABLE I: Amino Acid Composition of Ribulose Diphosphate Carboxylase from *H. eutropha*.

Amino Acid Residue	Recov after Hydrolysis (μmoles) for 12 hr	24 hr	48 hr	Extrapolated or Av Value (μmoles)[a]	Nearest Integral No. of Amino Acid Residues/ Molecule of Protein (mol wt 515,000)
Asp	0.11	0.11	0.11	0.11	346
Thr	0.064	0.060	0.057	0.068	214
Ser	0.049	0.046	0.040	0.052	164
Glu	0.10	0.099	0.098	0.099	311
Pro	0.060	0.058	0.055	0.058	182
Gly	0.093	0.089	0.090	0.091	286
Ala	0.11	0.12	0.11	0.11	346
Val	0.070	0.071	0.074	0.074	233
Ile	0.044	0.043	0.046	0.046	145
Leu	0.082	0.080	0.081	0.081	255
Tyr	0.038	0.037	0.037	0.037	116
Phe	0.046	0.045	0.045	0.045	142
Lys	0.040	0.039	0.042	0.040	126
His	0.024	0.024	0.027	0.025	79
Arg	0.072	0.070	0.075	0.072	226
NH_3	0.088	0.086	0.089	0.088	277
Cys				0.031	97
Met				0.034	107
Trp				0.018	57

[a] Protein sample size, 164 μg.

ultracentrifuge equipped with Rayleigh interference optics. The high-speed meniscus-depletion method of Yphantis (1964) was employed for all sedimentation equilibrium studies and sapphire windows were used in cells. Use of a special six-channel centerpiece (Yphantis, 1964) permitted simultaneous studies of three solution–solvent pairs. Experiments were conducted at 20° with 2.5-mm columns of solution corresponding to 0.1 ml of sample plus 0.01 ml of fluorocarbon. The duration of all experiments was up to 27 hr and the attainment of equilibrium was established by ascertaining whether additional fringe displacement had occurred over suitable intervals. Speeds were selected which resulted in equilibrium distribution in times well under 24 hr. Fringe displacements were measured with a Gaertner comparator for experiments in which there was no displacement in the interferogram representing the top half of the solution column. Only displacements greater than 150 μ were accepted as significant data. Analysis of the slopes of plots of the logarithm of fringe displacement, in microns, with respect to the square of the radius of centrifugation in centimeters was rendered by a linear least-squares program written by K. J. Johnson used with a GE 265 computer.

In sedimentation velocity studies, enzyme from *H. eutropha* was examined at 37,020 rpm and 20° in 0.1 M potassium phosphate (pH 7.0) at a concentration of 5.5 mg/ml. The sedimentation coefficient, $s_{20,w}$, was estimated by standard procedures.

For studies conducted in the presence of dissociating agents, separate 0.3-ml aliquots of protein (0.05% concentration) were dialyzed against 166 volumes of 3, 4, or 6 M guanidinium chloride containing 0.1 M 2-mercaptoethanol for 24 hr at 2°. Similar dialyses were also performed against 8 M urea containing 0.1 M mercaptoethanol. Densities of solutions outside of the dialysis membrane were determined pycnometrically at 20 ± 0.1° after equilibration.

The value of the partial specific volume of ribulose diphosphate carboxylase was determined by the sedimentation equilibrium method employing D_2O described by Edelstein and Schachman (1967). With this procedure, the parallel determination of the concentration distribution of a macromolecular solute in solutions of H_2O and D_2O at sedimentation equilibrium permits evaluation of the partial specific volume. Accordingly, two sedimentation equilibrium experiments are performed at an identical rotor speed and temperature such that the solvent density is the only variable. In the present study, these conditions were fulfilled by dialyzing 0.3-ml aliquots of enzyme against three changes of 20 volumes of 0.1 M potassium phosphate (pH 7.0) prepared in D_2O and H_2O. After equilibration, the solvent densities outside the membrane were determined pycnometrically at 20 ± 0.1°. Simultaneous studies were possible with the six-channel Yphantis centerpiece mentioned previously.

