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Incorporation of Molecular Oxygen into Glycine and Serine during Photorespiration in Spinach Leaves*

T. J. Andrews,† G. H. Lorimer, and N. E. Tolbert

ABSTRACT: When spinach leaves were exposed to an atmosphere of [^{18}O]oxygen the label was rapidly incorporated into the carboxyl groups of glycine and serine. This incorporation occurred only in the light. No label was incorporated into the hydroxyl group of serine. Under the same conditions, glycerate and phosphoglycerate did not become labeled. The data are consistent with a two-electron oxidation of a sugar phosphate intermediate of the photosynthetic carbon cycle to form phosphoglycolate or glycolate and an aldose phosphate. A considerable pool of unlabeled erythronic (or threonic) acid was detected by combined gas chromatography–mass spectrometry in phosphatase-treated extracts of spinach leaves.

The inhibition of net photosynthetic carbon dioxide fixation by oxygen, often referred to as the Warburg oxygen effect (Warburg, 1920), has been observed in a wide variety of algae and higher plants (Turner and Brittain, 1962) and isolated chloroplasts (Ellyard and Gibbs, 1969). It has become evident that this phenomenon is due to photorespiration; that is, the light-dependent uptake of oxygen and release of carbon dioxide which is thought to be associated with the glycolate pathway of metabolism (Jackson and Volk, 1970). Photorespiration is especially evident under conditions of high light intensity, limiting carbon dioxide, and high oxygen concentrations. In these circumstances a large part of the total carbon fixed during photosynthesis flows through the glycolate pathway (Tolbert, 1963). Recently, many of the enzymes of the glycolate pathway have been located in the peroxisomal (microbody) fraction, as distinct from both chloroplasts and mitochondria (Tolbert, 1971). The origin of phosphoglycolate and glycolate is one of the most interesting problems in photosynthetic carbon metabolism and one which is of fundamental importance in photorespiration. Glycolate may arise from phosphoglycolate by the action of a specific phosphatase, which is located in the chloroplast (Richardson and Tolbert, 1961), but whether it does so exclusively is not clear. Two radically different mechanisms have been proposed to account for the formation of uniformly labeled glycolate during photosynthetic [^{14}C]CO_2 fixation. One, proposed by Tanner *et al.* (1960), Stiller (1962), and Zelitch (1965), suggests that glycolate arises by means of a hitherto undiscovered reductive condensation of two molecules of carbon dioxide. However, the ^{14}C-labeling experiments of Hess and Tolbert (1966) and Coombs and Whittingham (1966) tend to discount this possibility. The other mechanism, proposed in various forms, suggests that glycolate and/or phosphoglycolate is formed as the result of the oxidation of one or more intermediates of the photosynthetic carbon cycle (Wilson and Calvin, 1955; Bassham and Kirk, 1962; Tolbert, 1963; Coombs and Whittingham, 1966; Gibbs, 1969). If the latter hypothesis is correct the nature of the oxidant is of considerable interest. To investigate the possibility that the oxidant is molecular oxygen, or is derived from it, we performed experiments in which detached spinach leaves were allowed to photorespire in an atmosphere of [^{18}O]oxygen. This report concerns the incorporation of ^{18}O into the products of the glycolate pathway, glycine and serine.

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† To whom to address correspondence at the Department of Biochemistry, Michigan State University.

Materials

Spinach (*Spinacia oleracea* L., var. Long Standing Bloomsdale) was grown in a growth chamber. During the day period of 11 hr, the light intensity was about 3000 ft-candles and the temperature was 21°. During the subsequent 13-hr darkness the temperature was 15°. Younger leaves, weighing about 2 g and about 10 cm long, were harvested from mature plants immediately before use.

[^{18}O]Oxygen (93.5 atoms %) and [^{18}O]H_2O (93 atoms %) were obtained from Miles Laboratories, Inc., Elkhart, Ind. Silylating reagents were obtained from Regis Chemical Co., Chicago, Ill. Solvents were redistilled where necessary.

