An Improved Method for the Isolation of Carboxydismutase. Probable Identity with Fraction I Protein and the Protein Moiety of Protochlorophyll Holochrome*

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ABSTRACT: An essentially homogeneous preparation of carboxydismutase has been obtained from spinach leaves by ammonium sulfate fractionation and repeated gel filtration on Sephadex G-200. The enzyme was stabilized during the purification by the presence of sulfate ions at a concentration of 0.1 M or greater, which reversibly inhibited its catalytic activity. Chloride ions at similar concentration caused no inhibition, but irreversibly deactivated the enzyme over a period of several days. The molecular weight of carboxydismutase was determined to be 515,000 by sedimentation equilibrium. Its sedimentation coefficient ($s_{20,w}$) was 18.57 Svedbergs.

These and other physical properties correspond closely with those of so-called fraction I protein present in many plants, and also with those of the protein moiety of protochlorophyll holochrome obtained from some etiolated plants. It is concluded that fraction I protein is crude carboxydismutase which may also make up the protein moiety of protochlorophyll holochrome. The purified enzyme showed no significant phosphoriboisomerase or phosphoribulokinase activities, these enzymes being readily separated from carboxydismutase by the procedure employed.

The isolation from *Chlorella* of a cell-free extract capable of catalyzing the carboxylation of RuDP[1] to give PGA was first reported by Quayle *et al.* (1954). Soon afterward, an enzyme, now named carboxydismutase or RuDP carboxylase, was isolated from spinach by Weissbach *et al.* (1956), and independently by Jakoby *et al.* (1956). The enzyme was purified about 10-fold from the crude extracts by Weissbach *et al.* It behaved as an almost homogeneous protein on ultracentrifugation and electrophoresis, and was estimated to have a molecular weight of about 300,000. Its sedimentation coefficient was 17 Svedberg units under the conditions used.

The similarity of these physical properties to those of an apparently homogeneous soluble protein obtained from many higher plants and known as "fraction I protein" (Wildman and Bonner, 1947) was noted by Dorner *et al.* (1957). Subsequent workers (Lyttleton and T'so, 1958; Park and Pon, 1961) confirmed this observation and demonstrated that carboxydismutase activity was associated with fraction I protein. More recently a highly purified protein-protochlorophyll complex, which had very similar physical properties to those of fraction I protein and carboxydismutase, was isolated from etiolated leaves (Boardman, 1962a). It therefore became of interest to determine the physical properties of highly purified carboxydismutase in order to test its possible identity with fraction I protein and also the protein moiety of protochlorophyll holochrome.

The mechanism of action of carboxydismutase has also been the subject of much investigation and speculation (Calvin, 1954b; Hurwitz *et al.*, 1956a; Pon, 1960; Pon *et al.*, 1963; Akoyunoglou and Calvin, 1963). Enzyme purified by the methods described in this paper was used in more recent studies in which the mechanism was partially elucidated (Rabin and Trown, 1964a,b; Trown and Rabin, 1964).

For investigation of its physical properties and the mechanism of its action, a large quantity of very pure, active, and, if possible, stable enzyme was required. The isolation procedure of Weissbach *et al.* (1956) was unsuitable because it was lengthy and gave low yield. An improved procedure was therefore sought.

Carboxydismutase has been shown to be located mainly in the chloroplasts of spinach (Heber *et al.*, 1963), and at least 90% of the carboxydismutase activity of chloroplasts was previously shown to be associated with the colorless supernatant protein obtained by high-speed centrifugation of chloroplasts which have been disrupted by sonication or by exposure to hypotonic solutions (Park and Pon, 1961). An extract of disrupted chloroplasts prepared by the latter method was therefore used as the starting material for the isolation, rather than a whole-leaf homogenate which contains many more impurities (Pon, 1960; Pon *et al.*, 1963). This communication describes the subsequent purification of the enzyme (by ammonium sulfate

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[1] Abbreviations used are: RuDP, ribulose-1,5-diphosphate; PGA, 3-phosphoglyceric acid; R-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; ATP, adenosine-5′-triphosphate; GSH, reduced glutathione.

fractionation and repeated gel filtration under special conditions) and the determination of some of its physical properties.

Experimental Procedures

Materials. Enzyme grade ammonium sulfate was purchased from Gallard-Schlesinger Chemical Corp., Garden City, N.Y.; it gave a negative reaction in the dithizone test for metal contaminants. Tris buffers at the desired *p*H were prepared from Trizma (Sigma Chemical Co., St. Louis, Mo.) and reagent grade hydrochloric acid or sulfuric acid. They were prepared as stock solutions (total Tris, 1.0 M) and freed from heavy-metal ion contaminants as described by Rabin and Trown (1964a). The water and dialysis tubing used in the investigations were freed of heavy-metal ion contaminants by standard procedures (Hughes and Klotz, 1956; Thiers, 1957), and polyethylene apparatus was used wherever possible.

RuDP was purchased from Calbiochem, Los Angeles, Calif., as the Ba salt. It was stated to contain 26% RuDP and was either used without purification in some of the earlier work, or was purified as described by Rabin *et al.* (1958) or by continuous electrophoresis in pyridine acetate buffer (0.25 M in pyridine, *p*H 5.0) using a Beckman Spinco Model CP continuous-flow paper electrophoresis cell (P. W. Trown, unpublished results). Where necessary, the Ba salt of RuDP was converted to the Na salt as previously described (Rabin and Trown, 1964a).

R-5-P was purchased from Calbiochem, Los Angeles, Calif. as the Ba salt. It was stated to be 98.3% pure, and was converted to the Na salt (0.1 M) as follows: Ten mg was suspended in water (750 μl) at 0°, then Na_2SO_4 (0.1 M, 250 μl) was added. The mixture was left at 0° for 15 minutes and then centrifuged to remove the precipitated $BaSO_4$.

ATP was purchased from Pabst Laboratories, Milwaukee, Wis., Sephadex G-200 from Pharmacia, Uppsala, Sweden, and Triton X-100 from Rohm and Haas, Philadelphia, Pa. All other chemicals used were reagent grade.