Aminoethylation of Ribulose Diphosphate Carboxylase. Aminoethylation of ribulose diphosphate carboxylase from *H. eutropha* was accomplished in 6 M urea or 0.5% sodium dodecyl sulfate as generally described for insulin by Raferty and Cole (1963). Prior to aminoethylation in urea, 1 mg of enzyme in 0.18 ml of 0.1 M Tris-SO_4 (pH 7.9, 25°) containing 0.14 M 2-

TABLE II: Amino Acid Composition of Ribulose Diphosphate Carboxylase from *H. facilis*.

Amino Acid Residue	Recov after Hydrolysis (μmoles) for 12 hr	24 hr	48 hr	Extrapolated or Av Value (μmoles)[a]	Nearest Integral No. of Amino Acid Residues/ Molecule of Protein (mol wt 551,000)
Asp	0.29	0.32	0.32	0.31	419
Thr	0.14	0.15	0.13	0.15	203
Ser	0.11	0.12	0.089	0.12	162
Glu	0.24	0.26	0.25	0.25	338
Pro	0.12	0.14	0.13	0.13	176
Gly	0.23	0.25	0.24	0.24	324
Ala	0.24	0.26	0.26	0.25	338
Val	0.16	0.18	0.19	0.19	257
Ile	0.11	0.12	0.13	0.13	176
Leu	0.20	0.21	0.22	0.21	284
Tyr	0.080	0.087	0.082	0.083	112
Phe	0.11	0.12	0.12	0.12	162
Lys	0.091	0.10	0.097	0.096	130
His		0.068	0.065	0.067	91
Arg	0.16	0.18	0.17	0.17	230
NH_3	0.25	0.23	0.23	0.24	324
Cys | Met				0.069 0.081	93 109
Trp				0.041	55

[a] Protein sample size, 408 μg.

mercaptoethanol was incubated 4 hr at 2°. Next, 0.25 ml of 10 M urea in 0.1 M Tris-SO_4 (pH 8.0, 25°) was added and the mixture was brought to 37° for 1 hr. Ethylenimine was added in twofold or tenfold excess of mercaptoethanol (effective concentrations 0.16 and 1.26 M, respectively) and the mixture was returned to 37° for the desired reaction time. All additions and incubations were performed under a N_2 atmosphere. After reaction, the mixture was dialyzed 5 hr against 0.01 M Tris-SO_4 (pH 8.0, 25°) containing 8 M urea and 0.1 M 2-mercaptoethanol.

Electrophoresis. Analytical polyacrylamide gel disc electrophoresis at pH 9.5 using native carboxylase was conducted in 7% cross-linked gels as described in the preceding paper (Kuehn and McFadden, 1969).

Gels containing Tris and 8 M urea were prepared as described by the method of Jovin *et al.* (1964). Aminoethylated protein solutions were diluted with 0.4 M Tris-Cl buffer (pH 8.0, 25°) containing 8 M urea and 0.1 M 2-mercaptoethanol and 0.1 ml was applied to each gel.

For studies of sodium dodecyl sulfate treated enzyme, standard 7% cross-linked gels were used with the run conducted at pH 9.5. Samples were applied to these gel columns in 0.1 ml of 0.4 M Tris-Cl buffer (pH 8.0 25°) containing 25% sucrose, 0.1 M 2-mercaptoethanol, and 0.03% sodium dodecyl sulfate (Rutner and Lane, 1967). The cathode buffer contained 10 mM mercaptoethanol and 0.03% sodium dodecyl sulfate.

Gels utilized in studies of dissociation were prerun before application of proteins in order to avoid oxidation of samples by ammonium persulfate.

Results

Amino Acid Composition. Tables I and II show the results of timed hydrolyses of ribulose diphosphate carboxylase from *H. eutropha* and *H. facilis*, respectively. The final values for amino acids demonstrating changes with hydrolysis time were obtained by appropriate extrapolation.