Methods

Photorespiration in [^{18}O]Oxygen. A spinach leaf was placed in a stoppered 2.2 × 10 cm test tube and the volume of the gas space determined by measuring the volume of water required to fill the tube completely. This volume was 31.0 ± 0.5 ml. The water was removed, except for the last 1 ml which covered the bottom of the petiole, and the leaf was allowed to photosynthesize in a stream of humidified air at 25° while being illuminated from two sides with white light of about 4000 ft-candles. The air entered and left the tube by means of narrow syringe needles passing through the stopper. After about 40 min the system was flushed with 50 ml of 100% oxygen, followed immediately by an injection of 6 ml of isotopic oxygen. Single leaves were killed at various times after introduction of the isotope by filling the tubes containing them with boiling 90% (v/v) ethanol.

Extraction of Glycine and Serine. After the leaf had remained in boiling 90% (v/v) ethanol for at least 3 min, the solution was decanted and the leaf further extracted for 3 min each with boiling 50% (v/v) ethanol and boiling absolute methanol. The extracts were combined and evaporated to dryness. The residue was dissolved in a minimum volume of chloroform–methanol–0.2 M formic acid (25:60:15, v/v). The chloroform phase was removed and washed twice with 0.2 M formic acid. The aqueous phases were combined and washed twice with chloroform. Sufficient Dowex 50 H^+ was added to the aqueous phase to render the supernatant solution colorless. This solution was removed and the resin was washed with 0.2 M formic acid. Glycine and serine were eluted from the resin with 1 M NH_4OH and the eluate was evaporated to dryness. The residue was dissolved in a minimum volume of water and applied as a streak to a 24 × 50 cm sheet of Whatman No. 3MM chromatography paper. Glycine and serine markers were applied on both sides of the main streak. Chromatography was performed for 17 hr at 25° with 1-butanol–propionic acid–water (10:5:7, v/v) as solvent. The chromatogram was dried and thin strips were cut from both sides and from the middle. These strips were sprayed with ninhydrin solution (0.2% w/v in water-saturated 1-butanol) and heated at 90° until color development was adequate. The area of the chromatogram containing both glycine and serine was located by comparison with the markers. Glycine and serine have very similar R_F values in this system. This area of the chromatogram was cut out and extracted with water. The resultant solution was evaporated to dryness in preparation for silylation.

Extraction of Organic Acids. The supernatant solution plus washings from the Dowex 50 step were combined and evaporated to dryness. The residue was dissolved in 8 ml of 0.05 M NH_4OH, the pH of the resultant solution being about 9.4. This solution was incubated with approximately 3 units of alkaline phosphatase (Sigma Type VII) at 25° for at least 30 min and then applied to a 0.7 × 6 cm column of Dowex 1 acetate. The column was washed with water and the organic acids were eluted with 4 M acetic acid. This fraction was evaporated to dryness and the residue used to prepare the Me_3Si derivatives.

Preparation of Me_3Si Derivatives. The dried residues obtained as described above were suspended in 50 μl of acetonitrile and 50 μl of bis(trimethylsilyl)trifluoroacetamide containing 1% (v/v) trimethylchlorosilane under dry conditions and heated at 150° for 15 min to facilitate the silylation reaction. Standards containing glycine, serine, glycerate, malate, and erythronate (1 μg/μl) were similarly prepared. Samples containing glycine were allowed to stand at room temperature for at least 48 hr before analysis to ensure the formation of the $(Me_3Si)_3$ derivative (Bergström *et al.*, 1970).

