Wålinder, O. (1968), *J. Biol. Chem. 243*, 3947.
Wålinder, O. (1969), *J. Biol. Chem. 244*, 1065.
Walkinshaw, C. H., and Van Lancker, J. L. (1964), *Lab. Invest. 13*, 513.

Ribulose Diphosphate Oxygenase. I. Synthesis of Phosphoglycolate by Fraction-1 Protein of Leaves†

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ABSTRACT: Preparations of ribulose diphosphate carboxylase (fraction-1 protein) from both soybean and spinach leaves catalyzed the formation of phosphoglycolate and 3-phosphoglycerate from ribulose 1,5-diphosphate in the presence of oxygen. A manometric assay was used, and the activity called ribulose diphosphate oxygenase. Fraction-1 protein was purified from spinach leaves by a two-step procedure involving DEAE-cellulose chromatography and sucrose density gradient centrifugation in a zonal rotor. The protein was electrophoretically homogeneous. The oxygenase and carboxylase activities co-purified, and other attempts to separate them were unsuccessful. However, the oxygenase was more stable than the carboxylase, and the activity ratio, oxygenase/carboxylase, increased from 0.25 in the crude extract to 0.59 in the final product. The oxygenase was also more stable than the carboxylase when the protein was stored as an $(NH_4)_2SO_4$ precipitate. The pH optimum of the oxygenase activity was about 9.3–9.5, being much more alkaline than that of the carboxylase. No activity was observed in the absence of Mg^{2+} ions. A gas phase of 100% oxygen was not sufficient to saturate the oxygenase and the activity in air was 37% of that in pure oxygen. The Michaelis constant for ribulose 1,5-diphosphate was about 0.18 mM. The purified protein did not catalyze the oxygenation of several other phosphate esters. It is probable that, during photosynthesis, ribulose diphosphate is carboxylated and oxygenated by the same protein and that the oxygenating activity is responsible for the supply of phosphoglycolate, the first intermediate in the glycolate pathway of photorespiration.

It is now established that the glycolate pathway (Tolbert, 1963, 1971) is the metabolic sequence responsible for the physiological phenomenon of photorespiration in plants. This light-dependent uptake of oxygen and release of recently fixed carbon, as CO_2, accompany photosynthesis in atmospheres containing oxygen (Jackson and Volk, 1970). While the reactions involved in the metabolism of glycolate and the release of CO_2 are reasonably well understood, the mechanism of synthesis of this two-carbon acid has remained enigmatic ever since it was recognized as one of the major radioactive products of photosynthetic $[^{14}C]CO_2$ fixation (Benson and Calvin, 1950). Hypotheses concerning possible synthetic mechanisms may be grouped into three categories, namely (a) those which involve a reductive condensation of two molecules of CO_2 (reviewed by Zelitch, 1971), (b) those which propose the oxidation of the two-carbon fragment of dihydroxyethylthiamine pyrophosphate which is involved in the transketolase reaction (reviewed by Gibbs, 1969), and (c) the proposal of Ogren and Bowes (1971) that RuDP[1] is oxidized by molecular oxygen in the presence of RuDP carboxylase (fraction-1 protein), thus producing phosphoglycolate and 3-phosphoglycerate. Data in this paper and in our other recent publications (Andrews *et al.*, 1971; Lorimer *et al.*, 1972, 1973) substantiate the last hypothesis, which is consistent with a wide range of experimental observations concerning photorespiration and glycolate biosynthesis (see Discussion). Bowes *et al.* (1971) had obtained indirect evidence for phosphoglycolate formation by assaying for the glyoxylate phenylhydrazone, after incubation with crude preparations of phosphoglycolate phosphatase and glycolate oxidase. In our investigations phosphoglycolate formation has been measured, and some enzymatic characteristics of the RuDP oxygenase are compared with the RuDP carboxylase activity of the same protein. Preliminary reports concerning this work have appeared (Lorimer, 1972; Lorimer *et al.*, 1972a).

Experimental Section

Materials

Spinach was grown in a growth chamber as previously described (Andrews *et al.*, 1971). RuDP (tetrasodium salt) was from Sigma Chemical Co., phosphoglycolate from General Biochemicals Inc., and other chemicals and enzymes were of the highest purity commerically available. Purified fraction-1 protein from soybean leaves was supplied by Dr. W. L. Ogren, U. S. Department of Agriculture Regional Soybean Laboratory, Urbana, III.

Methods

Preparation of [U-^{14}C]RuDP. This was prepared in a reaction mixture of 3 ml containing 0.5 mM [U-^{14}C]glucose

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[1] Abbreviations used are: RuDP, D-ribulose 1,5-diphosphate; Ammediol, 2-amino-2-ethyl-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid.

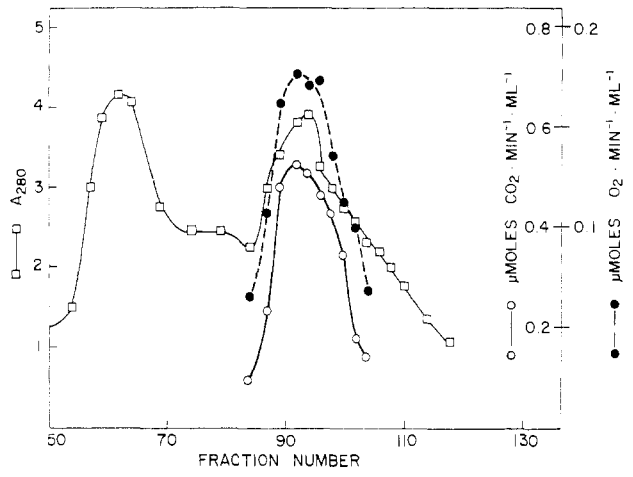

FIGURE 1: DEAE-cellulose chromatography of crude supernatant from spinach leaf homogenate. The beginning of the NaCl gradient would have emerged at approximately fraction 50.

