Plant Physiol. (1984) 74, 108–111
0032-0889/84/74/0108/04/$01.00/0

Incorporation of Oxygen into Glycolate, Glycine, and Serine during Photorespiration in Maize Leaves

Received for publication June 1, 1983 and in revised form September 25, 1983

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ABSTRACT

Glycolate, glycine, and serine extracted from excised *Zea mays* L. leaves which had been allowed to photosynthesize in the presence of $^{18}O_2$ were analyzed by gas chromatography-mass spectrometry. In each case, only one of the oxygen atoms of the carboxyl group had become labeled. The maximum enrichment observed in glycine and serine was attained after 5 minutes and 15 minutes of exposure to $^{18}O_2$ at the CO_2 compensation point; the labeling was very high, reaching 70 to 73% of that in the applied O_2. Thus, it appears that all or nearly all of the glycine and serine are synthesized in maize leaves via fixation of O_2. In the presence of CO_2 (380 or 800 microliters per liter), ^{18}O-labeling was markedly slower.

Glycolate enrichment was variable and much lower than that in glycine and serine. It is possible that there are additional pathways of glycolate synthesis which do not result in the incorporation of ^{18}O from molecular oxygen. An estimation of the metabolic flow of O_2 through the photorespiratory cycle was made. It appeared that less than 75% of the O_2 taken up by maize leaves is involved in this pathway. Therefore, other processes of O_2 metabolism must occur in the light.

The C_4 plants, and maize in particular, exhibit little evidence of photorespiratory CO_2 production (5). Thus, no post-illumination burst of CO_2 can be detected from maize leaves and no CO_2 evolution was observed in CO_2-free air. When maize was allowed to photosynthesize in the presence of $^{14}CO_2$ in a closed chamber, there was no isotopic dilution of external $^{14}CO_2$ with $^{12}CO_2$ of a photorespiratory origin (17). The CO_2 compensation point of maize leaves has been shown to be less than 10 $\mu l \cdot l^{-1}$ (11, 29). Apparent photosynthesis of maize leaves is relatively insensitive to changes in O_2 concentration (28); a slight inhibitory effect is observed when O_2 concentration is higher than its concentration in normal air (13).

Nevertheless, using the O_2 isotope ^{18}O and MS, a small light-dependent O_2 uptake by excised maize leaves (12, 19, 27) or by intact leaves (1) can be detected as for other photosynthetic organisms: algae (15, 16, 23, 24), C_3 plants (6, 14, 22), other C_4 plants (6, 12), and CAM (2). It has been shown that this O_2 uptake was accompanied by a rapid ^{18}O incorporation into the carboxyl group of the glycolate excreted by algae (9, 21). In the case of C_3 plants, ^{18}O labeling was observed at first in two metabolic derivatives of glycolate, glycine and serine (3), and subsequently in all intermediates of the photorespiratory pathway: glycolate, glycine, serine, glycerate, and 3-PGA[1] (4). We have observed ^{18}O labeling of glycine and serine formed by maize during exposure to $^{18}O_2$ in the absence of CO_2 (10, 18). Here we report results of experiments performed to examine the ^{18}O incorporation into glycolate and its derivatives in maize leaves allowed to photosynthesize in the presence of $^{18}O_2$. We also examined the effect of CO_2 on this incorporation and we estimated the flux of molecular O_2 through the photorespiratory pathway in maize.

MATERIALS AND METHODS

Plant Material. Maize (*Zea mays* L. var. INRA $F_7 \times F_2$) was grown in a greenhouse on natural soil. Mature leaves (5th to 7th from the bottom) were selected from 2-month-old plants.