Protein Determination. Dry-weight measurements on salt-free, highly purified carboxydismutase showed that a solution of the enzyme containing 0.71 mg/ml has an optical density of 1.00 (1 cm path) at 280 mμ. Protein concentrations were determined throughout, using this relationship.

Enzyme Assays. Carboxydismutase activity was determined by measurement of the acid-stable radioactivity produced in the reaction between RuDP and $NaH^{14}CO_3$ as described by Rabin and Trown (1964a). Approximately 5 μg of protein was used in each assay. A unit of carboxydismutase activity is defined as the amount that will fix 1 μmole of carbon in 10 minutes under the assay conditions used.

Phosphoriboisomerase activity was measured by the procedure of Axelrod and Jang (1954), in which Ru-5-P produced from R-5-P is determined by the cysteine-carbazole test. The modification described by Hurwitz *et al.* (1956b) was employed.

The combined activities of phosphoriboisomerase and phosphoribulokinase were determined by an assay using R-5-P, $NaH^{14}CO_3$, and ATP as substrates in the presence of an excess of carboxydismutase. The total acid-stable radioactivity present after the reaction was taken to be proportional to the RuDP produced by the two enzymes being assayed. A typical incubation mixture contained the following components in a total volume of 250 μl: Tris-chloride buffer, *p*H 8.0, 30 μmoles; $MgCl_2$, 4 μmoles; $NaH^{14}CO_3$, 25 μc/μmole, 3 μmoles; R-5-P, 1.25 μmoles; ATP, 5 μmoles; isomerase-kinase mixture, approximately 10 μg; purified carboxydismutase, 10 μg. The mixture was incubated at 25° for 10 minutes and then acetic acid (6 N, 50 μl) was added to stop the carboxydismutase reaction. Aliquots (25 μl) were plated on aluminum planchets for radioactivity counting. For preparations with very low activities, longer incubation periods (up to 6 hours) were employed. A unit of "isomerase-kinase" activity is defined as the amount of the combined enzymes phosphoriboisomerase and phosphoribulokinase required to produce 1 μmole of RuDP in 10 minutes under the conditions described. It is assumed that all of the RuDP produced by the isomerase-kinase mixture is converted to PGA by the excess carboxydismutase. The carboxydismutase used in the assay was a highly purified preparation with insignificant isomerase-kinase activity.

Isolation of Carboxydismutase. PREPARATION OF THE CHLOROPLAST EXTRACT. Chloroplasts were isolated by differential centrifugation from 10 kg of spinach leaves using a phosphate-buffered isotonic sucrose solution (*p*H 7.4) as described by Park and Pon (1961). The chloroplasts were disrupted with Tris-sulfate buffer (0.002 M, *p*H 7.4) and subsequently removed by centrifugation at 40,000 × *g* for 10 minutes. The resulting supernatant (the "chloroplast extract") was made 90% saturated ammonium sulfate ("saturated" is defined as 70 g/100 ml original volume at 0°) by the addition of solid ammonium sulfate (the *p*H being maintained at approx 7.5 by the addition of a concentrated solution of ammonia) and left overnight at 0°.

FRACTIONAL PRECIPITATION OF THE CHLOROPLAST EXTRACT. The material precipitated at 90% saturated ammonium sulfate from the chloroplast extract was collected by centrifugation at 40,000 × *g* for 15 minutes and then redissolved in Tris-sulfate buffer (0.01 M, *p*H 7.4). The final volume of the solution was 90 ml, and the concentration of ammonium sulfate, as determined by conductivity measurements, was 27.8% saturated. The concentration of protein in the solution was 28 mg/ml. Solid ammonium sulfate was then added to the solution to obtain consecutively 30, 35, 40, 45, 50, 60, and 70% saturated ammonium sulfate. After each addition the solution was stirred at 2° for at least 1 hour and was then centrifuged at 40,000 × *g* for 10 minutes to collect the precipitate which had formed. Each precipitated fraction was redissolved in Tris-sulfate buffer (0.01 M, *p*H 7.6), and dialyzed against the same

buffer at 0° overnight, by the method of Hospelhorn (1961).

GEL FILTRATION ON SEPHADEX G-200. After removal of the fines by decantation, Sephadex G-200 was equilibrated with a solution containing Tris-sulfate buffer, 0.1 M, *p*H 7.4; ammonium sulfate, 0.2 M; GSH, 0.001 M; EDTA Na salt, *p*H 7.4, 0.001 M. A gel column 44 × 4.7 cm was prepared from this material and cooled to 2°, at which temperature all subsequent operations were performed. The 35–40% and 40–45% saturated ammonium sulfate cuts from the fractional precipitation were combined and divided into two parts, one of which was retained for subsequent purification. The other half was dialyzed overnight against a solution containing Tris-sulfate, buffer, ammonium sulfate, GSH, and EDTA as before. The dialyzed solution (7.5 ml containing 580 mg protein) was applied to the top of the Sephadex gel bed (which was protected by a disk of filter paper) through a glass capillary tube as suggested by Porath (1964). Elution of the column was achieved with the same Tris-sulfate buffer/ammonium sulfate/GSH/EDTA mixture described above. After analysis of the column effluent by optical density and enzymic activity measurements, fractions containing material with the highest carboxydismutase activity were combined. The solution was concentrated by precipitation with ammonium sulfate followed by centrifugation and re-solution of the precipitate in Tris buffer (0.1 M, *p*H 7.4). After dialysis against more of the solution used as eluent of the first column, the enzyme solution was reapplied to a Sephadex G-200 column identical to that already described. The column was eluted in a similar manner and fractions containing the highest specific carboxydismutase activity were again combined. Carboxydismutase was stored until use as a suspension in 50% saturated ammonium sulfate at 2°.