6-phosphate (35 μCi/μmol from Calatomic), 25 mM Tris-HCl (pH 7.7), 8 mM $MgCl_2$, 2.5 mM dithiothreitol, 1 mM EDTA, 3 mM ATP, 3 mM NADP, 10 units of glucose phosphate dehydrogenase, 10 units of 6-phosphogluconate dehydrogenase, and 2 units of phosphoribulokinase. After incubation for 60 min at 30°, 500 mg of activated charcoal was added. The charcoal was removed by filtration and washed. The filtrate plus washings were reduced to 0.6 ml by lyophilization and applied as a streak to the origin of a Whatman 3 MM chromatogram. Descending chromatography was performed for 19 hr with butan-1-ol–propionic acid–water (10:5:7, v/v/v) as solvent. After drying, strips from both sides of the chromatogram were examined with a radiochromatogram scanner. A large peak of RuDP was observed near the origin, preceded by a smaller peak of monophosphates. The area corresponding to the RuDP peak was cut out of the chromatogram and eluted with water and the eluate neutralized by the addition of Tris-free base. Both rechromatography in the above system and high-voltage paper electrophoresis by the method of Bieleski and Young (1963) showed that all the radioactivity corresponded with a spot of authentic RuDP as visualized by the phosphate ester spray reagent of Bandurski and Axelrod (1951).

Reactions with [^{14}C]RuDP. The products of the oxygenase reaction were identified by paper chromatography. Reaction mixtures typically contained 100 mM Tris or Ammediol–HCl at pH 8.0, 5 mM $MgCl_2$, and 0.4 mM [U-^{14}C]RuDP (0.9 μCi/μmol) in a volume of 0.1 ml. They were sealed in small vials closed with a rubber septum. The vials were then gassed with oxygen by alternately drawing a vacuum and then admitting oxygen by means of a syringe needle. This procedure was carried out ten times. The reaction was started by injecting enzyme solution from either soybean or spinach leaves. After incubation for various times at 30°, the reactions were stopped either by placing in a boiling water bath for 2 min or by the addition of approximately 2 units of alkaline phosphatase to hydrolyze the substrate. When the reactions were stopped by boiling, an aliquot was subsequently taken and treated with alkaline phosphatase. Before chromatography, sufficient EDTA was added to be in excess of the Mg^{2+} concentration present. Aliquots of 25 μl were applied to the origin of 3MM Whatman chromatograms in parallel with appropriate markers and the chromatograms were developed in the descending manner for 14–16 hr. The solvent was either butan-1-ol–propionic acid–water (10:5:7, v/v/v) or 1-pentanol saturated with 5 M formic acid. As soon as dry, the chromatograms were sprayed with 0.1 M $NaHCO_3$ to neutralize glycolic acid which might otherwise be slightly volatile. After drying again, the chromatograms were cut into strips and scanned with a radiochromatogram scanner. Markers were located with the phosphate spray reagent or by the ^{14}C label in the cases of glycerate and glycolate samples.

Purification of Fraction-1 Protein. About 250 g of washed, spinach leaf tissue without midvein was ground in a Waring Blendor at full speed for 30 sec with 500 ml of 25 mM glycylglycine–KOH buffer solution, pH 7.7, containing 50 mM 2-mercaptoethanol and 1 mM EDTA. This and all subsequent steps were carried out at 4°. The homogenate was filtered through four layers of cheesecloth and one layer of miracloth, and the filtrate was centrifuged at 40,000*g* for 30 min. A 1-ml sample of the supernatant was freed of low-molecular weight compounds before assay by passage through a 0.7 × 16 cm column of Sephadex G-25, equilibrated with the above buffer with 1 mM dithiothreitol in place of 2-mercaptoethanol.

The crude supernatant was applied directly to a 6 × 24 cm column of DEAE-cellulose previously equilibrated with a solution containing 25 mM glycylglycine–KOH, pH 7.7, and 1 mM dithiothreitol. After all the supernatant had entered the column, one column volume of this buffer was allowed to pass through before elution was begun with a 2-l. gradient from 0 to 1 M NaCl added to the buffer. Fractions of about 22 ml were collected. RuDP oxygenase and RuDP carboxylase activities emerged together (Figure 1). Active fractions (84–104) were pooled and the protein in them precipitated by the addition of 1.5 vol of a saturated $(NH_4)_2SO_4$ solution at pH 7.6 (4°). The precipitate was collected by centrifugation and dissolved in 25 mM glycylglycine–KOH buffer, pH 7.7, containing 1 mM dithiothreitol and 1 mM EDTA. This solution was centrifuged at 40,000*g* for 30 min after which the volume of the supernatant was 30 ml. A small sample was taken for assay and passed through a small Sephadex G-25 column as described previously.

Further purification was carried out by a sucrose gradient centrifugation technique similar to that of Goldthwaite and Bogorad (1971) but adapted to the zonal rotor. A 600-ml linear (by volume) density gradient from 15 to 30% (w/w) sucrose in 25 mM glycylglycine–KOH buffer at pH 7.7 was loaded into a B-30 zonal rotor from the rim followed by sufficient 35% (w/w) sucrose to fill the rotor. The enzyme solution in 30 ml from the DEAE-cellulose column was adjusted (if necessary) by the addition of buffer so that it was less dense than 15% (w/w) sucrose and then pumped in from the rotor core followed by a 50-ml overlay of buffer solution. Centrifugation was conducted for 4.5 hr at 50,000 rpm. The gradient was displayed by pumping 35% (w/w) sucrose in from the rim and fractions of 25 ml were collected. The large peak of fraction-1 protein, which contained both the oxygenase and carboxylase activities, was well separated from some lower molecular weight contaminants (Figure 2). The apparent asymmetry of the fraction-1 protein peak is due to dilution incurred by the radial nature of the migration. Active fractions (8–14) were pooled and the protein was precipitated by $(NH_4)_2SO_4$ as before and stored at 4°. As required, portions of this suspension were centrifuged and the pellet was dissolved in 250 mM glycylglycine–NaOH buffer,

