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

G. H. Lorimer,‡ T. J. Andrews,§ and N. E. Tolbert*

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.

obtained when the reaction was conducted in a medium containing [^{18}O]water was also determined.

In other studies related to the mechanism of the RuDP oxygenase reaction, we measured the copper content of the purified enzyme, investigated the inhibition of its activity by cyanide, and determined the effect of superoxide dismutase and catalase on the oxygenation reaction. A preliminary communication regarding this work has appeared (Lorimer *et al.*, 1972).

Experimental Section

Materials

RuDP oxygenase was purified from spinach leaves as previously described (Andrews *et al.*, 1973). ^{18}O (93.5 atom %) and [^{18}O]water (20.6 atom %) were obtained from Miles Laboratories Inc., as well as superoxide dismutase (erythrocuprein, Pentax) which had about 10% of the specific activity of the preparations of McCord and Fridovich (1969). Catalase came from Worthington Biochemical Corp. Silylating reagents were purchased from Regis Chemical Co.

Methods

Assay for RuDP Oxygenase. The rate of oxygen uptake was determined manometrically (Andrews *et al.*, 1973).

Identification of Reaction Products. The manometric assay was allowed to continue until oxygen consumption ceased, and a control reaction which did not contain enzyme was run simultaneously. The protein was then coagulated by heating the solution in a boiling water bath for 40 sec. After cooling, the solution was passed through a 0.5 × 4 cm column of Dowex 50 H^+ form (200–400 mesh) to remove buffer and Mg^{2+}. The column was washed with 1.5 ml of water, and the eluate plus washings were immediately neutralized with KOH and then evaporated to dryness under vacuum. The residue was used to prepare the Me_3Si derivatives.

In this procedure the neutralization of the reaction products and completely anhydrous conditions before silylation were of critical importance. If neutralization were omitted, loss of label, as evidenced by less than theoretical incorporation of ^{18}O into phosphoglycolate, occurred probably due to an acid-catalyzed exchange of the carboxyl oxygen atoms with the medium during the drying procedure. In early experiments anhydrous conditions were ensured by the usual procedure of adding 0.1 ml of absolute ethanol to the dry residue, followed by re-evaporation to dryness. This step proved to be unnecessary and, in cases where neutralization of the Dowex 50 eluate had not been performed, it resulted in the formation of the ethyl ester of phosphoglycolate which appeared as an additional peak in the gas chromatogram and was identified by mass spectrometry of the silylated derivative. Thus, drying of residues with ethanol was omitted, and the dried residues from the first vacuum evaporation were directly silylated. Bis(trimethylsilyl)trifluouroacetamide (40 μl) containing 1% (v/v) trimethylchlorosilane were added to the residue and heated at 110° for 10 min. Standards containing 2.5 μmol/ml of phosphoglycolate or 3-phosphoglycerate were similarly silylated. Analysis by combined gas chromatography and mass spectrometry was carried out subsequently.

Experiments with ^{18}O. A standard RuDP oxygenase assay identical with that described above was used, except that the reaction was run in a 4 × 1 cm vial closed with a rubber septum. About 2 mg of enzyme was used. The vial was evacuated by means of a syringe needle and 6 ml of ^{18}O was injected, thus giving a positive pressure. The reaction was initiated by injection of 2 μmol of RuDP, allowed to run for 1 hr at 25° with shaking, and then stopped by freezing in acetone–Dry Ice bath. The ^{18}O content of the gas phase was measured, as described next, while the reaction mixture remained frozen. The vial was then transferred from the freezing mixture to a boiling water bath for 40 sec and the above procedure continued.

The ^{18}O content of the gas phase following a reaction was determined while the liquid phase was frozen at acetone–Dry Ice temperatures. Analyses were performed with a Varian MAT mass spectrometer GD-150, equipped with the HTE-DE inlet system. The instrument was operated at a pressure of 1.5×10^{-6} Torr. The ^{18}O content was calculated using the formula

$$^{18}\text{O content (atom \%)} = \frac{M_{36} + (M_{34}/2)}{M_{32} + M_{34} + M_{36}}$$

where M_{32}, M_{34}, and M_{36} are the net peak heights at masses 32, 34, and 36, respectively, after subtracting the background determined immediately before introduction of the sample. The contribution of ^{17}O–^{17}O to M_{34} was ignored, since there was a negligible amount of either ^{16}O–^{17}O (mass 33) or ^{17}O–^{18}O (mass 35).