The molar ratios of tyrosine:tryptophan determined spectrophotometrically in basic solution were 0.474 and 0.472 for carboxylase from *H. eutropha* and *H. facilis*, respectively. In all cases, the amino acid content shown is based upon the mass of protein estimated from amino acid analyses. The protein mass recovered on the basis of a comparison of amino acid analysis with dry weight determinations was 91 and 88% for ribulose diphosphate carboxylase from *H. facilis* and *H. eutropha*, respectively.

Table III shows a comparison of the amino acid composition, normalized with respect to phenylalanine of enzymes from *Hydrogenomonas* with that for the spinach enzyme.

5,5'Dithiobis(2-nitrobenzoic Acid) Titration. From the amino acid composition of both enzymes, it was evident that the enzymes contained 93–97 half-cystine residues/molecule. Furthermore, sulfhydryl reagents at *ca.* 0.5 mM such as *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, and iodoacetate rap-

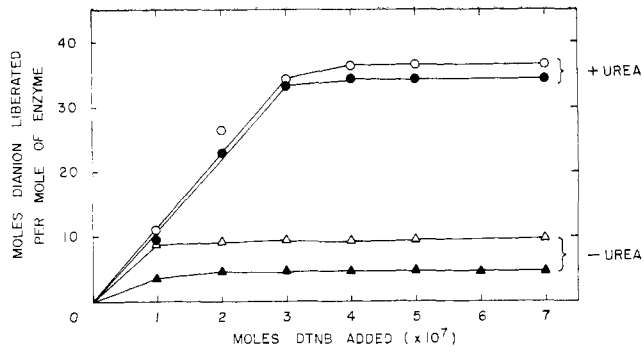

FIGURE 1: Reaction of ribulose diphosphate carboxylase with 5,5′-dithiobis(2-nitrobenzoic acid). Carboxylase was dialyzed overnight against 0.1 M phosphate (pH 8.0) at 2°. Reaction of carboxylase (1.0 mg in all cases) was carried out in a 1-ml cuvet at 25°. The reaction was allowed to proceed to completion for 5 min before reading the absorbance at 412 mμ. The molar extinction coefficient of the 5-thio-2-nitrobenzoate dianion at 412 mμ was taken to be 13,600 M^{-1} cm^{-1} (Ellman, 1959). Closed symbols denote carboxylase from *H. eutropha* in the absence (▲) and presence (●) of 8 M urea; open symbols denote carboxylase from *H. facilis* in the absence (△) and presence (○) of 8 M urea.

idly inactivated the enzyme (unpublished observation, G. D. Kuehn). Sulfhydryl titrations with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959) in the presence of 8 M urea revealed that the enzymes from *H. eutropha* and *H. facilis* contain 35 and 37 thiols per enzyme molecule, respectively (Figure 1). As evident, the availability of carboxylase SH groups for reaction with 5,5′-dithiobis(2-nitrobenzoic acid) was greatly increased by 8 M urea. Without this denaturing agent only five to ten groups were titratable.

Hydrodynamic Properties. The partial specific volume, $\bar{v}$, of the enzyme from *H. eutropha* determined by simultaneous sedimentation equilibrium runs in 0.1 M phosphate prepared in H_2O and D_2O was 0.729 ml/g. The values of the differentials $(d \ln c/dr^2)_{D_2O}$ and $(d \ln c/dr^2)_{H_2O}$ required for calculation of $\bar{v}$

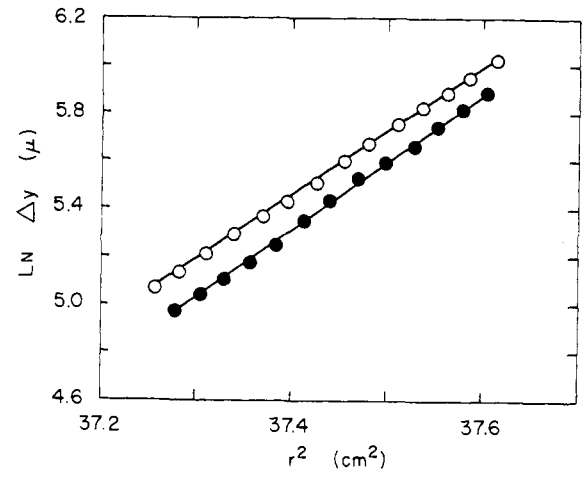

FIGURE 2: Plot of the natural logarithm of the fringe displacement, μ, *vs.* the square of the distance in centimeters from the center of rotation, r^2, derived from a sedimentation equilibrium experiment with native ribulose diphosphate carboxylase from *H. eutropha* (○) and *H. facilis* (●). The solvent was 0.1 M potassium phosphate (pH 7.0). The protein concentration in both experiments was 0.03%. The rotor speed was 9341 rpm and the temperature 20.0°.