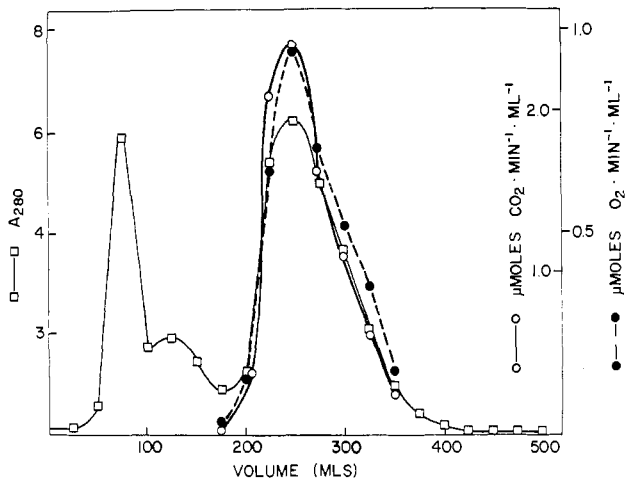

FIGURE 2: Sucrose density gradient centrifugation of pooled active fractions from DEAE-cellulose chromatography. Fractions were collected from the rotor core.

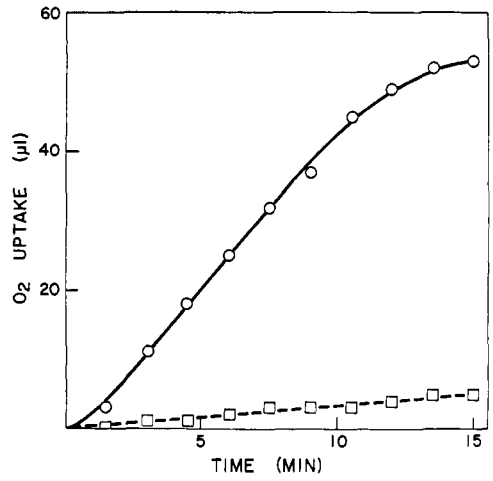

FIGURE 3: RuDP-dependent oxygen uptake catalyzed by fraction-1 protein: O, complete reaction (see Methods section); □, control, minus RuDP

pH 8.6, containing 10 mM dithiothreitol and 10 mM EDTA. This solution was then freed of $(NH_4)_2SO_4$ by passage through a small column of Sephadex G-25 equilibrated with a tenfold dilution of the buffer.

RuDP Oxygenase Assay. Methods which measured phosphoglycerate appearance or RuDP disappearance were considered unsatisfactory because of the complication caused by the competing carboxylation reaction. Measurement of phosphoglycolate by determining glycolate with the Calkins (1943) procedure was also unsatisfactory or very time consuming because of interference by unreacted ribulose. This interference was also encountered in the phenylhydrazine method used by Bowes *et al.* (1971). The method chosen employed the manometric measurement of oxygen uptake. Sensitivity was maximized by using small flasks (volume approximately 3 ml) and a Gilson constant volume respirometer. Assays with an oxygen electrode could also be used provided that the "dead space" of the electrode was not an appreciable fraction of the small reaction volumes, which were dictated by the expense of the substrate.

In the manometric assay the reaction mixture contained 100 μmol of Ammediol–HCl, pH 9.3, 10 μmol of $MgCl_2$, 1 μmol of EDTA, 0.4 μmol of dithiothreitol, and enzyme (0.5–2.0 mg of protein) in a volume of 0.92 ml. The side arm contained 2 μmol of RuDP in 80 μl. The final pH of the total mixture was 9.2. The center wells of the flasks were normally empty except for experiments where bicarbonate was added to the reaction mixture, in which case they contained wicks saturated with 9 M KOH. The flasks were attached to the apparatus (bath temperature 25°) and shaken (150 oscillations min^{-1}) for 3 min with pure oxygen passing through and were equilibrated for a further 9 min while closed. The reaction was then started by tipping in the RuDP from the side arm. Readings were taken every 90 sec. A short lag was usually observed, followed by a linear reaction which continued until RuDP became limiting (Figure 3). A control in which RuDP was omitted was run with each batch of assays. A significant rate of uptake was always observed in these controls due to leakage of oxygen through the Tygon tubing of the apparatus. This rate could be minimized by allowing oxygen to flow through the apparatus for at least an hour before use. The control rate could not be determined for each individual flask by observing the rate before adding substrate. If this were done a much lower net rate was observed, presumably due to inactivation of the enzyme in 100% oxygen at high pH during the extended period required to accurately determine the control rate. The net rate in microliters per minute was reduced to STP and converted to micromoles of O_2 uptake per minute by dividing by 22.4.

RuDP Carboxylase Assay. A spectrophotometric assay similar to that of Racker (1962) was employed. The reaction mixture contained the following components in a volume of 0.25 ml: 25 μmol of Tris-HCl at pH 7.8, 2.5 μmol of $MgCl_2$, 0.15 μmol of EDTA, 1.3 μmol of dithiothreitol, 0.13 μmol of $NADH_2$, 2.5 μmol of ATP, 12.5 μmol of $NaHCO_3$, approximately 1 unit each of phosphoglycerate kinase, phosphoglyceraldehyde dehydrogenase, triosephosphate isomerase, and glycerol phosphodehydrogenase, and sufficient carboxylase to give a rate of not more than 2 nmol min^{-1}. The temperature was 25°. After 10 min preincubation the reaction was initiated by the addition of 0.13 μmol of RuDP. A reaction blank, less carboxylase, was included. A rapid, nonlinear rate lasting about 2 min which occurred in all cases, including the blank, was ignored. Following this a linear rate proportional to carboxylase concentration was observed and this rate was used to calculate enzyme activity. A mixture of the coupling enzymes was prepared and freed of $(NH_4)_2SO_4$, which inhibits the carboxylase (Paulsen and Lane, 1966), by passage through a small Sephadex G-25 column equilibrated with a buffer solution containing 50 mM Tris-HCl at pH 7.8, 5 mM dithiothreitol, and 2 mM EDTA. Stored at 4° under nitrogen this coupling system remained active for at least 2 weeks.