Experiments with [^{18}O]Water. The reaction was performed in a 4 × 1 cm vial closed with a rubber septum. The following components were first mixed in a total volume of 45 μl: 5 μmol of Ammediol–HCl, pH 9.3, 1 μmol of $MgCl_2$, 0.02 μmol of dithiothreitol, 0.1 μmol of EDTA, and 0.4 mg of enzyme. To this was added 60 μl of [^{18}O]water (20.6 atom %). The vial was then flushed with 100% oxygen and the reaction initiated by the injection of 0.37 μmol of RuDP. The total volume was 120 μl and the final [^{18}O]water content was 10.3 atom %. The reaction was allowed to run for 1 hr at 25°, with shaking, and terminated by freezing. Identification of products and measurement of ^{18}O incorporation were by the same procedure as for ^{18}O oxygen experiments.

To investigate the possibility that the enzyme might catalyze an exchange of the carboxyl oxygens of the products with water, the enzyme was incubated with phosphoglycolate and 3-phosphoglycerate in the presence of [^{18}O]water. The procedure was the same as above, with RuDP being replaced by 0.5 μmol each of phosphoglycolate and 3-phosphoglycerate. These components were included in the reaction mixture, and the reaction was initiated by injection of 0.4 mg of enzyme.

Gas Chromatography–Mass Spectrometry. Aliquots (0.5–1.0 μl) of the silylated samples were analyzed using an LKB-9000 combined gas chromatograph and mass spectrometer equipped with a 1.4 m × 3 mm (i.d.) silanized glass column–packed with 3% (w/v) SE-30 on silanized Supelcoport (100–200 mesh, Supelco Inc., Bellefonte, Pa.). The column temperature was 150° and the flash heater temperature was 170°. Control of the flash heater temperature was critical, since at elevated temperatures the Me_3Si derivatives of phosphoglycolate and 3-phosphoglycerate were degraded. The flow rate of the helium carrier gas was 30 cm^3/min, the temperature of the ion source 290°, and the ionizing voltage 70 eV. Mass spectra were obtained in the form of computer-drawn, normalized bar graphs (Sweeley *et al.*, 1970). Incorporation of ^{18}O was calculated from the relative ion intensities obtained by measuring peak heights from these bar graphs or from oscillographic recordings. The arithmetic procedure described by Thorpe and Sweeley (1967) was used to compute the extent of ^{18}O incorporation.