Experimental Procedure. A maize leaf fragment (about 500 mg fresh weight) was placed in a chamber (35 cm^3) inserted in an open circuit. First it was swept for 5 min in the light (750 $\mu E \cdot m^{-2} \cdot s^{-1}$) with CO_2-free air (15 $l \cdot h^{-1}$). Then, after a short vacuum period (5 s), the atmosphere of the cell was replaced at a flow rate of 4 $l \cdot h^{-1}$ with a gas mixture of N_2 + $^{18}O_2$ (79:21, v/v) containing various concentrations of CO_2 (0, 380, and 800 $\mu l \cdot l^{-1}$). $^{18}O_2$ was highly enriched (75 to 98 atom %) and was purchased from Bureau des Isotopes Stables, CEA, Saclay, France. At the end of the feeding period (0 to 20 min), the chamber was opened and the leaf was immediately dipped into liquid N_2. This operation took about 2 s during which time the sample was exposed to $^{16}O_2$.

Extraction and Separation of Metabolites. The leaf fragment was ground in liquid N_2 with a VirTis homogenizer (Cenco Instruments). A small piece of filter paper impregnated with [1-^{14}C]glycolate (5.5 kBq; 13 nmol; Amersham), L-[^{14}C]serine (3.7 kBq; 0.8 nmol), and [^{14}C]glycine (1.8 kBq; 0.5 nmol; Service des Molécules Marquées, CEA, France) was added just before the grinding. The presence of these radiolabeled compounds allow location of glycolate, glycine, and serine during the subsequent purification procedure and control of the yield of recovery. The powdered leaf material was extracted at 0°C for 10 min with 5 ml of 0.1 N HCl and centrifuged at 7000*g* for 5 min. The supernatant was filtered through a 0.45-μm Millipore membrane and applied to a Dowex 1 column (0.9 × 9 cm). The column was washed with water and organic acids were eluted with 10% (v/v) HCOOH. Fractions containing glycolate were evaporated to dryness after neutralization. Glyceric, lactic, succinic, and malic acids were also present in the dried residue. Amino acids which passed through an anionic column were retained on a cationic resin (Dowex 50: 0.9 × 9 cm) and eluted with 0.5 N NH_4OH. Glycine and serine fractions were evaporated.

Gas Chromatography-Mass Spectrometry of Organic and Amino Acids. Residues were suspended in 70 μl of acetonitrile and 150 μl of bis(trimethylsilyl)trifluoroacetamide containing

[1] Abbreviations: 3-PGA, glyceric acid 3-phosphate; RuBP, ribulose-1,5-bisphosphate; TMS, trimethylsilyl.

1% (v/v) trimethylchlorosilane (Pierce reagents) under dry conditions. To facilitate the derivatization, the organic acid fraction was heated at 100°C for 15 min and the amino acid fraction was heated at 100°C for 60 min. Aliquots (1 μl) of the silylated extracts were injected into a Varian Mat 112 S combined gas chromatograph-mass spectrometer with a glass column (2 m × 1.5 mm) of 3% (w/w) SE-30 on Chromosorb W.AW.DMCS. The flow rate of the helium carrier gas was 30 ml·min^{-1}. For organic acids, the column temperature was maintained at 80°C for 3 min and then was programmed to increase at 6°C·min^{-1}. Under these conditions, TMS_2-glycolate eluted at 85°C and TMS_3-glycerate at 125°C. The derivatized amino acids were run isothermally for 3 min at 100°C and then the temperature was increased at 6°C·min^{-1}. TMS_3-glycine eluted at 120°C and TMS_3-serine at near 128°C. The ion source temperature of the mass spectrometer was 220°C and the ionizing voltage was 70 ev. A computer (Digital PDP 11/34) controlled the recording of several spectra as chromatogram peaks emerged. When the analysis was complete, the average value for each mass fragment was calculated and the background noise was subtracted.

Interpretation of Mass Spectra. The major ionic fragments of glycolate, glycerate, glycine, and serine have been described previously (3, 4, 9, 10) and are summarized in Table I. Of particular interest are the fragments at m/e = 161 for glycolate and m/e = 204 for serine which have lost both carboxyl oxygens and retain only oxygen of the hydroxyl group. For each mass fragment at m/e = p, there are significant peaks at m/e = $p + 1$, m/e = $p + 2$ . . . which are due to isotopes of C, H, N, O, and Si at their natural abundance. An increasing of the intensity of peaks at two mass units higher than the main peaks is due to substitution of ^{16}O with ^{18}O in some molecules.