Standardization of RuDP. Solutions of RuDP were standardized using the above spectrophotometric assay system with excess carboxylase and limiting RuDP. The concentration was determined from the total decrease in absorbancy at 340 nm which followed the addition of an aliquot of the RuDP solution.

Protein Determination. Protein was determined from absorbance at 260 and 280 nm by the method of Warburg and Christian (1941). In the case of the purified protein, values obtained by this method must be multiplied by 0.52 for comparison with those of Paulsen and Lane (1966) who used the formula: $A_{280} \times 0.61$ = milligrams/milliliter.

TABLE I: Purification of RuDP Oxygenase and RuDP Carboxylase.

Step	Protein (mg)	A_{280}/A_{260}	RuDP Oxygenase: Specific Activity (μmol min^{-1} mg^{-1})	RuDP Oxygenase: Yield (%)	RuDP Carboxylase: Specific Activity[d] (μmol min^{-1} mg^{-1})	RuDP Carboxylase: Yield (%)	Oxygenase/Carboxylase
Initial extract[a]	2460	0.93	0.051	100	0.21	100	0.25
DEAE-cellulose[a,b]	900	1.93	0.088	63	0.22	38	0.41
Sucrose density gradient centrifugation[c]	705	2.00	0.125	70	0.30	41	0.42
Redissolve $(NH_4)_2SO_4$ precipitate[a,b]	591	2.04	0.124	59	0.21	24	0.59

[a] Determinations performed after a small sample had been freed of 2-mercaptoethanol and other low molecular weight compounds by passage through a small column of Sephadex G-25 as described in the Methods section. [b] Redissolved $(NH_4)_2SO_4$ precipitate of the pooled active fractions. [c] Values obtained by summation of results of assays of individual fractions 8–14 of Figure 2. [d] These values must be multiplied by at least 3 for comparison with the specific activities of Paulsen and Lane (1966) who used a different formula to determine protein and assayed at 30° instead of 25°.

Results

Identification of Products Using [U-^{14}C]RuDP. The reaction and products were first explored with[^{14}C]RuDP and paper chromatography before and after dephosphorylation. Experiments were performed at pH 8, as used by Bowes *et al.* (1971), with a partially purified preparation of spinach fraction-1 protein. Before phosphatase treatment, chromatography of reaction mixtures with butan-1-ol–propionic acid–water as solvent showed a conversion of the [^{14}C]RuDP to products with R_F values in the area of 3-phosphoglycerate and phosphoglycolate, but these similar structures were not well separated by this system. When chromatography was performed on phosphatase-treated reaction mixtures with a solvent of pentan-1-ol saturated with 5 mM formic acid, three clearly separated peaks were detected by the radiochromatography scanner. They cochromatographed with authentic samples of ribulose, glycerate and glycolate in order of increasing R_F. No detectable reaction occurred when oxygen was replaced with nitrogen. It was also observed that the rate of glycolate formation was increased about fivefold if the pH was raised to 9.0–9.5. When 25 mM $NaHCO_3$ was included in the reaction mixture under oxygen, the amount of ribulose from the substrate, RuDP, was greatly reduced and the glycerate peak greatly enlarged. Under these conditions a small amount of phosphoglycolate was still formed. Essentially the same results were obtained with the enzyme from soybean leaves except that no glycolate was formed when bicarbonate was present. Since this chromatographic procedure was somewhat cumbersome, the manometric assay described in the Methods section was developed and used in all subsequent experiments. Subsequent proof of products with $^{18}O_2$ and the proposed reaction mechanism are presented in our next paper (Lorimer *et al.*, 1973).

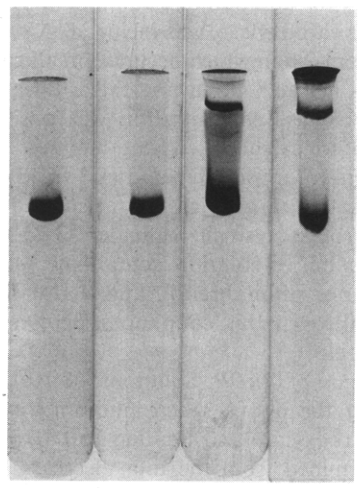

FIGURE 4: Polyacrylamide gel electrophoresis of purified fraction-1 protein. The method of Davis (1964) was used with a 6% gel. Samples containing 65 μg of protein were applied in a solution containing 10% glycerol. Electrophoresis was conducted at 5 mA/gel for 100 min. Subsequently the gels were stained for 3 hr in 0.5% Amido-Schwartz in 7.5% acetic acid and destained in 7.5% acetic acid. Electrophoresis was conducted immediately after purification (a) and after storage as an $(NH_4)_2SO_4$ precipitate for 12 (b), 26 (c), and 49 (d) days (a–d, left to right).