Effects of Various Compounds upon the Stability of Carboxydismutase. The compounds listed here were added at the stated concentrations to solutions of partially purified carboxydismutase (35–40% saturated ammonium sulfate cut) in Tris-chloride buffer (0.001 M, *p*H 7.6) containing EDTA (Na salt, 0.001 M, *p*H 7.4). The solutions were stored at 0°. Aliquots were withdrawn at various times and assayed for carboxydismutase activity. The compounds studied were: GSH, 0.001 M; sodium ascorbate, 0.001 M; cysteine, 0.001 M; sucrose, 1.0, 0.5, 0.25 M; Triton X-100, 0.05, 0.02, 0.01%; sodium dodecyl sulfate, 0.001 M; sodium chloride, 0.25, 0.083 M; ammonium sulfate, 0.25, 0.083 M; ammonium chloride, 0.25 M; sodium sulfate, 0.25 M; and potassium phosphate buffer *p*H 7.6, 0.2, 0.05, and 0.01 M. In the case of the inorganic salts, a sample was withdrawn from each solution between 48 and 96 hours after the start and dialyzed overnight against Tris-chloride buffer, 0.001 M, at 0°. The next day, the dialyzed and undialyzed solutions were assayed at the same time for carboxydismutase activity.

Inhibition of Carboxydismutase by Sulfate and Phosphate. The total fixation of CO_2 by carboxydismutase in 10 minutes at 25° was measured at six different concentrations of RuDP in the absence and presence of (a) ammonium sulfate, 0.02 M, (b) ammonium sulfate, 0.1 M, (c) potassium phosphate buffer, *p*H 7.6, 0.08 M, and (d) potassium phosphate buffer, 0.04 M. Under these conditions the reaction rates were linear for at least 30 minutes; values for total fixation of CO_2 were thus proportional to reaction velocity.

Ultracentrifugal Analysis of Purified Carboxydismutase. Sedimentation coefficients of purified carboxydismutase at various concentrations of protein ranging from 1 to 10 mg/ml were determined in a solution containing ammonium sulfate, 0.1 M, Tris-sulfate buffer, 0.01 M, *p*H 7.4, and EDTA, 0.001 M, at 4.75° in a Beckman Spinco Model E ultracentrifuge. Diffusion coefficients of carboxydismutase were determined in the same solvent at 4.75° and at protein concentrations within the same range by use of a synthetic-boundary cell in the ultracentrifuge. The Rayleigh interference optical system was employed and the data were computed by the method of Longsworth (1952).

The molecular weight of carboxydismutase was determined by the method of Archibald (1947), at a protein concentration of 7.3 mg/ml in the solvent already described. Data were obtained from the schlieren patterns and calculations were made as described by Ehrenberg (1957). A more accurate value of the molecular weight was obtained from a sedimentation equilibrium experiment at 2095 rpm and 4.75° with a solution of carboxydismutase (3.97 mg/ml) in the solvent described.

Miscellaneous. The partial specific volume of carboxydismutase was calculated from the densities of solutions of the enzyme at known concentrations and of the solvent. Densities were measured using a Gay-Lussac 5-ml density bottle at 25° ± 0.05°. Purified carboxydismutase was subjected to electrophoresis on 2.5% (final concn) polyacrylamide gel at *p*H 7.9. Protein bands were detected by staining the gel columns with Nigrosin in 5% acetic acid.

Results

Stabilization of Carboxydismutase. ORGANIC COMPOUNDS. Experiments with partially purified carboxydismutase showed that, of a number of organic compounds tested, only GSH, 1.0 M sucrose, and Triton X-100 at a concentration of 0.05% protected the enzyme against deactivation to any significant extent. The results of these experiments are summarized in Table I. Although values of remaining activity are only given for one time (160 hours), assays were performed approximately every 24 hours, and indicated a steady trend toward the final values given in Table I. The activity remaining in the control solutions varied considerably between the various experiments. The cause of this variation is unknown. The ionic detergent, sodium dodecyl sulfate, completely destroyed carboxydismutase in less than 1 minute in contrast with the stabilizing effect shown by the nonionic detergent Triton X-100.

INORGANIC COMPOUNDS. The comparable effects upon the stability of carboxydismutase of sodium chloride, sodium sulfate, ammonium chloride, and ammo-

TABLE I: Stabilization of Carboxydismutase.

Compound Added	Per Cent Initial Activity Remaining after 160 Hours	Compound Added	Per Cent Initial Activity Remaining after 160 Hours
GSH, 0.001 M	95	Triton X-100, 0.05%	75[a]
Sodium ascorbate, 0.001 M	70	Triton X-100, 0.01%	40
		Triton X-100, 0.002%	40
Cysteine, 0.001 M	70	Sodium dodecylsulfate, 0.001 M	0[b]
Control—no additions	70	Control—no additions	40
Sucrose, 1.0 M	60		
Sucrose, 0.5 M	45		
Sucrose, 0.75 M	45		
Control—no additions	40		

[a] No loss of activity during first 72 hours. [b] No carboxydismutase activity remained after 1 minute.

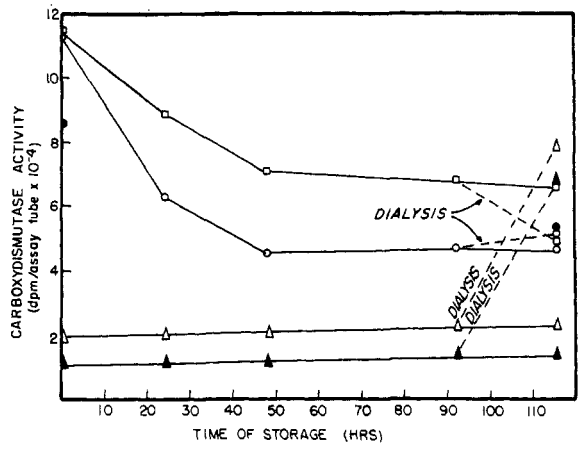

FIGURE 1: Effects of ammonium sulfate, ammonium chloride, sodium sulfate, and sodium chloride on the stability and activity of carboxydismutase. ▲, 0.25 M $(NH_4)_2SO_4$; ○, 0.25 M NH_4Cl; △, 0.25 M Na_2SO_4; □, 0.25 M NaCl; ●, control.