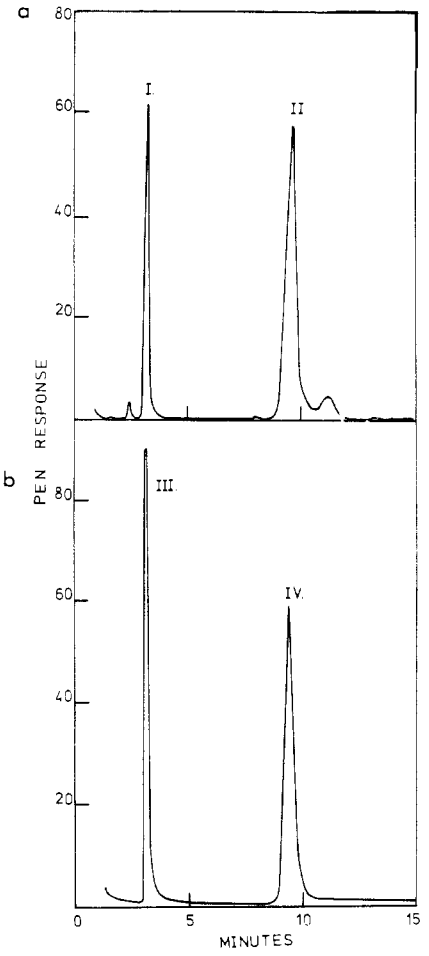


FIGURE 1: Gas–liquid chromatographic separation of (a) the silylated reaction products of the RuDP oxygenase reaction and (b) a standard mixture containing the Me_3Si derivatives of authentic phosphoglycolate and 3-phosphoglycerate. The tracing shown is the response of the total ion current detector. The column temperature was maintained at 150°. Mass spectra showed that peaks I and III were $(Me_3Si)_3$-P-glycolate and peaks II and IV were $(Me_3Si)_4$-3-P-glycerate.

Determination of Copper Content of Purified Enzyme. The $(NH_4)_2SO_4$ precipitate of the purified enzyme was dissolved in a buffer solution containing 0.25 M glycylglycine–NaOH at pH 8.6, 0.01 M dithiothreitol, and 0.01 M EDTA, and passed through a 0.7 × 36 cm column of Sephadex G-25 equilibrated with 5 mM Tris–HCl buffer solution at pH 8.3, and collected in a volume of 3 ml. The protein concentration was 12–13 mg/ml when calculated by the formula of Paulsen and Lane (1966). The copper concentration was determined with a Perkin-Elmer Model 303 atomic absorption spectrophotometer using $CuSO_4$ standards. A reading of zero was obtained for the buffer solution which emerged from the Sephadex column just before the enzyme. The readings for the enzyme solution were in the range 0.15–0.20 μg of Cu/ml.

Results

Identity of the Reaction Products. Gas chromatography readily separated a mixture of authentic $(Me_3Si)_3$-P-glycolate and $(Me_3Si)_4$-3-P-glycerate (Figure 1b). Analysis of the silylated reaction products from the RuDP oxygenase reaction, prepared as described in the Methods section, revealed two major components, one of which cochromatographed with

TABLE I: Summary of Results of Studies with Oxygen-18.

Substrate	Labeled Compd	Degree of Labeling (Atom %)	Degree of Labeling of Products (Mol %)	
			Phosphoglycolate	3-Phosphoglycerate
RuDP	[^{18}O]Oxygen	82[a]	75	0
RuDP	[^{18}O]Water	10.3[b]	10.3	9.8
Phosphoglycolate + 3-phosphoglycerate	[^{18}O]Water	10.3[b]	0	0.5

[a] Determined as described in the Methods section. [b] Calculated from the analysis supplied by the manufacturer.

$(Me_3Si)_3$-P-glycolate and the other with $(Me_3Si)_4$-3-P-glycerate (Figure 1a). These compounds were not present in a control from which the enzyme had been omitted. The mass spectrum of the compound with the shorter retention time (Figure 2) was identical with that of $(Me_3Si)_3$-P-glycolate, while the mass spectrum of the compound emerging later (Figure 3) was identical with that of $(Me_3Si)_4$-3-P-glycerate.

Mass Spectra of $(Me_3Si)_3$-P-glycolate and $(Me_3Si)_4$-3-P-glycerate. The mass spectrum of $(Me_3Si)_3$-P-glycolate (Figure 2) contained the molecular ion at *m/e* 372. However, the ion at *m/e* 357, resulting from the loss of a methyl group from the parent compound, was much more intense. Therefore, isotope analyses were performed by comparing the intensities of the ions at *m/e* 357 and 359. In addition, this mass spectrum contained an ion at *m/e* 328 (M − 44). This ion probably arose by migration of the Me_3Si from the carboxyl group and then the loss of the carboxyl group. The ability of Me_3Si groups to migrate in the mass spectrometer has been well documented (McCloskey *et al.*, 1968; DeJongh *et al.*, 1969). The most probable structure of this ion is $CH_2{=}OP(OMe_3Si)_3$. This ion did not contain the carboxyl oxygens, but it did represent the α-hydroxyl group of phosphoglycolate. Therefore, incorporation of isotope into this ion was also studied in experiments where ^{18}O was used (see below). Ions at *m/e* 299 and 315 were also prominent. These ions are common in the mass spectra of Me_3Si derivatives of phosphate esters. The ion at *m/e* 315 has been assigned the structure $(Me_3SiO)_2P(OH){=}O^+{-}SiMe_3$ and the ion at *m/e* 299, $(MeSiO)_2P(O){-}^+O{=}SiMe_3$ (Zinbo and Sherman, 1970). When the $(Me_3Si)_3$-P-glycolate was labeled with ^{18}O in the carboxyl group these ions were also found to contain ^{18}O. Such results suggest that the rearrangement leading to the formation of these ions involves the migration of the $-OSiMe_3$ group from the carboxyl position.