Purification of Fraction-1 Protein. The purification of fraction-1 protein from the leaves of higher plants to apparent homogeneity was facilitated by the fact that this protein usually constitutes 30% or more of the total soluble protein of the leaf. A preparation greatly enriched with fraction-1 protein, although not pure, could be obtained by sucrose density gradient centrifugation of crude homogenates from spinach leaves in a zonal rotor (Methods section). This partially purified enzyme preparation, together with the purified enzyme from soybean, was used to investigate the oxygenase reaction using [^{14}C]RuDP and to develop the manometric assay. Subsequent attempts to separate the oxygenase activity from the carboxylase activity by various combinations of $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography, hydroxylapatite chromatography, and zonal centrifugation in a sucrose density gradient were unsuccessful. Whichever combination of techniques was used, the final product, which was invariably homogeneous as judged by disc gel electrophoresis, contained both carboxylase and oxygenase activities. The abbreviated procedure finally adopted (see Methods section and Table I) was rapid and gave a homogeneous product (Figure 4a) and a good yield of the oxygenase. When oxygenase assays were performed in

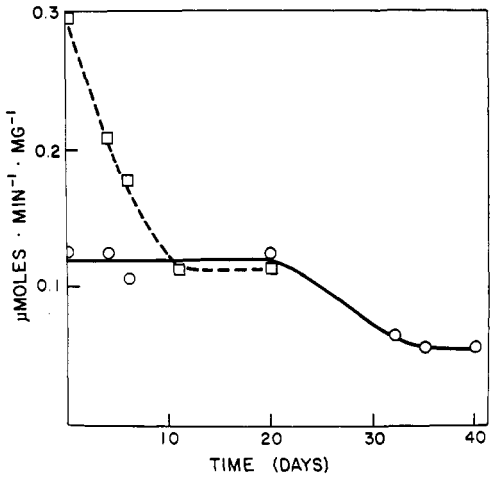

FIGURE 5: Stabilities of carboxylase (□) and oxygenase (O) activities during storage of the purified enzyme in 60% saturated $(NH_4)_2SO_4$.

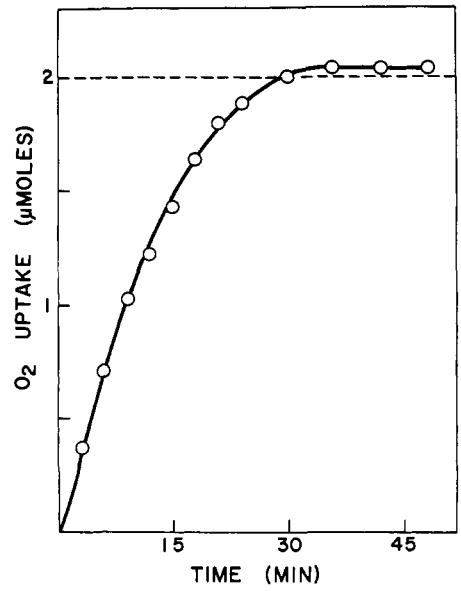

FIGURE 6: Stoichiometric consumption of oxygen by RuDP oxygenase. The reaction was initiated with 2 μmol of RuDP and standardized by the procedure given in the Methods section. Uptake occurring in the control, minus RuDP, has been subtracted for each point.

parallel with carboxylase assays on individual fractions from DEAE-cellulose chromatography and zonal centrifugation, it was apparent that the two activities followed one another (Figures 1 and 2). This evidence suggested that the two activities were due to the same protein. However, in all preparations the final yield of the carboxylase was considerably lower than that of the oxygenase. Apparently the carboxylase was less stable than the oxygenase during one or more steps of the purification procedure. As a result the activity ratio, oxygenase/carboxylase, increased during the course of the purification (Table I).

Stability During Storage. When the $(NH_4)_2SO_4$ precipitate of the purified enzyme was redissolved as described in the Methods section and stored under nitrogen at 4°, both activities decreased by only about 20% during a 2-week period. No activation occurring soon after the precipitate was redissolved, as described by Bowes and Ogren (1972), was observed. However, as an $(NH_4)_2SO_4$ precipitate, the carboxylase was less stable than the oxygenase (Figure 5). Over a period of 3 weeks immediately after preparation, the carboxylase lost almost two-thirds of its activity while the oxygenase remained fully active. However, in the subsequent 3 weeks the oxygenase also lost about half of its original activity. It was apparent that polymerization of the protein was occurring during storage of the $(NH_4)_2SO_4$ precipitate. While polyacrylamide gel electrophoresis of the purified enzyme immediately after preparation showed only one band (Figure 4a), similar gels for the same preparation after various times of storage showed a progressive appearance of another band with a lower mobility and eventually a cluster of bands which barely moved into the gel (Figure 4b–d). Analytical ultracentrifugation also showed the progressive appearance of species with higher molecular weights (data not shown). Similar evidence has been interpreted by Kleinkopf *et al.* (1970) to indicate polymerization. However, any correlation between the extent of polymerization and the loss in carboxylase or oxygenase activities is tenuous at best.

Requirements for Oxygen Uptake. The requirements for oxygen uptake are given in Table II. As well as the substrate, Mg^{2+} ions were absolutely required. Dithiothreitol was required for full activity and the concentration used, 0.4 mM, was sufficient to satisfy this requirement while causing negligible uptake of oxygen due to its own slow oxidation. A lag period of about 90 sec after addition of RuDP was routinely observed (Figure 3). Similar lag periods have sometimes been observed in the assay of RuDP carboxylase (*e.g.*, Paulsen and Lane, 1966). The activity measured after the lag period was a linear function of enzyme concentration over the range tested of 0.2–1.1 mg of protein per assay (data not shown).

Stoichiometry of Oxygen Uptake. A stoichiometry of 1 was observed between RuDP consumption and oxygen uptake. When the reaction was allowed to proceed to completion with a known amount of RuDP present, 1 mol of oxygen was consumed per mole of RuDP consumed (Figure 6).

pH Optimum. Using the manometric assay, the pH *vs.* rate profiles shown in Figure 7 were obtained for the purified spinach enzyme. A sharp optimum in the region pH 9.3–9.5 was observed. Since Ammediol has poor buffering capacities above pH 9.5, the assays were also run with glycine as a buffer. To minimize the possibility of inactivation, while the enzyme was maintained at high pH during the gassing and equilibration period, the usual procedure was reversed for experiments with the glycine buffer in that the

TABLE II: Requirements of RuDP Oxygenase Reaction.