The extent of labeling was calculated from the measured peak heights I_p, I_{p+1}, I_{p+2}, I_{p+3}, and I_{p+4}, corresponding to a molecular fragment at m/e = p. These intensities were corrected for the presence of natural isotopes using mass spectra obtained from authentic unlabeled glycolate to calculate the constants $a_1 = I_{p+1}/I_p$, $a_2 = I_{p+2}/I_p$ . . . If corrected intensities of I_i are called J_i, ^{18}O labeling of the carboxyl group of studied compounds can be calculated from the formula

$$\text{atom \% } ^{18}O = \frac{J_{p+2} + 2J_{p+4}}{n(J_p + J_{p+2} + J_{p+4})}$$

where n is the number of oxygen atoms present in the ion at m/e = p.

^{18}O Exchange during Preparation and Analysis. The possible exchange of oxygen between the labeled compound and water during the experimental procedure was estimated as follows. Standard [^{18}O]glycolate, [^{18}O]glycine, and [^{18}O]serine were prepared by exchange with $H_2{}^{18}O$, incubated for 10 min in 0.1 N HCl, purified by ion exchange chromatography and analyzed for their ^{18}O content. The procedure resulted in very little loss of ^{18}O: near 3%.

On the other hand, to be sure that glycolate was not produced by hydrolysis during extraction, a leaf sample was extracted with HCl into $H_2{}^{18}O$ (16.5 atom %). During this procedure, ^{18}O labeling of glycolate reached only 0.64 atom %. Thus, we concluded that insignificant formation of glycolate occurred during extraction.

Determination of Glycolate, Glycine, and Serine Pool Sizes. The content of glycolate, glycine, and serine in maize leaves was determined by isotopic dilution, with the addition to the leaf extract of known quantities of [^{13}C]glycolate, [^{15}N]glycine, and [^{15}N]serine. The three considered compounds analyzed by GC-MS were in this case doubly labeled. This method is quite independent of the different yields of recovery during the experimental procedure. In the mature leaves used in these experiments, the pool sizes of glycolate, glycine, and serine were 67 ± 18, 103 ± 13, and 107 ± 38 μmol·m^{-2}, respectively.

RESULTS

Incorporation of ^{18}O into Photorespiratory Intermediates. Analysis of the mass spectra of the TMS derivatives of glycolate, glycine, and serine from maize leaves which had been exposed to $^{18}O_2$ in the light confirmed that molecular oxygen is incorporated into the carboxyl oxygens as reported previously in C_3 plants (3, 4) and algae (9, 21). Only one of the oxygen atoms of the carboxyl group of these three acids becomes labeled and additionally, no incorporation into the hydroxyl groups of glycolate and serine occurs. Hence, to calculate ^{18}O labeling, only the peak heights I_p, I_{p+2}, and the corresponding constants a_1 and a_2 (Table I) were used and the results are expressed as percentage of molecules containing an ^{18}O:

$$\text{Molecule \% } ^{18}\text{O-labeled compound} = 2 \times \text{atom \% } ^{18}O$$

The enrichment of the metabolites is reported as a percentage of that in $^{18}O_2$ applied to the leaves:

$$\text{Relative enrichment (\%)} = \frac{\text{molecule \% } ^{18}\text{O-labeled compound}}{\text{atom \% } ^{18}O_2 \text{ applied}} \times 100$$

Table II summarizes the results of 5-min feeding experiments conducted at the CO_2 compensation point on mature maize leaves. The relative enrichment observed in glycine was very high (average = 70 ± 6%); serine was less enriched than glycine (average = 35 ± 8%). A significant ^{18}O label was detected in glycolate. Nevertheless, the enrichment of glycolate was variable and not very high compared to glycine and serine. One explanation for this low enrichment is that there may be more than one pool of glycolate, and that incorporation of ^{18}O does not occur equally in every pool. Another possibility is that a ^{16}O chase could have happened at the end of the feeding when the leaf was exposed briefly to $^{16}O_2$ just prior to freezing in liquid N_2. In the case of glycerate, no consistent enrichment was observed (Table II). Other amino and organic acids were identified and analyzed during experiments: alanine, threonine, aspartate, glutamate, lactate, succinate, malate, citrate; in no case was any incorporation of ^{18}O detected (data not shown).