TABLE III: Relative Comparison of Amino Acid Compositions of the Respective Ribulose Diphosphate Carboxylases of *Hydrogenomonas* and Spinach.

Amino Acid	*H. facilis*	*H. eutropha*	Spinach[a]
Asp	2.58	2.44	2.01
Thr	1.25	1.51	1.37
Ser	1.00	1.16	0.68
Glu	2.08	2.20	2.18
Pro	1.08	1.29	1.24
Gly	2.00	2.02	2.05
Ala	2.08	2.44	1.89
Val	1.58	1.64	—
Ile	1.08	1.02	0.84
Leu	1.75	1.80	2.00
Tyr	0.69	0.82	1.07
Phe	(1.00)	(1.00)	(1.00)
Lys	0.80	0.88	1.15
His	0.56	0.56	0.67
Arg	1.42	1.60	1.34
Cys	0.58	0.69	0.46
Met	0.68	0.76	0.45
Trp	0.34	0.40	0.64

[a] From Rutner and Lane (1967).

by the Edelstein and Schachman (1967) equation were 2.048 and 2.663, respectively. $\bar{v}$ calculated from the weight percentages of the amino acid residues and their respective specific volumes was 0.728 ml/g (Cohn and Edsall, 1943). A similar calculation from the amino acid composition of ribulose diphosphate carboxylase from *H. facilis* yielded 0.726 ml/g. The directly measured value of 0.729 ml/g was used for enzymes from both sources in calculations of all molecular weights below.

Data from sedimentation equilibrium studies partially depicted in Figures 2 and 3 were used to calculate the apparent molecular weight of the two native ribulose diphosphate carboxylates. These results and others pertaining to native and dissociated ribulose diphosphate carboxylase from both sources are tabulated in Table IV. The apparent molecular weight for ribulose diphosphate carboxylase from *H. eutropha* was 515,000 and from *H. facilis* was 551,000. These values are uncorrected for the small net charge of the protein possessed at pH 7.0, about one pH unit above the crude isoelectric point estimated from the composition. The magnitude of this correction is quite small and uncertain (Tanford, 1961).

The apparent subunit molecular weights were essentially invariant in 3, 4, and 6 M guanidinium chloride solution suggesting complete dissociation of each enzyme into its monomeric subunits (Table IV). The average subunit molecular weights (uncorrected for preferential interaction of small molecules) for the enzymes from *H. eutropha* and *H. facilis* were 40,700 and 38,000, respectively.

Sedimentation velocity studies of the enzymes each revealed a single symmetrical peak sedimenting at similar rates. A value of 20×10^{-13} sec was generated for the $s_{20,w}$ of the protein from *H. eutropha*. The diffusion coefficient, $D_{20,w}$, as calculated from $s_{20,w}$, the M_W, and $\bar{v}$ was 3.7×10^{-7} cm^2/sec. The

TABLE IV: Molecular Weights of Ribulose Diphosphate Carboxylase and Its Subunits from *H. eutropha* and *H. facilis*.