Composition of Reaction Mixture	Rel Activity (% of Control)
Complete[a]	100
Minus RuDP	0
Minus O_2[b]	0
Minus $MgCl_2$	0
With boiled enzyme (2 min, 100°)	0
Minus dithiothreitol	55
With 50 μM dithiothreitol	87

[a] The complete reaction is described in the Methods section.
[b] Gassed with nitrogen instead of oxygen.

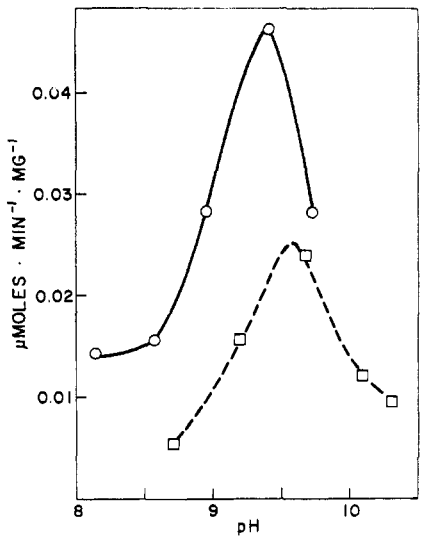

FIGURE 7: pH *vs.* activity profiles of RuDP oxygenase: ○, 0.1 M Ammediol–HCl buffer, standard procedure; □, 0.1 M glycine–NaOH, enzyme in side arm.

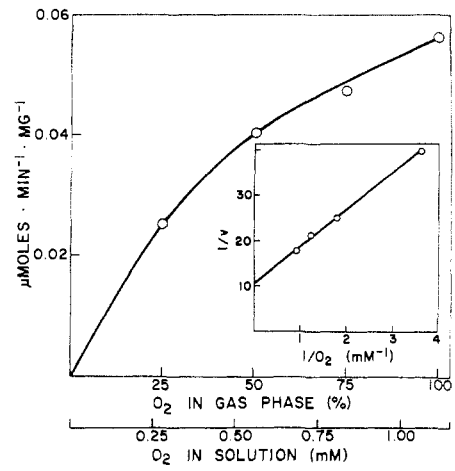

FIGURE 8: RuDP oxygenase activity as a function of oxygen concentration. The standard assay procedure was used. The various gas mixtures were obtained by mixing nitrogen and oxygen with gear-driven displacement pumps.

enzyme was placed in the side arm and the substrate in the main reaction mixture. The lower activity observed with glycine is somewhat misleading. A decrease in activity was also observed with Ammediol buffer if the enzyme was placed in the side arm. Apparently a preactivation period in the presence of the other components of the reaction mixture is required for full oxygenase activity. A similar observation has been made concerning the carboxylase reaction (Pon *et al.*, 1963) and was confirmed by us. However, extended preincubation of the enzyme in the reaction mixture was also undesirable. Not only was the maximum oxygenase activity greatly reduced (see Methods section), but the pH optimum was shifted to the region of pH 8.6 with very little activity being observed above pH 9.2.

Oxygenase Activity as a Function of Oxygen and RuDP Concentrations. Oxygenase activity responded to oxygen concentration in a hyperbolic manner but was not saturated by 100% oxygen (Figure 8). The rate at an oxygen concentration equivalent to that in air was 37% of that in pure oxygen, the Michaelis constant being about 0.75 mM.

The response to RuDP concentration also appeared to be hyperbolic. However, the points obtained at the lower concentrations were regarded as approximate, since the accuracy of the assay declined when the complete reaction involved less than about 20 μl of oxygen uptake (*i.e.*, about 0.3 mM RuDP in an expanded 3-ml reaction volume used). A K_m(RuDP) of 0.18 mM and a V_{max} of about 0.1 μmol min^{-1} mg^{-1} of protein were observed, but the values should be regarded as approximate. A similar value has been observed for the K_m(RuDP) for the carboxylase activity (Paulsen and Lane, 1966).

Substrate Specificity. The oxygenase reaction appeared to be specific for RuDP. With the manometric assay, no activity could be detected with fructose 1,6-diphosphate (3 mM), fructose 6-phosphate (3 mM), 3-phosphoglycerate (3 mM), or ribulose 5-phosphate (2 mM). The presence of alkaline phosphatase completely inhibited the standard assay, thus showing that free ribulose was not a substrate. However, the manometric assay would not have been able to detect a rate which was less than 5% of the rate observed with RuDP, so a more sensitive ^{14}C method was also used for the fructose phosphates. The procedure was the same as used for the manometric assay with 1 mM [U-^{14}C]fructose 1,6-diphosphate (1 μCi/μmol) and 1 mM [U-^{14}C]fructose 6-phosphate as substrates. The reaction mixtures were half-scale and 4 mg of purified enzyme was used in each reaction. After 60 min the reactions were stopped and chromatographed by the procedures described in the Methods section for ^{14}C studies. In no instance was any [^{14}C]glycolate formed.

The lack of activity with 3-phosphoglycerate as substrate eliminates the possibility that RuDP was first carboxylated, and the 3-phosphoglycerate thus formed was subsequently oxidized to phosphoglycolate.

Effect of Bicarbonate. The addition of 50 mM $NaHCO_3$ to the reaction mixture for the manometric assay caused a large reduction in the extent of the oxygenase reaction due to removal of RuDP by carboxylation. However, no decrease in the initial rate of oxygen uptake was observed. Since these experiments were conducted at pH 8.7–9.0 where the concentration of CO_2 is less than 1% of that of the bicarbonate supplied, no conclusions concerning any possible inhibitory properties of CO_2 can be drawn. Similar experiments at lower pH values where the CO_2 concentration would be higher were not technically feasible because of the very rapid rate of substrate removal by carboxylation.