It must be noted that little or no label occurred when $^{18}O_2$ was supplied to the leaf for 10 to 15 min in darkness: atom % ^{18}O = about 0 for glycine, 0.69 for serine.

Kinetics of ^{18}O Incorporation into Glycine and Serine. The kinetics of ^{18}O incorporation into glycine and serine after feedings with a gas mixture containing $^{18}O_2$ and various concentrations of CO_2 (0, 380, and 800 μl·l^{-1}) are shown in Figure 1. The relative enrichment of these two amino acids increased with the length of the exposure to $^{18}O_2$. In the presence of a CO_2-free atmosphere, saturation in glycine was reached within 5 min. The final enrichment of serine was identical to that of glycine but the appearance of label into this compound was slightly slower. This observation is consistent with the expected sequence glycine → serine. In the presence of CO_2 (380 or 800 μl·l^{-1}), ^{18}O labeling of these amino acids was markedly slower.

The kinetic of ^{18}O incorporation into glycine at the CO_2 compensation point may be used to estimate the flux of O_2 involved in glycine synthesis and more generally in the glycolate pathway in these conditions.

Estimation of the Metabolic Flux of O_2 through the Glycolate Pathway. The rate of O_2 fixation into glycine can be determined from the knowledge of the pool size of this amino acid in maize leaves (103 ± 13 μmol·m^{-2}) and from the kinetics of glycine labeling characterized with the maximum enrichment (73 ± 6%) and the rate constant (0.72 ± 0.09 min^{-1}). This indicates a flux

Table I. *Mass and Formula of the Major Ions Observed in the Mass Spectra of TMS Derivatives of Glycolate, Glycerate, Glycine, and Serine*

The constants $a_1 = I_{p+1}/I_p$ and $a_2 = I_{p+2}/I_p$ were calculated from mass spectra of authentic unlabeled compounds.

Compound	Mass	Formula	a_1	a_2
Glycolate-(TMS)$_2$	205	(TMS-CH$_2$O-COOTMS)$^+$ (-CH$_3$)	0.181	0.109
	177	(TMS-CH$_2$O-OTMS)$^+$ (-CH$_3$)	0.163	0.075
	161	(TMS-CH$_2$O-TMS)$^+$ (-CH$_3$)	0.168	0.080
Glycerate-(TMS)$_3$	292	(TMS-CHOTMS-COOTMS)$^+$	0.282	0.148
Glycine-(TMS)$_3$	276	((TMS)$_2$NCH$_2$-COOTMS)$^+$ (-CH$_3$)	0.311	0.176
	248	((TMS)$_2$NCH$_2$-OTMS)$^+$ (-CH$_3$)	0.249	0.139
Serine-(TMS)$_3$	306	(TMS-CH$_2$O-CHNHTMS-COOTMS)$^+$ (-CH$_3$)	0.281	0.141
	278	(TMS-CH$_2$O-CHNHTMS-OTMS)$^+$ (-CH$_3$)	0.270	0.136
	218	(TMS-CHNH-COOTMS)$^+$	0.188	0.090
	204	(TMS-CH$_2$O-CHNHTMS)$^+$	0.191	0.087

Table II. *Incorporation of ^{18}O into the Carboxyl Group of Intermediates of the Glycolate Pathway*

Maize leaves were exposed during 5 min in the light (750 $\mu E \cdot m^{-2} \cdot s^{-1}$) to N_2 + $^{18}O_2$ (79:21; v/v). The results obtained from seven separate experiments are reported; they are expressed as enrichment relative to that in $^{18}O_2$ applied to the leaves. Each value represent the mean (±SE) calculated from at least eight mass spectra.