Isotope Analysis. Aliquots (1–4 μl) of the above silylated samples were analyzed using an LKB-9000 combined gas chromatograph–mass spectrograph equipped with a 1.4 m × 3 mm i.d. silanized glass column packed with 3% (w/v) SE-30 on silanized Supelcoport (100–120 mesh, Supelco Inc., Bellefonte, Pa.). The column temperature was 100° for glycine and serine and programmed at a rate of 5°/min from 80 to 200° for the organic acids. The flow rate of the helium carrier gas was 30 cc/min. The temperature of the ion source was 290° and the ionizing voltage 70 eV. Isotope incorporation was measured by determining the ratio $I_{p+2}/(I_p + I_{p+2})$, where I_p is the intensity of the ion at $m/e = p$ and I_{p+2} is the intensity of the ion at $m/e = p + 2$. For most determinations the intensities of the various ions were determined by direct measurement of peak heights either from oscillographic recordings or from normalized bargraphs (Sweeley *et al.*, 1970). When more accurate data were required for detection of any possible incorporation into the hydroxyl group of serine, the accelerating voltage alternator unit (multiple ion analyzer) was used. This technique involves the continuous recording, at constant magnetic field, of the intensities of two ions formed by electron impact on the gas chromatographic effluent. The ions were carefully focused by manual setting of the magnetic field strength (for the ion at the lower m/e value) and by decreasing the accelerating voltage (for the ion at the higher m/e value) (Sweeley *et al.*, 1966).

Investigation of Extraction Procedures. To test whether any isotope incorporated into glycine or serine during the *in vivo* experiment might be wholly or partly lost during the subsequent extraction procedure, a sample containing both glycine and serine labeled with ^{18}O in the carboxyl groups was prepared and subjected to the same procedure used to isolate these amino acids from the spinach leaf. A mixture of glycylglycine (1.0 mg) and serylglycine (1.1 mg) was dissolved in 190 μl of 6 N HCl and 10 μl of [^{18}O]H_2O (93 atom %). This solution was heated at 110° for 20 hr in a sealed vial. An aliquot was then removed for analysis and the remainder taken through the entire extraction procedure for glycine and serine. Both samples were analyzed for ^{18}O content as described above. Results (Table I) showed that no label was lost during the extraction procedure. It is interesting that the hydrolysis procedure resulted in an observed incorporation very near the theoretical value expected if incorporation occurred only by hydrolysis of the peptide bonds of the dipeptides. Even under these rigorous conditions, exchange between the medium and the carboxyl oxygens of the dipeptides and amino acids apparently did not occur.

A similar control experiment was performed with glycerate.

TABLE I: Retention of ^{18}O during Extraction Procedures.

Sample	^{18}O Content (mole %)[a]		
	Glycine	Serine	Glycerate
Before extraction	1.6	4.3	5.0
After extraction	2.0	4.2	5.2

[a] Intensity ratios were determined by measuring the appropriate peaks in the mass spectra. The values shown were obtained by subtracting the intensity ratios determined for authentic samples of the compounds from those obtained for the same compound containing ^{18}O. Each result is an average of three or more determinations.

Calcium DL-glycerate (3.5 mg) was dissolved in 90 μl of 2 N HCl and 10 μl of [^{18}O]H_2O (93 atom %). This solution was heated at 90° for 17 hr. An aliquot was removed for analysis and the remainder was taken through the extraction procedure for organic acids, omitting the Dowex 50 step and the alkaline phosphatase treatment. Both samples were then analyzed for ^{18}O content as described above. Again, no label was lost during the extraction procedure (Table I). The incorporation achieved was 30% of that predicted for complete exchange of both oxygen atoms of the carboxyl group with the medium.

Results

Incorporation of ^{18}O into Glycine and Serine. Since the amounts of phosphoglycolate and glycolate in leaves are too small for isolation from small leaf samples, it was necessary to investigate photorespiratory incorporation of ^{18}O, supplied as molecular oxygen, by analyzing the metabolic products of glycolate, glycine and serine. Both of these compounds accumulate in relatively large pools. The Me_3Si derivatives were formed, rather than the more commonly used *N*-trifluoroacetyl-*n*-butyl esters, since the latter esterification would result in the loss of at least half of any ^{18}O incorporated into the carboxyl groups.