Experimental Condition[a]	Protein Concn (%)	Mol Wt[b]
Ribulose Diphosphate Carboxylase from *H. eutropha*		
Native (pH 7.0)	0.03	521,000
	0.04	512,000
	0.05	514,000
	0.05	515,000
Guanidine hydrochloride (3 M)	0.05	39,700
Guanidine hydrochloride (4 M)	0.05	42,400
Guanidine hydrochloride (6 M)	0.05	42,100
Urea (8 M)	0.05	38,600
Ribulose Diphosphate Carboxylase from *H. facilis*		
Native (pH 7.0)	0.03	558,000
	0.04	550,000
	0.05	544,000
Guanidine hydrochloride (3 M)	0.05	38,400
	0.05	37,000
	0.05	38,400

[a] All studies were carried out at 20.0° for 24–26 hr. The rotor speed setting was 9341 rpm for trials with native enzyme; trials conducted in guanidine and urea solutions employed a speed of 42,040 rpm. [b] The standard deviations expressed as percentages of each value from top to bottom were 2.3, 12.5, 8.9, 13.8, 9.1, 8.8, 7.8, 9.2, 11.9, 4.4, 3.9, 4.2, 6.2, and 3.2, respectively.

approximate frictional ratio, f/f_0, was estimated to be 1.04. This value is in the usual range for a globular protein having typical hydration characteristics (Tanford, 1961).

In none of these studies was any evidence for heterogeneity obtained.

Electrophoretic Studies. Strict weight homogeneity of the subunits observed from guanidine- and urea-dissociated carboxylase in the sedimentation equilibrium studies was corroborated by electrophoretic studies.

Figure 4 displays polyacrylamide electrophoretograms of native ribulose diphosphate carboxylase from *H. eutropha* and aminoethylated enzyme dissociated by urea and sodium dodecyl sulfate. Because of the sieving effect of the polyacrylamide gel, the electrophoretic mobility of an anionic–sodium dedecyl sulfate complex is primarily a function of the molecular weight of the polypeptide (Shapiro *et al.*, 1967). Dissociation in both sodium dodecyl sulfate and urea resulted in a single protein zone after exhaustive aminoethylation of reduced enzyme. Reduced enzyme subjected to aminoethylation for 4 hr at lower ethylenimine concentrations and then dissociated yielded more than one protein zone, however (Figure 4). Column chromatographic efforts to separate polypeptides differing in charge in 8 M urea (Friedland and Hastings, 1967) or size in 0.5% sodium dodecyl sulfate were unsuccessful (Rutner and Lane, 1967).

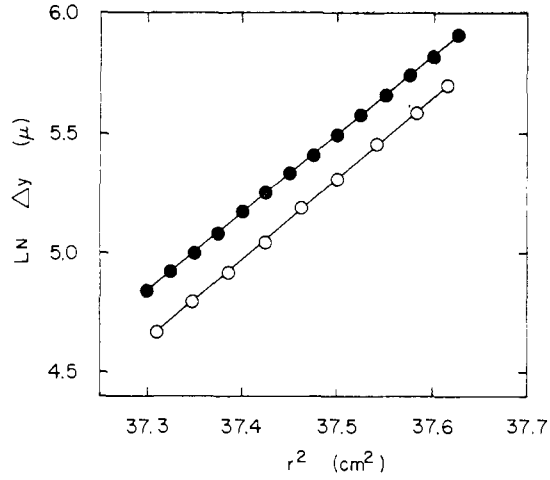

FIGURE 3: Plot of the natural logarithm of the fringe displacement, μ, *vs.* the square of the distance in centimeters from the center of rotation, r^2, derived from a sedimentation equilibrium experiment with dissociated ribulose diphosphate carboxylase from *H. eutropha* (○) and *H. facilis* (●). Protein solutions (0.05%) were equilibrated in 3 M guanidinium chloride containing 0.1 M mercaptoethanol prior to ultracentrifugal analysis. The rotor speed was 42,040 rpm and the temperature 20.0°.