Relative Enrichment			
Glycolate	Glycine	Serine	Glycerate
	%		
ND[a]	65.2 ± 2.5	40.0 ± 4.5	ND
ND	72.7 ± 4.3	ND	ND
ND	75.4 ± 2.7	47.2 ± 4.0	ND
10.4 ± 0.2	62.7 ± 3.9	28.2 ± 3.3	−0.8 ± 1.0
16.8 ± 1.5	58.8 ± 2.0	28.3 ± 2.4	−2.1 ± 0.6
12.3 ± 3.1	65.4 ± 3.0	31.6 ± 1.2	3.1 ± 0.7
17.5 ± 2.0	ND	ND	ND

[a] ND, not determined.

of O_2 into glycine of about 54 ± 18 $\mu mol \cdot m^{-2} \cdot min^{-1}$ or 0.9 ± 0.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$. If we consider the glycolate pathway, several steps need O_2 uptake. The first is the synthesis of glycolate for which 1 mol of O_2 is taken up per mol of glycolate produced. The second is the peroxisomal oxidation of glycolate into glyoxylate by glycolic acid oxidase during which 0.5 mol of O_2 is incorporated into water per mol of glycolate oxidized. A third reaction which could involve O_2 uptake is the conversion of glycine to serine in mitochondria via electron transport linked to ATP conversion; 0.5 mol of O_2 would be necessary per mol of serine synthesized. It is also possible that NADH oxidation occurs via an internal malate-oxaloacetate shuttle (7). Of these three reactions, the only one which incorporates O_2 into organic material is the synthesis of glycolate. When incorporation of ^{18}O into H_2O is included, however, a total of 1.75 mol (or 1.5 mol) of $^{18}O_2$ are consumed per mol of glycolate entering the photorespiratory carbon oxidation cycle. The photorespiratory flux of O_2 will be then equal to 1.75 or 1.5 × flux of O_2 into glycine, *i.e.* 1.6 ± 0.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (or 1.35 ± 0.45 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and can be compared to the rate of light-dependent $^{18}O_2$ uptake by maize leaves measured at the CO_2 compensation point (19): 3.0 ± 0.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$. It appears that only 53 ± 24% (or 45 ± 21%) of O_2 taken up by *Zea mays* can be accounted for by the glycolate pathway. In spite of the evident variability, the rate of light-dependent O_2 uptake still appears to be higher than the estimated photorespiratory flux of O_2.

DISCUSSION

The ^{18}O labeling of the carboxyl group of glycolate, glycine, and serine extracted from maize leaves exposed to $^{18}O_2$ cannot be explained by an isotopic exchange via intracellular water (3,

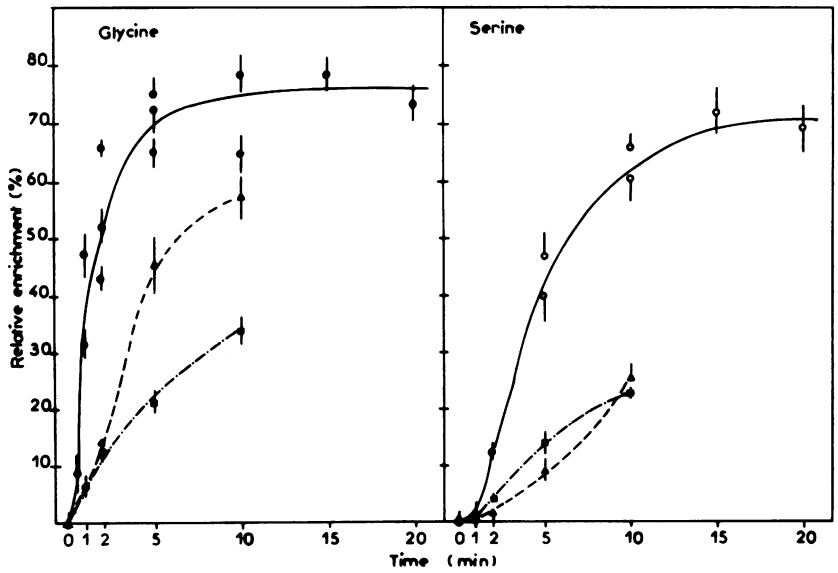

FIG. 1. Kinetics of ^{18}O labeling of glycine and serine as a function of CO_2 concentration in the feeding gas mixture N_2 + $^{18}O_2$. Zero CO_2 (——), 380 $\mu l \cdot l^{-1}$ CO_2 (– – –), 800 $\mu l \cdot l^{-1}$ (–·–). Each point represents a feeding experiment and ^{18}O labeling is calculated from at least five mass spectra.