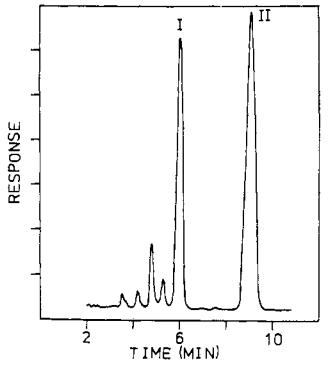


FIGURE 1: Gas chromatography of a silylated extract from spinach leaves containing glycine and serine. The column temperature was maintained at 100°. The tracing shown is the response of the total ion current detector. Mass spectra showed that peak I was $(Me_3Si)_3$-glycine and peak II was $(Me_3Si)_3$-serine. This tracing was obtained for a leaf exposed to 100% oxygen for 5 min in the light. Smaller peaks for glycine and serine were observed for shorter exposure times or when exposure occurred in the dark.

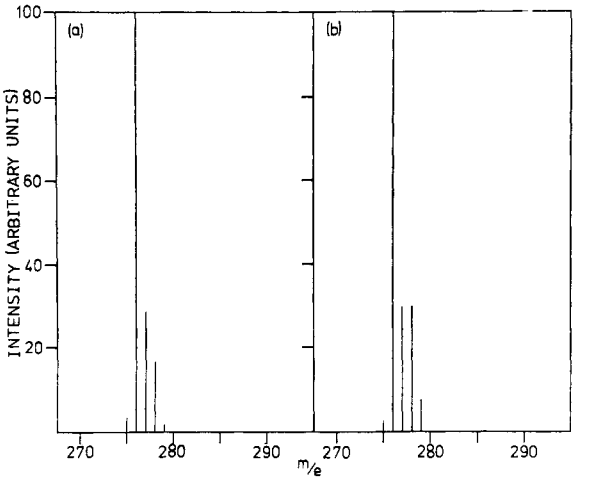


FIGURE 2: Comparison of part of the mass spectrum of authentic $(Me_3Si)_3$-glycine (a) with that of the same compound extracted from a leaf which had been exposed to 100% oxygen containing 18.1 atom % ^{18}O for 2 min in the light (b).

$(Me_3Si)_3$-glycine and $(Me_3Si)_3$-serine were readily separated by gas chromatography from each other and from several other compounds present in the sample prepared as described in the Methods section (Figure 1). The mass spectra of authentic samples of $(Me_3Si)_3$-glycine and $(Me_3Si)_3$-serine were similar to those previously reported (Vandenheuvel and Cohen, 1970; Bergström *et al.*, 1970). The only ion in the mass spectrum of $(Me_3Si)_3$-glycine suitable for measuring ^{18}O incorporation was that at *m/e* 276, which results from the loss of a methyl group from the parent compound and thus contains both carboxyl oxygen atoms. The spectrum of $(Me_3Si)_3$-serine contains prominent ions at *m/e* 204 and 218. The ion at *m/e* 204, which may be regarded as $Me_3Si—O—CH_2—CH=N^+H—Me_3Si$, contains the hydroxyl oxygen atom only, while that at *m/e* 218, $Me_3Si—N^+H=CH—COO—Me_3Si$, contains only the carboxyl oxygen atoms. In Figures 2 and 3 the mass spectra, in the region of these ions, for the Me_3Si