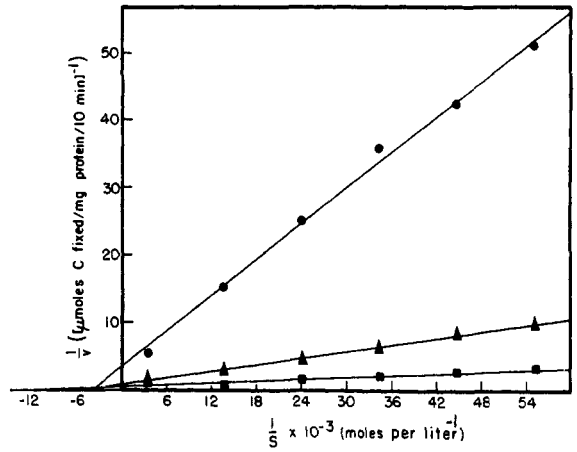

FIGURE 2: Lineweaver-Burk plot for the inhibition of carboxydismutase by ammonium sulfate. Here V = reaction velocity (μmoles of C fixed per mg protein per 10 min), S = concentration of RuDP (mM). ■, no $(NH_4)_2SO_4$; ▲, 0.02 M $(NH_4)_2SO_4$; ●, 0.1 M $(NH_4)_2SO_4$. Lines were drawn by the method of least squares.

nium sulfate at a concentration of 0.25 M are summarized in Figure 1. Sodium sulfate and ammonium sulfate had similar effects in that they inhibited carboxydismutase approximately 80% immediately upon addition to the enzyme solution. This inhibition was maintained with time but could be completely reversed by dialysis with restoration of essentially all the initial activity. In contrast, ammonium chloride and sodium chloride caused an apparent activation of about 25% above that of the control solution immediately upon their addition to the enzyme solutions. However, this activation was not maintained since the activity decreased with time and could not be recovered by dialysis. Potassium phosphate buffer at *p*H 7.6 protected carboxydismutase activity in a similar manner to sodium and ammonium sulfates, except that only 80% of the initial activity was recovered after 120 hours on removal of the phosphate by dialysis. Phosphate also inhibited carboxydismutase activity: at zero time, solutions containing 0.01, 0.05, and 0.2 M potassium phosphate at *p*H 7.6 had, respectively, 80, 40, and 5% of the control solution activity.

Inhibition of Carboxydismutase by Sulfate and Phosphate. Figures 2 and 3 show the Lineweaver-Burk plots for the inhibition of carboxydismutase by sulfate and phosphate, respectively, each at two different concentrations.

Purification of Carboxydismutase. The results of the

TABLE II: Fractionation of the 90% Saturated Ammonium Sulfate Precipitate from the Chloroplast Extract.

Per Cent Saturated Ammonium Sulfate	OD_{280} Units Recovered (% total)	λ_{max} (mμ)	Specific Carboxydismutase Activity (units/mg)[a]	Specific "Kinase-Isomerase" Activity (units/mg)	Carboxydismutase/Kinase-Isomerase Ratio
30	4.0	260	0.13	0.02	6.5
35	7.7	257	0.23	0.06	3.8
40	54.5	276	0.81	0.22	3.7
45	21.6	277	1.20	0.36	3.3
50	5.9	278	0.35	0.45	0.78
60	2.4	275	0.08	0.12	0.66
Supernatant from 60% saturated ammonium sulfate	3.6	273	0.009	0.02	0.45
Chloroplast extract		269	0.74	0.21	3.5

[a] Units as defined in experimental section. This unit is not the same as that defined by Weissbach *et al.* (1956), since very different conditions of incubation with RuDP were employed in the assay.

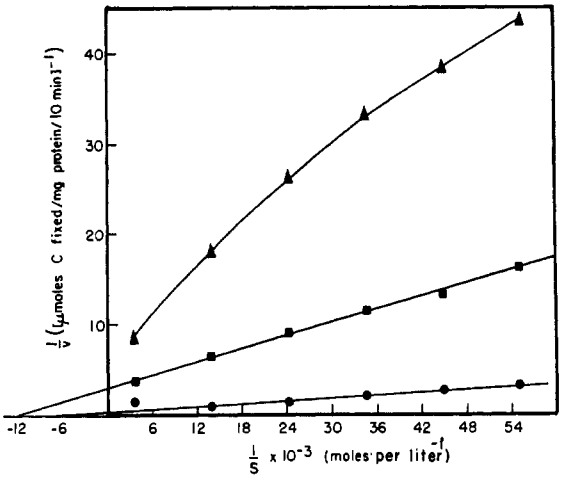

FIGURE 3: Lineweaver-Burk plot for the inhibition of carboxydismutase by potassium phosphate buffer, *p*H 7.6. Here V = reaction velocity (μmoles C fixed per mg protein per 10 min), S = concentration of RuDP (mM). ●, no phosphate; ■, 0.04 M phosphate; ▲, 0.08 M phosphate.

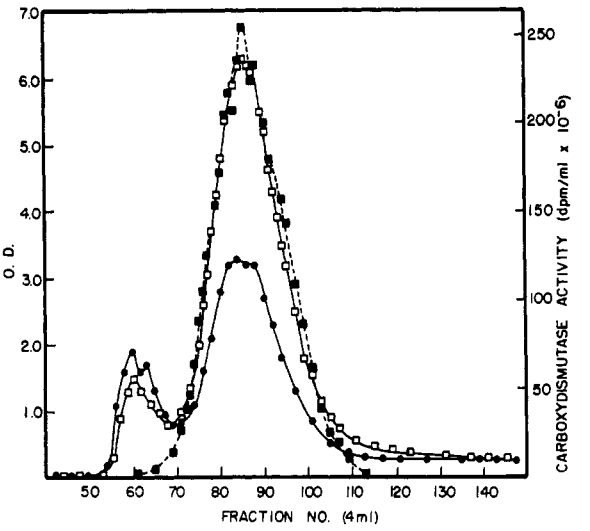

FIGURE 4: Fractionation of carboxydismutase on Sephadex G-200 column (44 × 4.7 cm). ○, OD at 260 mμ; □, OD at 280 mμ; ■, carboxydismutase activity [(dpm/ml) × 10^{-6}].

fractionation of the 90% saturated ammonium sulfate precipitate from the chloroplast extract are summarized in Table II. More than 75% of the total protein was precipitated in the range 35–45% saturated ammonium sulfate where the highest specific carboxydismutase activity was also found. A 60% increase in the specific carboxydismutase activity was achieved during the fractionation. The highest kinase-isomerase activity was found in the 40–50% saturated ammonium sulfate range. The ratio of carboxydismutase to kinase-isomerase activities decreased with increasing ammonium sulfate concentrations from a value of 6.5:1 in the 30% saturated ammonium sulfate cut to 0.45:1 in the supernatant from the 60% saturated ammonium sulfate precipitation.