The mass spectrum of $(Me_3Si)_4$-3-P-glycerate (Figure 3) contained no molecular ion (*m/e* 474). However, the ion at *m/e* 459, which resulted from the loss of a methyl group from the parent compound, was prominent and thus suitable for measuring ^{18}O incorporation.

Experiments with [^{18}O]Oxygen. In Figures 4 and 5, the relevant regions of the mass spectra of the Me_3Si derivatives of authentic samples of phosphoglycolate and 3-phosphoglycerate are compared with similar spectra obtained for the

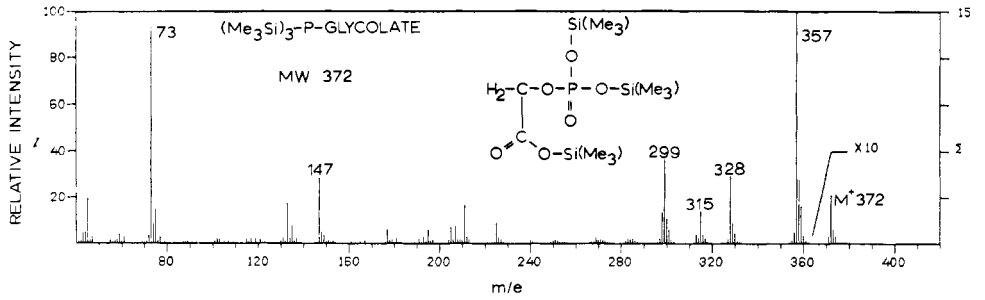


FIGURE 2: The mass spectrum of the compound emerging as peak I in Figure 1. The mass spectrum of authentic $(Me_3Si)_3$-P-glycolate was identical in every respect. The ionizing voltage was 70 eV.

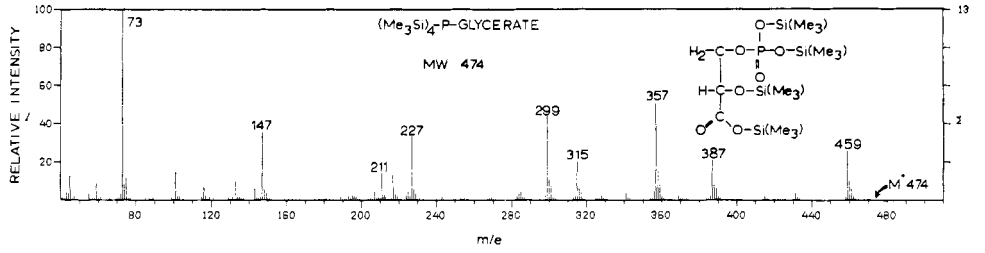


FIGURE 3: The mass spectrum of the compound emerging as peak II in Figure 1. The mass spectrum of authentic $(Me_3Si)_4$-3-P-glycerate was identical in every respect. The ionizing voltage was 70 eV.