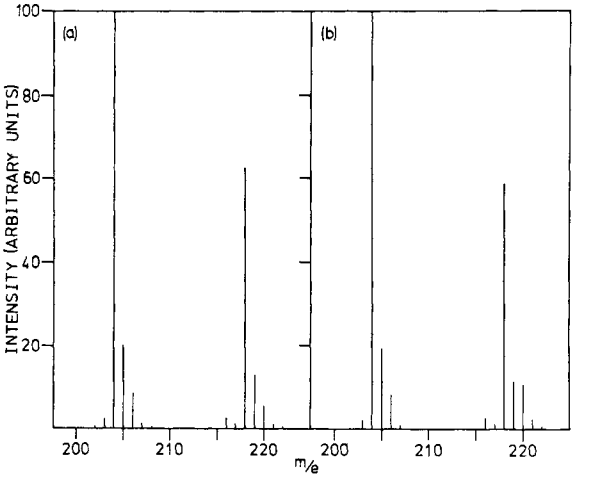


FIGURE 3: Comparison of part of the mass spectrum of authenitc $(Me_3Si)_3$-serine (a) with that of the same compound extracted from a leaf which had been exposed to 100% oxygen containing 18.1 atom % ^{18}O for 2 min in the light (b).

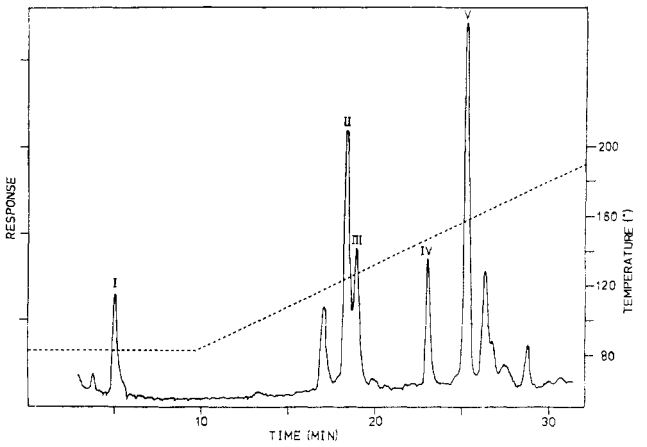


FIGURE 4: Gas chromatography of a silylated extract from spinach leaves containing organic acids. (——) Response of total ion current detector, (– – –) column temperature. Subsequent mass spectrometry identified the various peaks as follows: I, $(Me_3Si)_2$-oxalate; II, $(Me_3Si)_3$-glycerate; III, $(Me_3Si)_2$-succinate; IV, $(Me_3Si)_3$-malate; and V, $(Me_3Si)_4$-erythronate (or $(Me_3Si)_4$-threonate).

derivatives of authentic samples of glycine and serine are compared with similar spectra obtained for glycine and serine extracted from a spinach leaf which had been exposed to 100% oxygen containing 18.1 atom % ^{18}O for 2 min in the light. In the case of the glycine spectra, a comparison of the relative intensities of the ions at *m/e* 276 and 278 clearly shows that ^{18}O has been incorporated into the carboxyl group. The lack of any measurable change in the ion at *m/e* 280 indicates that only one of the oxygen atoms of the carboxyl group has become labeled. For the serine spectra a similar comparison of the intensities of the ions at *m/e* values 218, 220, and 222 shows that here also one, and only one, of the carboxyl oxygens has become labeled. Additionally, in the case of serine, a comparison of the intensities at *m/e* 204 and 206 reveals that no incorporation into the hydroxyl group has occurred. More accurate data concerning the incorporation of ^{18}O into serine were obtained using the accelerating voltage alternator unit (multiple ion analyzer) of the mass spectrometer (Table II). The greater sensitivity of this technique made it possible to be more certain that no incorporation into the hydroxyl group had occurred.

Experiments were performed in which the incorporation of ^{18}O into glycine and serine was followed as a function of time (Table III). The leaf's pool of glycine became labeled more rapidly than did the pool of serine and the level of incorporation at saturation, in moles per cent, was greater for glycine than for serine. In both cases this value was considerably less than the ^{18}O enrichment, in atoms per cent, of the oxygen supplied. Once again, no incorporation into the hydroxyl group of serine was observed. When the leaf was placed in darkness immediately before the labeled oxygen was supplied, insignificant incorporation into either amino acid occurred. It was apparent that a net synthesis of both glycine and serine occurred when the leaves were exposed to 100% oxygen in the light. Qualitative comparison of the total ion current tracings (equivalent to gas chromatograms) for the samples in this experiment revealed that the leaf's content of these amino acids at least doubled during the course of the 5-min experiment. No increase was observed for the dark control.