Discussion

The present demonstration of the conversion of [U-^{14}C]-RuDP to labeled 3-phosphoglycerate and phosphoglycolate and the stoichiometry of oxygen uptake provide evidence for enzymatic activity in spinach and soybean leaves which catalyzes the reaction

$$\text{RuDP} + O_2 \longrightarrow \text{3-}P\text{-glycerate} + P\text{-glycolate}$$

Further identification of the products by combined gas–liquid chromatography and mass spectrometry is reported in the accompanying paper (Lorimer *et al.*, 1973). While it is probable that this oxygenation is catalyzed by the same protein as RuDP carboxylase (fraction-1 protein), this is not established beyond doubt. The final enzyme preparations were electrophoretically homogeneous and attempts to separate the two activities by a variety of purification techniques

failed. However, in view of the very low specific activity, that is a characteristic property of RuDP carboxylase, an undetectable contaminant with a high specific activity might account for the oxygenase. The difference in stabilities of the carboxylase and oxygenase activities during purification and storage in $(NH_4)_2SO_4$ does not establish nonidentity. It is conceivable that the two activities could be due to the same RuDP binding site, with the oxygenase being less strict in its requirements for an active site than the complete carboxylation reaction. For example, the observed difference in stability could be consistent with a situation where oxidation of one or more sulfhydryl groups caused cessation of carboxylation, while oxygenation continued unaffected.

Regardless of whether or not RuDP oxygenase is the same protein as the carboxylase, the fact that such enzymatic activity exists is of major importance regarding photosynthesis and photorespiration. This becomes a proven reaction by which phosphoglycolate, and thus glycolate, may be synthesized during photosynthesis. The characteristics of the oxygenase reaction are consistent with its being the primary photorespiratory reaction. A wide range of experimental observations concerning photorespiration and glycolate biosynthesis can be rationalized in terms of this reaction and are listed as follows.

(i) RuDP oxygenase constitutes a site of involvement of oxygen in photorespiration, the others being glycolate oxidase and glycine oxidation. While glycolate oxidase becomes effectively saturated at oxygen concentrations of about 60% (Lorimer, 1972), RuDP oxygenase is not saturated even at 100% oxygen (Figure 8). This observation correlated with the oxygen saturation characteristics of photorespiration (Forrester *et al.*, 1966).

(ii) The physiological conditions of low CO_2 and high oxygen concentrations, known to promote photorespiration, and the inhibitory effect of high CO_2 concentrations on glycolate biosynthesis (Tolbert, 1963; Jackson and Volk, 1970), may be rationalized in terms of a competition between CO_2 and oxygen for RuDP. Since the K_m for RuDP of the oxygenase appears to be similar to that of the carboxylase, the relative rates of carboxylation and oxygenation *in vivo* should depend on the prevailing concentrations of CO_2 and oxygen, provided that they are both in the subsaturating range, as is the case. Therefore, competition between carboxylation and oxygenation exists regardless of whether or not competition between CO_2 and oxygen also occurs in the strictly enzymatic sense. The present studies do not provide evidence either for or against the latter possibility.

(iii) The light dependency of glycolate synthesis and of photorespiration can be explained in terms of the regeneration of the substrate, RuDP, in the same manner that the continuous fixation of CO_2 demands the regeneration of RuDP. Thus, photorespiration is truly a dark reaction, the light being required merely to supply ATP and $NADPH_2$ involved in the regeneration of RuDP. The promotion of glycolate synthesis by high light intensity (Tolbert, 1963) may be related to the high pH optimum of the RuDP oxygenase reaction (Figure 7). It is possible that, *in vivo*, the chloroplast stroma becomes increasingly alkaline with increasing light intensity. This may be inferred from the rise in the pH of the medium which occurs when unbuffered chloroplasts are illuminated (Jagendorf and Neumann, 1965). This phenomenon is interpreted as being due to transport of protons into the space enclosed by the lamellar membrane. It is not known whether the pH of the stroma ever rises into the region of optimal oxygenase activity but any shift in this direction would favor oxygenation at the expense of carboxylation which proceeds optimally at about pH 7.8. These ideas are supported by the experiments of Orth *et al.* (1966) with intact algae and Dodd and Bidwell (1971) with chloroplast preparations which showed a large increase in the degree of labeling of glycolate from $[^{14}C]CO_2$ as the pH of the medium was raised. It is even conceivable that the balance between photosynthesis and photorespiration could be governed by subtle shifts in the pH of the chloroplast stroma.

(iv) Glycolate produced during $[^{14}C]CO_2$ fixation is uniformly labeled (Schou *et al.*, 1950), as are the top two carbon atoms of RuDP, which constitute the fragment producing the phosphoglycolate product of the oxidation.

(v) A reaction which produces phosphoglycolate as the primary photorespiratory substrate is consistent with the presence of a very active and specific phosphoglycolate phosphatase (Richardson and Tolbert, 1961) in chloroplasts (Randall *et al.*, 1971).

(vi) *In vivo*, the synthesis of glycolate involves the incorporation of an atom of oxygen from molecular oxygen into the carboxyl group (Andrews *et al.*, 1971). Oxygen incorporation into the carboxyl group of phosphoglycolate is catalyzed *in vitro* by RuDP oxygenase (Lorimer *et al.*, 1972, 1973).

Finally, the question must be raised concerning whether or not the level of RuDP oxygenase present in crude extracts is sufficient to account for the observed rates of photorespiration in the intact leaf. Although few would doubt that RuDP carboxylase has an obligatory role in photosynthesis, the observed level of this enzyme has often been found to be insufficient to account for the rate of CO_2 fixation *in vivo* (Kawashima and Wildman, 1970; Hatch and Slack, 1970). Therefore, perhaps the most meaningful approach to this question can be made by comparing the oxygenase rate with that of the carboxylase. In crude extracts the oxygenase/carboxylase activity ratio was 0.25 when each activity was assayed under optimum conditions. However, if the assays were to be carried out at a pH intermediate between the two optima, say 8.5, which may be close to that prevailing *in vivo*, and under the normal atmospheric conditions of 21% oxygen and 0.03% CO_2, oxygenation would far outstrip carboxylation. This is perhaps an unfair comparison since, anomalously, the K_m for CO_2 of the carboxylase is one to two orders of magnitude greater than the apparent K_m for CO_2 of photosynthesis *in vivo*. Nevertheless, the weight of evidence appears to be in favor of the activity of RuDP oxygenase being at least sufficient to account for photorespiration.