products of the RuDP oxygenase reaction carried out in an atmosphere containing [^{18}O]oxygen. In the case of phosphoglycolate, a comparison of the relative intensities of the ions at *m/e* 357, 359, and 361 clearly showed that one, and only one, atom of ^{18}O has been incorporated (Figure 4). The increase in the relative intensity of the ion at *m/e* 361 can be entirely explained by incorporation of one atom of ^{18}O into the ion normally occurring at *m/e* 359 along with the naturally occurring isotopes of silicon, ^{29}Si and ^{30}Si. It cannot be due to the incorporation of two atoms of oxygen into the ion occuring at *m/e* 357. Additionally, a comparison of the ions at *m/e* 328 and 330, ions lacking the carboxyl oxygens, shows the absence of ^{18}O. In the case of the 3-phosphoglycerate, a comparison of the relative intensities of the ions at *m/e* 459 and 461 clearly shows that no isotope was incorporated into the molecule (Figure 5). These results establish that the enzymatic oxidation of RuDP by molecular oxygen proceeds with the incorporation of an atom of oxygen into the carboxyl group of only one of the products, namely phosphoglycolate. The calculated extent of incorporation of label into phosphoglycolate, in mole per cent, was close to the isotope content, in atom per cent, of the oxygen in the gas phase (Table I).

Experiments with [^{18}O]Water. When the RuDP oxygenase reaction was carried out in the presence of [^{18}O]water, one atom of ^{18}O was incorporated into the carboxyl group of each of the products. An interpretation of this result is mentioned in the Discussion. When a similar experiment was performed with phosphoglycolate and 3-phosphoglycerate

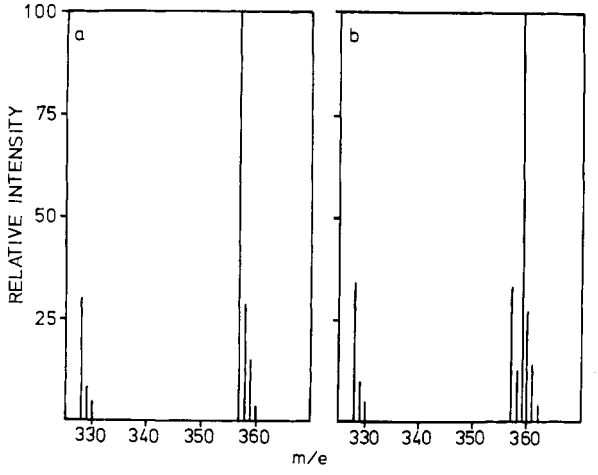


FIGURE 4: Comparison of part of the mass spectrum of authentic $(Me_3Si)_3$-P-glycolate (a) with that of the same compound isolated from an incubation of RuDP with the enzyme in 82 atom % [^{18}O]-oxygen (b).

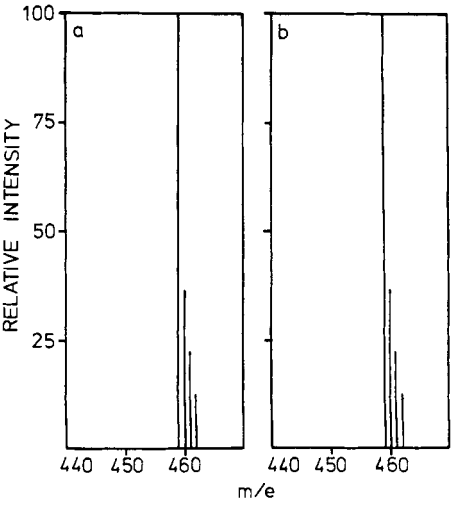


FIGURE 5: Comparison of part of the mass spectrum of authentic $(Me_3Si)_2$-3-P-glycerate (a) with that of the same compound isolated from an incubation of RuDP with the enzyme in 82 atom % [^{18}O]-oxygen (b).

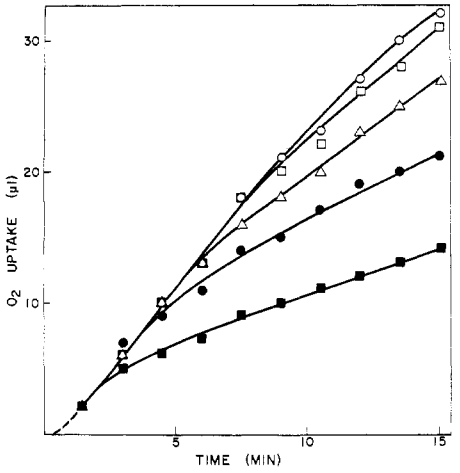


FIGURE 6: Inhibition of RuDP oxygenase by cyanide. The manometric assay was used with no additions (○) or 20 μM (□), 50 μM (△), 100 μM (●), and 200 μM (■) KCN. The KCN was added to each flask 15 min before the reaction was started by tipping in the RuDP. Because of variations in the lag period, the curves were superimposed by aligning the points at 90 sec rather than at zero time.