TABLE II: Incorporation of ^{18}O into Serine.[a]

Sample	Ratio of Intensities (%)[d] $\frac{I_{220}}{I_{218}+I_{220}}$ (Carboxyl)	$\frac{I_{206}}{I_{204}+I_{206}}$ (Hydroxyl)
Control[b]	8.10 ± 0.03	8.73 ± 0.20
Test[c]	15.47 ± 0.26	8.43 ± 0.11

[a] Data obtained using accelerating voltage alternator. [b] Sample of authentic serine. [c] Serine extracted from a spinach leaf exposed to 100% oxygen containing 18.1 atom % ^{18}O for 2 min in the light. [d] Average of at least three determinations. The error quoted is the standard deviation.

TABLE III: Incorporation of ^{18}O into Glycine and Serine as a Function of Time.[a]

Time (min)[b]	Incorporation of ^{18}O (mole %)[c] Glycine	Serine Carboxyl	Serine Hydroxyl
0.5 (light)	6.9	2.3	−0.2
2.0 (light)	9.5	6.9	0.0
5.0 (light)	9.3	6.8	0.0
5.0 (dark)	0.1	0.2	0.2

[a] The gaseous phase was 100% oxygen containing 18.1 atom % ^{18}O. [b] A separate leaf was used for each time. [c] Calculated as described for Table I.

The kinetics of incorporation of label into glycine and serine are dependent on the sizes of the pools which are associated with photorespiration and the activities of the glycolate-pathway enzymes. These factors may vary with the age of the plant. In an experiment where a leaf from a young plant (about 21 days from germination) was exposed to [^{18}O]oxygen for 20 sec, the incorporation into the carboxyl group of serine was 5.1 mole %, a value higher than that obtained at 30 sec in the previous experiment where leaves from a fully mature plant were used. The incorporation into glycine, 5.8 mole %, was close to that observed previously at 30 sec.

Lack of Incorporation of ^{18}O into Glycerate and Other Organic Acids. Organic acids, including phosphorylated organic acids, were extracted from a leaf that had been exposed to [^{18}O]oxygen for 5 min in the light. $(Me_3Si)_3$-glycerate was readily separated by gas chromatography from other compounds occurring in the extract (Figure 4). Several of these compounds were identified by their mass spectra. In addition to $(Me_3Si)_3$-glycerate, the Me_3Si derivatives of oxalate, malate, succinate, and erythronate (or threonate) were identified. Isotope analysis was conducted on glycerate, malate, and erythronate. In no case was any incorporation of ^{18}O detected.

Discussion

To date, the only well-documented reaction which consumes oxygen during photorespiration is the oxidation cat-

alyzed by glycolate oxidase. This flavin enzyme is known to produce hydrogen peroxide (Kenten and Mann, 1952; Zelitch and Ochoa, 1953). The reaction does not involve the carboxyl group of glycolate and should not lead to the incorporation of molecular oxygen. The enzymatic reactions leading to the formation of glycine and serine from glyoxylate are not known to involve oxygen. Therefore, the observed *in vivo* incorporation of ^{18}O supplied as molecular oxygen into the carboxyl groups of glycine and serine indicates that oxygen is involved in the photorespiratory process at a step earlier than glycolate oxidase. We conclude that oxygen, or a species derived from oxygen, is involved in the synthesis of glycolate and/or phosphoglycolate. Furthermore, this oxygen is incorporated into the carboxyl group in the process and subsequently appears in glycine and serine. The absence of incorporation in the dark confirms that the process is a photorespiratory event. The possibility that this incorporation could occur *via* $[^{18}O]H_2O$ formed in the leaf from [^{18}O]oxygen is eliminated by the observation that the hydroxyl group of serine did not become labeled. Since serine is formed from glycine in plant tissues by the combined action of glycine decarboxylase and serine hydroxymethyltransferase (Kisaki *et al.*, 1971), the hydroxyl oxygen atom must arise from water. Thus any labeling of water during the course of the experiment would be reflected in the hydroxyl oxygen of serine.