The elution of carboxydismutase from the first of

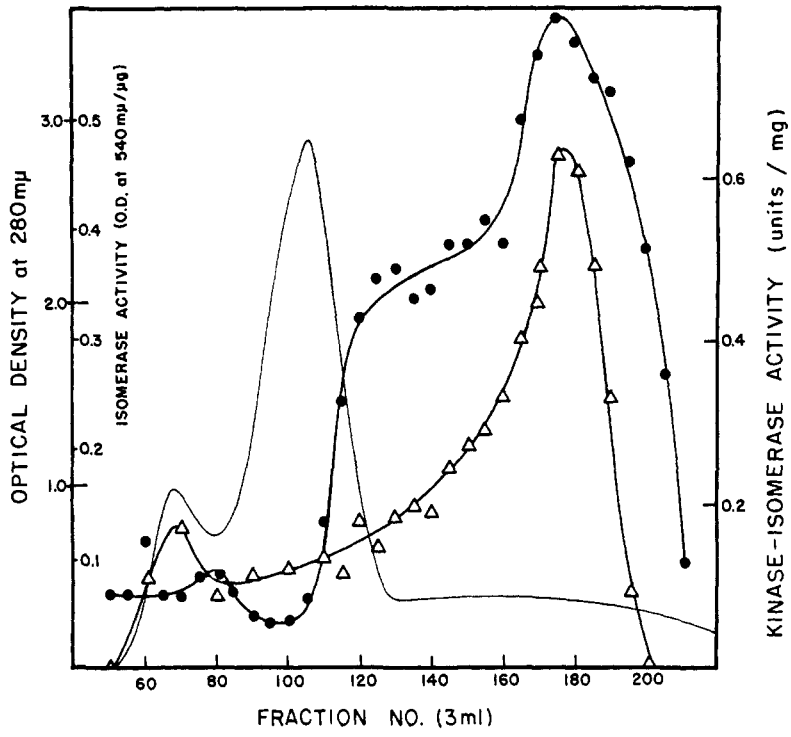

FIGURE 5: Elution of phosphoriboisomerase and phosphoribulokinase from Sephadex G-200 column (44 × 4.7 cm). △, phosphoriboisomerase; ●, kinase-isomerase (see experimental section); line without points, OD at 280 mμ.

the two columns of Sephadex G-200 used is shown in Figure 4. Material with an ultraviolet absorption maximum at 260 mμ was separated from carboxydismutase and was eluted both before the main protein peak and also after it in a long "tail" (not shown in Figure 4). The specific carboxydismutase activity for fractions 75–100 was constant at 1.26 units/mg, but values up to 2.15 units/mg have been obtained in subsequent similar purifications. Fractions 75–100 were combined, concentrated, and then applied to the second Sephadex G-200 column as described in the experimental section. The elution pattern of the second column was similar to that of the first except that the first peak was very small and there was almost no "tail." Material from the peak fractions of the second column had a specific carboxydismutase activity of 1.54 units/mg, but activities up to 2.58 units/mg have been obtained in subsequent similar purifications. This activity remained constant over a period of several months when the enzyme was stored as a precipitate in 50% saturated ammonium sulfate.

The eluate from the first Sephadex G-200 column of a different isolation from that shown in Figure 4 was also assayed for phosphoriboisomerase and "isomerase-kinase" activities. The results of these assays are shown in Figure 5. While some phosphoriboisomerase and phosphoribulokinase activity was eluted with the carboxydismutase, the two former enzymes reached their peak specific activities at fractions 180 and 190, respectively, where no carboxydismutase activity could be detected. The elution of phosphoriboisomerase and isomerase-kinase activities from the second Sephadex G-200 column in this experiment was similar to that shown in Figure 5, except that none of either activity was detected until fraction 150 had been eluted. The maximum specific activities of both enzymes again occurred at about fractions 180–190.

The purified carboxydismutase from the peak fractions of the second Sephadex G-200 column was assayed for isomerase-kinase activity for 6 hours at 25° in order to obtain enough radioactivity for a count. The equivalent of 1 unit of kinase-isomerase activity was detected per 20,000 units of carboxydismutase activity.

Properties of Carboxydismutase. The sedimentation coefficient of carboxydismutase was proportional to the concentration of the enzyme. A plot of $1/s$ versus concentration was used for the extrapolation to zero concentration, and a value of 11.56 S was obtained for $s°$ under the conditions employed. Corrections for temperature, viscosity, and density to the standard state gave $s°_{20,w}$ = 18.57 S. The constant k in the equation $s°/s = 1 + kc$ was found to have a value of 0.091 (s = sedimentation coefficient at concentration c in g/100 ml and $s°$ = the value of s at infinite dilution). A solution of carboxydismutase (7.3 mg/ml) which had been exposed to some unknown factor not normally encountered in isolation or storage of the enzyme was examined for sedimentation characteristics. Three distinct schlieren peaks were visible with sedimentation coefficients of 18, 26, and 32 S. The areas under the schlieren peaks were in the ratios 6:2:1, respectively.