10). Molecular oxygen or a compound directly derived from O_2 is involved in the biosynthesis of these three photorespiratory compounds. The absence of any labeling in the dark confirms that the process is a light-driven event. Glycolate has been previously reported to become labeled with $^{18}O_2$ in algae (9, 21) or C_3 plants (4) but never in maize leaves. Our results support those of Berry *et al.* (4) who observed in C_3 plants that ^{18}O enrichment in glycolate was variable and slightly lower than for glycine and serine. It is possible that glycolate can be synthesized by several pathways, some of which are not closely related to light-dependent O_2 uptake and incorporation of molecular oxygen. The maximum relative enrichment observed in glycine and serine was high (73 ± 6% and 70 ± 5%, respectively, of that in $^{18}O_2$ applied) but less than 100%. Incomplete saturation of isotope labeling was also observed by Berry *et al.* (4) in studies with *Spinacia*, *Atriplex*, and *Helianthus*: relative enrichment varied from 46 to 79% for glycine and from 50 to 74% for serine after 20- to 40-min exposure to $^{18}O_2$. In fact, the relative abundance of $^{18}O_2$ in the chloroplast must have been lower than that present in the gas phase due to the concurrent production of $^{16}O_2$. Therefore, since the ^{18}O enrichment in glycine and serine cannot reach 100%, it appears likely that all or nearly all glycine and serine synthesized by maize leaves occurred with fixation of O_2. Berry *et al.* (4) observed a low label in glycerate and 3-PGA, indicating that ^{18}O probably enters the photosynthetic carbon reduction cycle. In maize, it has not been possible to measure any consistent enrichment of glycerate, perhaps because glycerate produced from serine is only a small fraction of that in equilibrium with 3-PGA. No ^{18}O incorporation was detected in compounds other than those associated with photorespiration. Our results demonstrate that light-dependent O_2 uptake by maize was accompanied by an incorporation of this O_2 in glycolate, glycine, and serine as for C_3 plants, both during CO_2 fixation and at the CO_2 compensation point.

The experiments described here do not permit a choice between the two mechanisms proposed for glycolate formation. Oxygenation of RuBP catalyzed by RuBP carboxylase/oxygenase is known to result in the incorporation of one oxygen atom in the carboxyl group of phosphoglycolate, the precursor of glycolate (20). On the other hand, the peroxidation of dihydroxyethylthiamine pyrophosphate, intermediate of the transketolase reaction, according to the scheme proposed by Coombs and Whittingham (8) and Shain and Gibbs (25) could explain an incorporation of O_2 in glycolate since we have shown *in vitro* that oxidation with $H_2{}^{18}O_2$ of phosphorylated sugars (fructose 6-P, RuBP) and of a keto acid as hydroxypyruvate gave [^{18}O]glycolate or [^{18}O]phosphoglycolate, and the pattern of labeling was identical to that obtained with biologically synthesized glycolate (18, 26). The study of the kinetics of ^{18}O incorporation in glycine and serine in the absence or in the presence of CO_2 supports the existence of a competition between O_2 and CO_2 either for RuBP as a substrate of RuBP carboxylase/oxygenase or via a competition between NADP and O_2 for reducing equivalents associated with PSI activity (18).

Berry *et al.* (4) reported that the rate of O_2 uptake by C_3 plants corresponded to the flux of O_2 through the glycolate pathway. In contrast, it appears that the rate of O_2 uptake by maize leaves in the light is higher than the photorespiratory flux of O_2, and that other processes, which do not lead to O_2 incorporation in glycolate and its derivatives, must occur.

Acknowledgments—We thank Drs. C. Costes and E. Creach for helpful discussions as well as critical reading of this manuscript and Mrs. A. Lohou for technical assistance.

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