The incorporation of isotopic oxygen occurred very rapidly. After as short a time as 30 sec, incorporation into glycine reached a value equal to 73% of the saturation level. However, the observed saturation levels of incorporation of ^{18}O into glycine and serine, 9.4 and 6.8 mole %, respectively, were below the theoretical maximum of 18.1 mole %. Control experiments indicated that ^{18}O was not lost by exchange during the extraction procedures. Dilution of the isotopic oxygen with unlabeled oxygen formed in the photochemical act is considered to be negligible owing to the short duration of the experiments. Besides such a dilution would have reduced the saturation level of incorporation into glycine and serine by equal amounts, which was not the case. An explanation which cannot be dismissed entirely is that there is more than one mechanism for the synthesis of glycolate, only one of which involves the incorporation of molecular oxygen. However, again it would be expected that the saturation level of incorporation into both amino acids would be reduced equally. A more likely explanation for the failure to reach the maximum theoretical incorporation lies in the possibility that more than one pool of both glycine and serine might exist. Only that pool which is closely associated with photorespiration would become rapidly labeled with ^{18}O. The level of incorporation at saturation would depend on the relative sizes of these pools. This explanation probably accounts for the higher rate of incorporation into serine observed when a leaf from an immature plant was used. Support for this interpretation comes from ^{14}C-labeling experiments with *Chlorella* (Bassham *et al.*, 1964) and with wheat leaves (Hellebust and Bidwell, 1963). There exist in both organisms pools of glycine and serine which are closely associated with photosynthetic carbon metabolism but which are not in rapid equilibrium with other pools of glycine and serine.

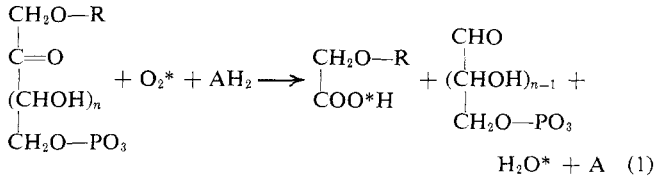
The aim of this study was to gain insight into the mechanism whereby an intermediate of the photosynthetic carbon reduction cycle is oxidized to glycolate and/or phosphoglycolate. As shown here, this oxidation proceeds with incorporation of oxygen into the carboxyl group. Ogren and Bowes (1971) recently showed that oxygen is an inhibitor of ribulose diphosphate carboxylase and that it is competitive in this respect with CO_2. They suggested that oxygen may substitute for CO_2 in the reaction of this enzyme so that ribulose diphosphate is oxidized to phosphoglycerate and phosphoglycolate. If such a four-electron oxidation occurred, one would expect to find incorporation of ^{18}O into phosphoglycerate as well as phosphoglycolate. However, our analysis of the combined glycerate and phosphoglycerate pool did not detect any incorporation of ^{18}O. Again a control experiment ruled out the possibility of loss of incorporated isotope during the extraction procedure. Therefore we suggest that, *in vivo*, neither ribulose 1,5-diphosphate nor any phosphorylated pentose is subject to a four-electron oxidation by molecular oxygen to yield phosphoglycerate and phosphoglycolate or glycolate.

Gas chromatography of the acidic fraction extracted from spinach leaves showed that, in addition to such expected organic acids as glycerate, malate, succinate, and oxalate, an unknown was present in large quantities (peak V in Figure 4). This compound was indentified by mass spectrometry as $(Me_3Si)_4$-erythronic (or -threonic) acid. Since the extraction procedure involved treatment with alkaline phosphatase it is possible that this acid exists *in vivo* as 4-phosphoerythronate. It is also possible that this acid is an artifact formed during the extraction procedure. It seems unlikely that a compound present in such large quantities could have escaped detection before now if it became labeled during $[^{14}C]CO_2$ fixation studies. Nothing is known of the formation or metabolism of erythronic acid or its phosphate ester. In any case, isotopic analysis failed to detect any incorporation of ^{18}O into erythronic acid. Therefore we suggest that, *in vivo*, neither fructose 1,6-diphosphate nor any phosphorylated hexose is subject to a four-electron oxidation by molecular oxygen to yield phosphoerythronate and phosphoglycolate or glycolate.