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References

Andrews, T. J., Lorimer, G. H., and Tolbert, N. E. (1971), *Biochemistry 10*, 4777.

Bandurski, R. S., and Axelrod, B. (1951), *J. Biol. Chem. 193*, 405.

Benson, A. A., and Calvin, M. (1950), *J. Exp. Bot. 1*, 63.

Bieleski, R. L., and Young, R. E. (1963), *Anal. Biochem. 6*, 54.

Bowes, G., and Ogren, W. L. (1972), *J. Biol. Chem. 247*, 2171.
Bowes, G., Ogren, W. L., and Hageman, R. H. (1971), *Biochem. Biophys. Res. Commun. 45*, 716.
Calkins, V. P. (1943), *Ind. Eng. Chem. Anal. Ed. 15*, 762.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Dodd, W. A., and Bidwell, R. G. S. (1971), *Plant Physiol. 47*, 779.
Forrester, M. L., Krotkov, G., and Nelson, C. D. (1966), *Plant Physiol. 41*, 422.
Gibbs, M. (1969), *Ann. N. Y. Acad. Sci. 168*, 356.
Goldthwaite, J. J., and Bogorad, L. (1971), *Anal. Biochem. 41*, 57.
Hatch, M. D., and Slack, C. R. (1970), *Annu. Rev. Plant Physiol. 21*, 141.
Jackson, W. A., and Volk, R. J. (1970), *Annu. Rev. Plant. Physiol. 21*, 385.
Jagendorf, A. T., and Neumann, J. (1965), *J. Biol. Chem. 240*, 3210.
Kawashima, N., and Wildman, S. G. (1970), *Annu. Rev. Plant Physiol. 21*, 325.
Kleinkopf, G. E., Huffaker, R. C., and Matheson, A. (1970), *Plant Physiol. 46*, 204.
Lorimer, G. H. (1972), Ph.D. Thesis, Michigan State University, East Lansing, Mich.
Lorimer, G. H., Andrews, T. J., and Tolbert, N. E. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 461.
Lorimer, G. H., Andrews, T. J., and Tolbert, N. E. (1973), *Biochemistry 12*, 18.
Ogren, W. L., and Bowes, G. (1971), *Nature (London), New Biol. 230*, 159.
Orth, G. M., Tolbert, N. E., and Jiminez, E. (1966), *Plant Physiol. 41*, 143.
Paulsen, J. M., and Lane, M. D. (1966), *Biochemistry 5*, 2350.
Pon, N. G., Rabin, B. R., and Calvin, M. (1963), *Biochem. Z. 338*, 7.
Racker, E. (1962), *Methods Enzymol. 5*, 266.
Randall, D. D., Tolbert, N. E., and Gremel, D. (1971), *Plant Physiol. 48*, 480.
Richardson, K. E., and Tolbert, N. E. (1961), *J. Biol. Chem. 236*, 1285.
Schou, L., Benson, A. A., Bassham, J. A., and Calvin, M. (1950), *Physiol. Plant 3*, 487.
Tolbert, N. E. (1971), *Annu. Rev. Plant Physiol. 22*, 45.
Tolbert, N. E. (1963), *Nat. Acad. Sci. Nat. Res. Counc. Publ. No. 1145*, 648.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.
Zelitch, I. (1971), Photosynthesis, Photorespiration and Plant Productivity, New York, N. Y., Academic Press.

Ribulose Diphosphate Oxygenase. II. Further Proof of Reaction Products and Mechanism of Action†

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ABSTRACT: The identities of the products of the ribulose diphosphate oxygenase reaction were confirmed by combined gas chromatography and mass spectrometry to be phosphoglycolate and 3-phosphoglycerate. Oxygen-18, supplied as molecular oxygen, was incorporated into one of the carboxyl oxygen atoms of phosphoglycolate. No label appeared in 3-phosphoglycerate, the other reaction product. When the reaction was carried out in a medium containing [^{18}O]-water, the carboxyl groups of both products were singly labeled. A reaction mechanism is proposed. Cyanide inhibited the ribulose diphosphate oxygenase reaction in a manner consistent with the formation of an inactive enzyme–cyanide–substrate complex. Neither catalase nor superoxide dismutase (erythrocuprein) had any effect on the oxygenation reaction. In contrast to previous reports, purified preparations of spinach leaf fraction-1 protein were found to contain less than 14% of the copper required for a stoichiometry of one atom of copper per molecule of enzyme.

In a previous study we concluded that enzymatic activity associated with fraction-1 protein from both spinach and soybean leaves catalyzed the oxidation of RuDP[1] by molecular oxygen to form phosphoglycolate and 3-phosphoglycerate, and this activity was called RuDP oxygenase (Andrews *et al.*, 1973). The products were identified by paper chromatography of the radioactive products obtained when [U-^{14}C]-RuDP was the substrate. Here we report further confirmation of these results using combined gas chromatography–mass spectrometry.

We have also shown that when [^{18}O]oxygen was supplied to spinach leaves in the light the glycolate-pathway intermediates, glycine and serine, became labeled in one of the oxygen atoms of the carboxyl groups, while several organic acids, including glycerate (and 3-phosphoglycerate), were not labeled (Andrews *et al.*, 1971). To determine if this pattern of labeling could be explained in terms of the RuDP oxygenase reaction, we investigated the incorporation of [^{18}O] by this enzyme. As a corollary, the labeling pattern

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[1] Abbreviations used are: RuDP, D-ribulose 1,5-diphosphate; Ammediol, 2-amino-2-methylpropane-1,3-diol; EDTA, ethylenediaminetetraacetic acid.