Discussion

The present sedimentation equilibrium studies provide additional evidence (Kuehn and McFadden, 1969) for homogeneity of the ribulose diphosphate carboxylases isolated from *H. eutropha* and *H. facilis*. Enzymes from these two sources have average molecular weights of 515,000 and 551,000, respectively, and the enzyme from *H. eutropha* has an $s_{20,w}$ of 20×10^{-13} sec. These values are similar to those reported for the spinach protein (Trown, 1965; Paulsen and Lane, 1966; Ridley *et al.*, 1967). The sedimentation coefficient estimated by sucrose density centrifugation for ribulose diphosphate carboxylase from two chemoautotrophs, *Thiobacillus thioparus* and *Thiobacillus neapolitanus*, is also similar to that of the spinach enzyme (MacElroy *et al.*, 1968). Thus the molecular weights may be similar. It should be emphasized, however, that the enzymes from the thiobacilli contain phosphoribulokinase activity, an activity not detectable in the hydrogenomonad enzymes (Kuehn and McFadden, 1969). In recent work, sedimentation in sucrose density gradients has been used to estimate molecular weights of ribulose diphosphate carboxylase in extracts from various autotrophs (Anderson *et al.*, 1968). It is of interest that the values, which must be regarded as crude, varied from 120,000 for the enzyme from *Rhodospirillum rubrum* to 660,000 for that from green and blue-green algae and from *Chromatium*.

In the present work, average subunit molecular weights of 40,700 and 38,000 for ribulose diphosphate carboxylase from *H. eutropha* and *H. facilis*, respectively, were obtained in three concentrations of guanidinium chloride and in 8 M urea. The results are compatible with subunit homogeneity as are the results from gel electrophoretic studies of dissociated enzyme from *H. eutropha*. These observations clearly establish a major difference in quaternary structure between the hydrogenomonad carboxylases and that from higher plants even though the amino acid compositions are similar (Table III) as are the molecular weights. Rutner and Lane (1967) have presented

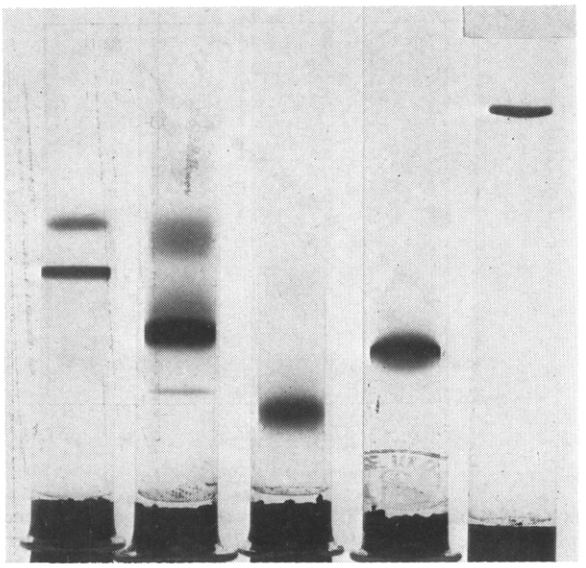

FIGURE 4: Polyacrylamide electrophoretograms (pH 9.5) of dissociated and native ribulose diphosphate carboxylase isolated from *H. eutropha* displayed in test tubes. The display has been cut at the position corresponding to the origin of each running gel with the bottom of the gel resting on the stopper. From left to right: (a) 100 μg of enzyme treated for 4 hr with 0.16 M ethyleneimine in the presence of 0.5% sodium dodecyl sulfate and run in 0.03% sodium dodecyl sulfate, (b) enzyme treated as in part a but in the presence of 6 M urea and run in 8 M urea, (c) 60 μg of enzyme treated for 12 hr with 1.26 M ethyleneimine in the presence of 6 M urea and run in 8 M urea, (d) enzyme treated as in part c but in the presence of 0.5% sodium dodecyl sulfate and run in 0.03% sodium dodecyl sulfate, and (e) 60 μg of native enzyme.

convincing evidence that spinach ribulose diphosphate carboxylase is composed of two distinct kinds of noncovalently bonded subunits which differ both in amino acid composition and molecular weight. The present data on molecular weights suggest ribulose diphosphate carboxylases from *Hydrogenomonas* contain 12–14 subunits of uniform size and closely similar or identical electrophoretic properties.

In conclusion, the carboxylases described in the present study resemble the catalyst from higher plants in several respects. If increasing molecular weight of an enzyme can be considered a consequence of later evolutionary development, the hydrogen bacteria and higher plants may represent a later form of autotrophy than do the purple, nonsulfur photosynthetic bacteria typified by *R. rubrum*.

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