It has been suggested that glycolate is formed as a result of the oxidation of α,β-dihydroxyethylthiamine pyrophosphate formed as an intermediate in the transketolase reaction (Wilson and Calvin, 1955) and it has been further suggested that the oxidant in this process, *in vivo*, is hydrogen peroxide, generated from oxygen in a Mehler-type reaction with reduced ferredoxin (Coombs and Whittingham, 1966; Plaut and Gibbs, 1970). Indeed, Gibbs (1969) has stated that this reaction would result in the incorporation of an oxygen atom from hydrogen peroxide into the glycolate formed. Since there is no precedent for the formation of a phosphorylated thiamine pyrophosphate addition compound, this mechanism would not account for the formation of phosphoglycolate. The above proposal is based on observations that the α,β-dihydroxyethylthiamine pyrophosphate is enzymatically oxidized to glycolate in the presence of such electron acceptors as ferricyanide (Bradbeer and Racker, 1961; Holzer and Schröter, 1962) or 2,6-dichlorophenolindophenol (daFonseča-Wollheim *et al.*, 1962). The equation written for the hydrogen peroxide dependent reaction by both Coombs and Whittingham (1966) and by Plaut and Gibbs (1970), in which oxygen appears as a product, does not balance chemically. If we assume that hydrogen peroxide can indeed oxidize dihydroxyethylthiamine pyrophosphate, water rather than oxygen will be formed. In the oxidation of dihydroxyethylthiamine pyrophosphate by ferricyanide, it is thought that the ferricyanide first oxidizes the dihydroxyethylthiamine pyrophosphate to the α-keto derivative which then undergoes spontaneous hydrolysis to yield glycolate and thiamine pyrophosphate (Holzer and Schröter, 1962). If hydrogen peroxide were to act in a similar manner, that is, as an electron acceptor as it does in the peroxidase reaction, the carboxyl oxygen of the glycolate so formed would come from water, not from hy-

drogen peroxide. For these reasons we feel that this mechanism cannot account for the incorporation of ^{18}O into glycolate.

A hypothesis which is consistent with our observations involves reaction 1 which is a two-electron oxidation. The



$$H_2O^* + A \quad (1)$$

R = H or PO_3; n = 2, 3, or 4

reductant, AH_2, might be reduced ferredoxin or $NADPH_2$. A special case of the reaction could involve a preliminary Mehler-type reaction between the reductant and oxygen to produce labeled hydrogen peroxide. This species could then perform the oxidation. The substrate could be any phosphorylated ketose intermediate of the photosynthetic carbon cycle. The products would be (phospho)glycolate and an aldose phosphate which could be glyceraldehyde 3-phosphate, erythrose 4-phosphate, or ribose 5-phosphate. The aldehyde oxygen of this aldose phosphate would not retain label since it exchanges with the medium and this compound would be readily absorbed into the photosynthetic carbon cycle. The above reaction might proceed either with or without catalysis by an enzyme. Recent experiments in this laboratory (unpublished results) have shown that ribulose 1,5-diphosphate can be oxidized by hydrogen peroxide, nonenzymatically, in the presence of Mn^{2+} ions to give phosphoglycolate and phosphoglyceraldehyde. It is also possible to envisage reactions similar to the above and consistent with our observations where the oxidant is one of the very reactive oxygen radicals, such as the superoxide anion radical, $O_2\cdot^-$, or the hydroxyl free radical, $OH^\cdot$.

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