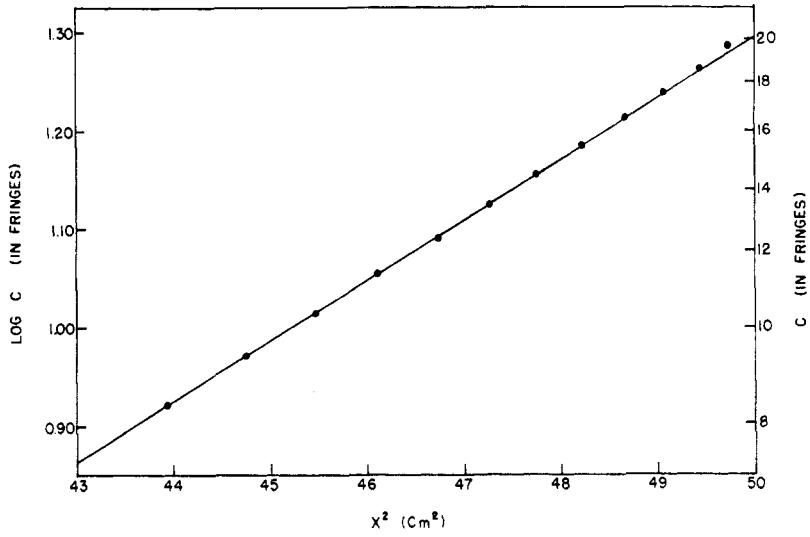

FIGURE 6: Functions of protein concentration (c) and distance from center of rotation (x) in a sedimentation equilibrium experiment with purified carboxydismutase (3.97 mg/ml) in 0.1 M $(NH_4)_2SO_4$, 0.01 M Tris-sulfate buffer *p*H 7.4, 0.001 M EDTA. The rotor was overspeeded at 3397 rpm for 8 hours and then run at 2095 rpm until equilibrium was attained. The data were obtained from a photograph of the Rayleigh interference pattern taken 120 hours after reaching 2095 rpm. The rotor temperature was 4.75°.

Electron micrographs of such a solution showed the presence of monomers, dimers, and trimers in approximately the same ratio. The trimers consisted of monomers arranged in a linear fashion.

The diffusion coefficient of carboxydismutase also varied linearly with concentration, and extrapolation of a plot of D versus c gave $D^\circ = 1.77 \times 10^{-7}$ cm²/sec. Corrections for temperature, viscosity, and density to the standard state gave $D^\circ_{20,w} = 2.93 \times 10^{-7}$ cm²/sec. The partial specific volume of carboxydismutase, as determined from measurements of the densities of enzyme solutions and solvent, was 0.730 ml/g.

The molecular weight of carboxydismutase, as determined by Archibald's method, was 560,000 ± 60,000. Another value of the molecular weight, 559,000, was obtained using the equation $M = RTs/D(1 - \bar{v}\rho)$, where R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume, and ρ is the density of the enzyme solution. The most accurate determination of the molecular weight was obtained from a sedimentation equilibrium experiment. A plot of the logarithm of protein concentration versus the square of the distance from the center of rotation (x^2) derived from this experiment is shown in Figure 6. The molecular weight derived from these data was 515,000 ± 10,000. The slight heterogeneity of the preparation of carboxydismutase is indicated by the departure from linearity of the log c versus x^2 plot at high values of x^2, i.e., near the bottom of the centrifuge cell. This heterogeneity was confirmed when the enzyme was subjected to electrophoresis on polyacrylamide gel at *p*H 7.9. A small band of contaminant which contained less than 5% of the total fixed Nigrosin color was observed to move approximately half as fast toward the anode as did the main protein band.

Electron micrographs of the purified enzyme indicated that the molecule is compact and nonlinear. Micrographs at 80,000× magnification, in which the protein had been shadowed with Nichrome, showed that many of the particles had a depression in the center of the upturned face. This finding was confirmed by higher-resolution pictures (magnification factor 480,000×) using sodium phosphotungstate (*p*H 7.6) in the negative staining technique. Almost every particle had an electron-dense center, i.e., there was a hole or depression in the molecule which could be penetrated by the phosphotungstate. Some particles had an electron-dense line across them instead of the hole.

Some of the physical properties described above are presented in Table III, together with values obtained by other workers for fraction I protein and protochlorophyll holochrome.

Discussion

Carboxydismutase activity has long been known to be inhibited by sulfhydryl blocking agents such as *p*-mercuribenzoate and iodoacetamide (Mayaudon *et al.*, 1957), and it has been shown recently that a sulfhydryl group is probably an essential part of the active site of the enzyme (Rabin and Trown, 1964a,b; Trown and Rabin, 1964). It was not surprising therefore that reduced glutathione protected the enzymic activity during purification, presumably by inhibition of the oxidation of the essential sulfhydryl group on the enzyme. That cysteine was not able to act in a similar

manner may be related to its higher reduction potential (Calvin, 1954a), making it less readily oxidized than the essential enzyme sulfhydryl group.

The protection, though small, afforded the enzyme by 1.0 M sucrose is in agreement with the results of Heitefuss *et al.* (1959), who found that polyhydroxy compounds in general stabilized cabbage leaf proteins. The stabilization of carboxydismutase by the nonionic detergent Triton X-100 may be a related phenomenon. Carboxydismutase is evidently a very hydrophilic protein, as is also evident from its very high solubility in aqueous solvents (solutions containing up to 200 mg/ml have been obtained in the present investigation). This hydrophilicity must be owing in part to the large number of charged groups the enzyme carries; its mobility on electrophoresis at *p*H 7.7 (Weissbach *et al.*, 1956) is remarkable considering its high molecular weight. This high charge may account for the dramatic effect which the ionic detergent sodium dodecyl sulfate has upon the enzyme, contrasting sharply with that of the nonionic detergent Triton X-100. Deactivation by sodium dodecyl sulfate is immediate and irreversible. Interaction of charged species on the detergent and enzyme could lead to disruption of the tertiary structure, and indeed carboxydismutase is broken down into subunits with sedimentation coefficients of approximately 2 S by sodium dodecyl sulfate at the concentration used in the present investigations (P. W. Trown, unpublished results).