replacing RuDP no significant incorporation occurred (Table I). This latter result eliminated the possibility of exchange of the carboxyl oxygens of these phosphorylated products with the medium, either during the course of the reaction or during subsequent work-up procedures. It also follows that the RuDP oxygenase reaction must be essentially irreversible.

Copper Content of Purified Enzyme. Two preparations of fraction-1 protein purified by the two-step procedure (Andrews *et al.*, 1973) were assayed for tightly bound copper as described in the Methods section. Values of 0.14 and 0.11 g-atoms of copper per mole (560,000 g) of enzyme were obtained. The formula of Paulsen and Lane (1966) was used to calculate the protein concentration. The presence of $CuSO_4$ (0.1 mM) in the standard RuDP oxygenase assay did not cause a stimulation of the rate of oxygen uptake.

Inhibition of Oxygenase Activity by Cyanide. The result of adding various concentrations of KCN to the standard manometric assay for RuDP oxygenase is shown in Figure 6. When cyanide was present the progress of the reaction became biphasic. An initial linear rate was similar to that in the absence of cyanide, but the rate subsequently declined to a lower value which also became linear. As the cyanide concentration was raised the magnitude of the second rate decreased and the discontinuity occurred earlier.

Effect of Superoxide Dismutase and Catalase on Oxygenase Activity. When superoxide dismutase (erythrocuprein, 1 mg) or catalase (0.1 mg) was added to the standard assay solution, no decrease in activity was observed relative to controls with these inactive proteins which had been boiled for 2 min at 100°.

Discussion

From this study a mechanism for the RuDP oxygenase catalyzed oxidation of RuDP by molecular oxygen is proposed in Figure 7. One atom of the [^{18}O]oxygen molecule was incorporated into the carboxyl group of the phosphoglycolate formed but not into the other product, 3-phosphoglycerate. The other oxygen atom is apparently lost to the medium. The present results are entirely in agreement with

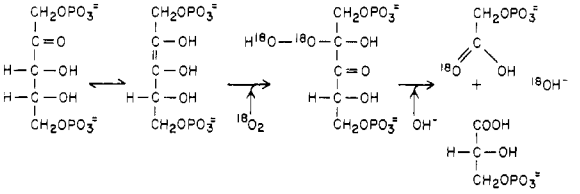


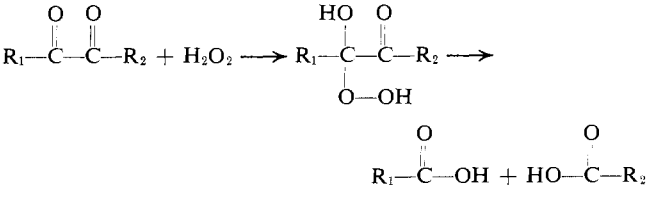
FIGURE 7: Proposed reaction sequence of RuDP oxygenase.

the results of our *in vivo* experiments in which glycine and serine, but not glycerate, became labeled when [^{18}O]oxygen was supplied to photorespiring spinach leaves (Andrews *et al.*, 1971). The discussion of that previous paper expressed a reservation about the RuDP oxygenase reaction *in vivo* since no ^{18}O was found in the phosphoglycerate. However, since ^{18}O incorporation *in vitro* was the same as *in vivo*, this reaction now seems well established.