Experiments with the four possible salts of the cations Na^+ and NH_4^+ and the anions SO_4^{2-} and Cl^- indicated that the anionic content of the salts controls their effect on the enzyme. Sulfate ions reversibly inhibit carboxydismutase and also protect it against deactivation. In contrast, chloride ions caused an apparent activation of the enzyme, which, however, was not maintained, and the long-term result was permanent deactivation. Phosphate buffer at *p*H 7.6 had a similar effect to sulfate; Weissbach *et al.* (1956) reported both phosphate and arsenate to be inhibitors of carboxydismutase. On the basis of these limited results, it seems that divalent anions are able to inhibit and protect the enzyme, whereas the monovalent anion Cl^- causes deactivation. Such a result is not without precedent; London *et al.* (1962) found that prostatic acid phosphatase was protected by sulfate ions against thermal denaturation, whereas the ions Cl^-, Br^-, and CNS^- accelerated the process. Inhibition of enzymic activity by inorganic anions is also fairly common; for example, transaldolase is inhibited by inorganic phosphate (Bonsignore *et al.*, 1960). In all these cases, it is possible that the anions are able to inhibit and/or protect the enzyme by virture of their being similar in structure and/or charge distribution to the substrate, which, in the cases cited, is an organic phosphate. Protection of enzymes by substrates or substrate analogs has often been reported (e.g., Delory and King, 1943; Burton, 1951). It was therefore surprising to find, in this case, that sulfate and phosphate do not seem to be competitive with RuDP as indicated in Figures 2 and 3, respectively. However it should be

TABLE III: Physical Properties of Carboxydismutase, Fraction I Protein, and Protochlorophyll Holochrome.

	Sedimentation Coefficient (S)	Diffusion Coefficient ($cm^2/sec \times 10^{-7}$)	Partial Specific Volume (ml/g)	Molecular Weight	*p*H at Which Electrophoretic Mobility toward Anode Was Observed	Particle Dimensions (from electron micrographs) (A)
Fraction I protein	$s_{20,w} = 17.9$–19.5[a] $s_{20} = 16.2$–16.6[b] $s^\circ_{20} = 18.5$[g]	$D^\circ_{20,w} = 2.60$–2.75[b]	0.69[e]	600,000 (est)[d,e] 375,000[a] 595,000–620,000[b,d]	*p*H 7.2[a]	100×200[f]
Carboxydismutase	$s_{obs} = 17$[h] $s_{20,w} = 18$[j] $s^\circ_{20,w} = 18.57$[i]	$D_{obs} = 5.5$[h] $D_{obs} = 1.6$[j] $D^\circ_{20,w} = 2.93$[i]	0.73[i]	300,000 (est)[h] 515,000 ± 10,000[i]	*p*H 7.7[h] *p*H 6.9[j] *p*H 7.9[i]	100×200[f] ~100[i]
Protochlorophyll holochrome	$s_{20} = 15.3$–16.2[k] $s^\circ_{20,w} = 18.0$[l]	$D^\circ_{20,w} = 2.7$[l]	0.73[l]	400,000–700,000[m] 600,000 ± 50,000[l]	*p*H 9.6[l]	100–110[l]

[a] Eggman *et al.* (1953). [b] Lyttleton (1956). [c] Sample contained 11.2% nucleic acid. [d] Molecular weights calculated assuming $\bar{\nu} = 0.75$ ml/g. [e] Singer *et al.* (1952). [f] Park and Pon (1961). [g] Kupke (1962). [h] Weissbach *et al.* (1956). [i] Present study. [j] Pon (1960). [k] Smith and Kupke (1956). [l] Boardman (1962a). [m] Smith (1960).

pointed out that, since high concentrations of RuDP are inhibitory to carboxydismutase (Weissbach *et al.*, 1956), it is difficult to obtain meaningful results in the crucial kinetic region. An added complication is that the other substrate is probably the charged species HCO_3^- and thus sulfate and phosphate may be competitive with it. At present, it is therefore not possible to decide whether or not sulfate and phosphate inhibit and protect carboxydismutase by virtue of being substrate analogs.

The curvature of the upper line in Figure 3 is unusual; a possible explanation is that phosphate is able in some way to relieve the inhibition of the enzyme by high RuDP concentrations. However in tests of the linearity of the reaction velocity, there were no indications of excess substrate inhibition at the concentrations employed, with the possible exception of the highest concentration, 0.29 mM.

As previously suggested (Dorner *et al.*, 1957; Lyttleton and Ts'o, 1958; Park and Pon, 1961), and also strongly indicated by results to be discussed later, so-called "fraction I protein" is a crude form of carboxydismutase. We may therefore regard attempts to purify fraction I protein and carboxydismutase as synonymous. Such attempts have always been complicated by the tendency of the enzyme to bind other substances to itself to varying degrees. Among the substances which have at some time been considered to be integral parts of fraction I protein are auxin and a phosphatase (Wildman and Bonner, 1947), nucleic acid (Eggman *et al.*, 1953; Mendiola and Akazawa, 1964), and the enzymes phosphoriboisomerase and phosphoribulokinase (Van Noort *et al.*, 1961; Mendiola and Akazawa, 1964). However, Weissbach *et al.* (1956) were able to obtain a preparation of carboxydismutase which did not contain auxin or nucleic acid and which had no other enzymic activity but that of carboxydismutase. More recently, Van Noort and Wildman (1964) were also able to prepare, by precipitation with specific rabbit antibody, fraction I protein which contained carboxydismutase activity, but not phosphoriboisomerase or phosphoribulokinase activity. The present work supports these findings and furthermore shows that the enzymes phosphoriboisomerase and phosphoribulokinase may be partially separated from carboxydismutase by careful ammonium sulfate fractionation and completely separated by repeated gel filtration on Sephadex G-200. This latter technique was employed by Mendolia and Akazawa (1964) for the purification of fraction I protein from rice leaves, but these authors assayed the column eluent for catalysis of the sequence of reactions R-5-P → Ru-5-P → RuDP → PGA using a radioactive substrate (CO_2) in the final stage, and therefore would not have detected phosphoriboisomerase and phosphoribulokinase if they had been separated from carboxydismutase.

The position at which phosphoriboisomerase and phosphoribulokinase were eluted from the Sephadex G-200 column indicates that these enzymes have molecular weights smaller than 200,000, which is normally thought to be the exclusion limit for this particular Sephadex. Since the molecular weight of carboxydismutase is much greater than 200,000 it is excluded from Sephadex G-200, but it is nevertheless retarded somewhat on the column compared with a material containing nucleic acid, which comes through with the front. The retardation of carboxydismutase must be caused by specific physical interaction between the enzyme and the column material. This may be yet another manifestation of the hydrophilic nature of carboxydismutase, resulting in interaction with the polyhydroxy compound dextran.