Experiments with [^{18}O]water were complicated by the likelihood that the keto oxygen of the RuDP exchanged with water by formation of the hydrate. Even though RuDP exists in solution predominantly in the free keto form (Gray and Barker, 1970), this exchange probably occurred very rapidly. This possibility is supported by studies on the rate of exchange of label from [2-^{18}O]dihydroxyacetone phosphate (Model *et al.*, 1968). At 25° and pH 7, the half-time for this exchange was less than 1 min, due in part to catalysis by the phosphate group α to the keto oxygen. It is therefore suggested that the observed labeling of one of the carboxyl oxygens of phosphoglycolate from [^{18}O]water during the course of the RuDP oxygenase reaction was due to prior labeling of the substrate by this exchange reaction. However, the labeling of one of the carboxyl oxygens of 3-phosphoglycerate by [^{18}O]water must have occurred during the actual oxidation of RuDP, and thus water must be involved in this reaction. This conclusion is supported by the lack of incorporation of label from [^{18}O]oxygen into 3-phosphoglycerate.

The reaction sequence of Figure 7 is closely analogous to the sequence proposed for the RuDP carboxylase reaction (Calvin, 1956; Rabin and Trown, 1964; Kawashima and Wildman, 1970) with the intermediate peroxide replacing the C-6 intermediate and a cleavage occurring between C_2 and C_3. The catalytic involvement of a hydroxyl ion may be partly responsible for the rather alkaline pH optimum observed for the RuDP oxygenase reaction (Andrews *et al.*, 1973).

The most analogous nonenzymatic reaction is the alkaline oxidation of hexoses and pentoses by molecular oxygen studied first by Nef (1914) and more recently by Dubourg and Naffa (1959), DeWilt and Kuster (1971), and Gleason and Barker (1971a,b). Unfortunately no mechanistic studies with ^{18}O have been reported. However, the oxidation of α-diketones by hydrogen peroxide is thought to proceed *via* the following intermediate peroxide, the structure of which is exactly similar to the proposed ribulose diphosphate peroxide.



Mechanistic studies with [^{18}O]water by Bunton (1961) in-

dicate that the intermediate peroxide decomposes by the addition and elimination of a hydroxyl ion, rather than by an intramolecular concerted mechanism. Thus, the mechanism of an analogous nonenzymatic reaction supports the proposed mechanism of the RuDP oxygenase reaction.

The results do not permit us to be more specific regarding the mode of formation of the intermediate peroxide. The simplest mechanism involves the attack of oxygen on the enediol form of RuDP. More complex mechanisms involving oxygen free radicals may be conceived. Recently there has been interest in the possible involvement of the superoxide free radical in oxygenase reactions (Hirata and Hayaishi, 1971; Strobel and Coon, 1971). Our inability to demonstrate any inhibiting effect of superoxide dismutase on RuDP oxygenase does not eliminate the possibility of involvement of the superoxide free radical, but it does suggest that if such a species is involved it must remain enzyme bound.

Since some other oxygenases have required copper as a prosthetic group, the report that spinach RuDP carboxylase contained 1 g-atom of tightly bound copper per mole (Wishnick *et al.*, 1969) was of considerable interest. However, our purified RuDP carboxylase or oxygenase preparations contained less than 14% of the copper required for each enzyme molecule to contain one atom of the metal. Furthermore, the addition of $CuSO_4$ did not stimulate the oxygenase rate. Apart from an absolute dependence on Mg^{2+} ions (Andrews *et al.*, 1973) the oxygenase reaction does not appear to require metals. However, the possibility remains that the reagents could have contained traces of copper sufficient to activate the enzyme.

The pattern of inhibition of oxygenase by cyanide (Figure 6) may be explicable in terms of the formation of a ternary inactive complex between enzyme, cyanide, and RuDP. Such a complex would have to be formed in a reaction which reached an equilibrium on a time scale of the order of minutes. Hence, both the rate of inactivation and the fraction of enzyme remaining active (*i.e.*, not complexed) at equilibrium would be dependent on the cyanide concentration, as was observed. The inhibition of RuDP carboxylase by cyanide is also thought to be due to the formation of an enzyme–cyanide–RuDP complex (Wishnick and Lane, 1969) and this similarity provides further circumstantial evidence that both oxygenase and carboxylase activities are due to the same active site.

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