The purity of the carboxydismutase prepared by the method described was very high. The slight departure from linearity of the plot of log c versus x^2 in a sedimentation equilibrium experiment was an indication of the presence in the sample of a small amount of a contaminant of higher molecular weight. This finding was confirmed by electrophoresis on polyacrylamide gel where a very small amount of slower-moving (i.e., less charged or of higher molecular weight) contaminant was detected. This contaminant may well be polymerized carboxydismutase which was encountered in a preparation of the enzyme which had been stored under non-optimal conditions for extended periods.

The physical properties of carboxydismutase are very similar to those of fraction I protein, as may be seen in Table III. The sedimentation coefficients of the two substances, particularly those of the more purified preparations, are virtually identical. The properly corrected diffusion coefficient ($2.60–2.75 \times 10^{-7}$ cm²/sec) of the fraction I protein prepared by Lyttleton (1956) compares well with that determined for purified carboxydismutase (2.93×10^{-7} cm²/sec). The values determined by Weissbach *et al.* (1956) and Pon (1960) were not corrected to the standard state. Probably the most reliable molecular weight of fraction I protein, determined by Lyttleton (1956), was calculated for an assumed partial specific volume of 0.75 ml/g. If the value determined for carboxydismutase (0.73 ml/g) is used in the calculation, the molecular weight obtained (550,000–574,000) compares well with that determined for purified carboxydismutase (515,000 ± 10,000). The values obtained by Singer *et al.* (1952) and Weissbach *et al.* (1956) were stated by the authors to be estimates, and that obtained by Eggman *et al.* (1953) was determined for material containing 11.2% nucleic acid. In all cases where it was investigated for both fraction I protein and carboxydismutase, the direction of electrophoretic mobility was toward the anode in the *p*H range 6.9–7.9. Both fraction I protein and carboxydismutase were stated by most of the authors cited under Table III to be unstable below *p*H 6, both being precipitated and irreversibly denatured. Electron micrographs of both fraction I protein and a crude preparation of carboxydismutase showed particles 100 × 200 A in each preparation which were indistinguishable from each other (Park and Pon, 1961). The present preparation appears to contain similar particles with an average diameter of 100 A.

The foregoing evidence, in conjunction with the reported finding that material prepared as fraction I

protein showed carboxydismutase activity (Lyttleton and T'so, 1958; Park and Pon, 1961; Van Noort and Wildman, 1964), strongly indicates that carboxydismutase and fraction I protein are identical. Certainly the obvious characteristic of fraction I protein, i.e., its high molecular weight and consequent high sedimentation coefficient, is due to carboxydismutase. Any other enzymatic activity present in fraction I protein would appear to be owing to absorption on carboxydismutase of the enzymes involved. Further evidence to support this hypothesis was recently reported by Trown and Rabin (1964), who found that the equivalent weight of carboxydismutase on the basis of the sulfhydryl group at the active site of the enzyme was 267,000. There are thus two active sites per molecule of 515,000 mw. It is still possible, though not likely on the basis of the present evidence, that a large protein molecule exists in the plant to which the various enzymes of the carbon cycle, including carboxydismutase, are attached. It seems more likely that the large protein molecule is carboxydismutase and that the other enzymes are either bound to it in some kind of organized structure in the plant or become bound, as did auxin and nucleic acid, during the isolation process.

The similarities of the physical properties of carboxydismutase and those of protochlorophyll holochrome are also striking (see Table III). The most purified preparations of each have virtually identical sedimentation coefficients, diffusion coefficients, partial specific volumes, and molecular weights. Although the stability of the holochrome to *p*H changes has not been studied extensively, a lyophilized preparation of the protein is soluble only above *p*H 6.0 (below which carboxydismutase is denatured). Electron micrographs of carboxydismutase and protochlorophyll holochrome are indistinguishable from each other. Both show particles with holes or depressions in the centers of the molecules, particularly evident on negative staining with sodium phosphotungstate, and the particle dimensions obtained from the micrographs are the same. Another property reported by Boardman (1962a) for the holochrome is also shown by carboxydismutase. After freezing and thawing a solution of the holochrome, the single schlieren peak corresponding to 18 S is split into three peaks corresponding to 18, 26, and 32 S. A similar splitting of the single schlieren peak into 18, 26, and 32 S peaks was observed in one preparation of carboxydismutase which had been exposed to some unknown factor not normally encountered in isolation or storage of the enzyme. In this case the phenomenon was demonstrated, by electron microscopy, to be owing to aggregation into dimers and trimers, a conclusion also reached by Boardman (1962a) for protochlorophyll holochrome.

In reporting the isolation of protochlorophyll holochrome, Boardman (1962a) pointed out the similarity of the holochrome to fraction I protein and confirmed that the two substances are indistinguishable in the ultracentrifuge. The present findings, in confirming the identity of fraction I protein and carboxydismutase, and taken in conjunction with the striking similarities in physical properties between carboxydismutase and holochrome, make it likely that carboxydismutase makes up the protein moiety of protochlorophyll holochrome. This is not unlikely in view of the ability, already discussed, of carboxydismutase to bind other substances to itself. In this connection it is of interest that the amount of active protochlorophyll per protein molecule varies considerably from one preparation of protochlorophyll holochrome to another and also during the isolation process (Boardman, 1962a). In addition, it has been shown that probably not all of the active protochlorophyll in the holochrome preparations is bound to the protein in the same way (Smith and Coomber, 1960; Boardman, 1962b). Ultraviolet light converts only 30% of the transformable protochlorophyll to chlorophyll a; the remainder is converted by visible light (McLeod and Coomber, 1960). These two pieces of evidence do not support the hypothesis that protochlorophyll is bound to a site specifically designed for this purpose in the plant, and it seems that the binding of protochlorophyll to the protein may be fortuitous, possibly occurring during the isolation procedure. Isolation of a protochlorophyll holochrome preparation which showed carboxydismutase activity will shed considerable light on this question, and work is proceeding along these lines.

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