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Photorespiratory Rates in Wheat and Maize as Determined by ^{18}O-Labeling[1]

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ABSTRACT

A method was devised to quantify short-term photorespiratory rates in terrestrial plants using ^{18}O-intermediates of the glycolate pathway, specifically glycolate, glycine, and serine. The pathway intermediates were isolated and analyzed on a GC/MS to determine molecular percent ^{18}O-enrichment. Rates of glycolate synthesis were determined from ^{18}O-labeling kinetics of the intermediates, derived rate equations, and nonlinear regression techniques. Glycolate synthesis in wheat (*Triticum aestivum* L.), a C_3 plant, and maize (*Zea mays* L.), a C_4 plant, was stimulated by high O_2 concentrations and inhibited by high CO_2 concentrations. The synthesis rates were 7.3, 2.1, and 0.7 micromoles per square decimeter per minute under a 21% O_2 and 0.035% CO_2 atmosphere for leaf tissue of wheat, maize seedlings, and 3-month-old maize, respectively. Photorespiratory CO_2 evolution rates were estimated to be 27, 6, and 2%, respectively, of net photosynthesis for the three groups of plants under the above atmosphere. The results from maize tissue support the hypothesis that C_4 plants photorespire, albeit at a reduced rate in comparison to C_3 plants, and that the CO_2/O_2 ratio in the bundle sheath of maize is higher in mature tissue than in seedling tissue. The pool size of the three photorespiratory intermediates remained constant and were unaffected by changes in either CO_2 or O_2 concentrations throughout the 10-minute labeling period. This suggests that photorespiratory metabolism is regulated by other mechanism besides phosphoglycolate synthesis by ribulose-1,5-bisphosphate carboxylase/oxygenase, at least under short-term conditions. Other mechanisms could be alternate modes of synthesis of the intermediates, regulation of some of the enzymes of the photorespiratory pathway, or regulation of carbon flow between organelles involved in photorespiration. The glycolate pool became nearly 100% ^{18}O-labeled under an atmosphere of 40% O_2. This pool failed to become 100% ^{18}O-enriched under lower O_2 concentrations.

One of the remaining controversies surrounding photorespiration is its rate within terrestrial plants (6, 36). Depending upon the assay used, photorespiratory rates from as low as 6% to as high as 70% of net photosynthesis have been reported for C_3 plants (17, 36). The methods have been criticized as either inherently overestimating photorespiration or underestimating it (6, 36). The photorespiratory rate in C_4 plants is unknown. It is assumed to be low, since these plants exhibit little or no light-dependent, oxygen-sensitive CO_2 evolution (6, 10, 34).

The ^{18}O isotope has been used for more than 20 years to assay photorespiration (33). The oldest and most common application of the isotope has been to determine light-dependent O_2 consumption (11–13, 33, 34). The use of $^{18}O_2$ has many advantages over other assays. Photorespiration can be measured under normal O_2 and CO_2 concentrations (11–13). Photosynthetic cells do not discriminate significantly against the ^{18}O isotope, at least for short-term studies (12). The biggest problem with using $^{18}O_2$ to estimate photorespiratory consumption is that it is difficult to correct for other O_2 consumption reactions, such as mitochondrial respiration and the Mehler reaction (11–13, 15, 34). To get around this problem, $^{18}O_2$ can be used to selectively label glycolate pathway intermediates (1). This is another advantage to the ^{18}O-isotope. Those researchers using $^{14}CO_2$ to isotopically label glycolate pathway intermediates have found that photorespiratory generated $^{12}CO_2$ is photosynthetically refixed, which eventually results in a substantial underestimation of photorespiration (6, 14, 25). However, the photorespiratory refixation of photosynthetically generated $^{16}O_2$ is two orders of magnitude lower than the above $^{12}CO_2$ refixation and, under most circumstances, $^{16}O_2$ refixation is considered negligible (25). Photorespiratory rates can thus be determined through knowledge of the pool sizes of the intermediates and examination of the intermediates' ^{18}O-labeling kinetics.

The use of ^{18}O-labeling to determine photorespiratory rates was first suggested by Berry *et al.* (5). They reported that spinach had a photorespiratory glycine synthesis rate of about 7.8 μmol/dm^2/min under an atmosphere of 21% O_2 and a CO_2 concentration at the compensation point using this method. However, they did not explain how they obtained this rate from the labeling kinetics of glycine. Jolivet-Tournier and Gerster (15) used an ^{18}O-labeling method to determine photorespiration in maize. They reported a photorespiratory glycine synthesis rate of about 0.6 μmol/dm^2/min under normal concentrations of O_2 and CO_2. However, they determine this rate from the ^{18}O-labeling assuming that the isotopic content changes linearly with time, whereas in reality it changes in exponential fashion (24, 27, 37).

We attempted to expand the work of Berry *et al.* (5) and Jolivet-Tournier and Gerster (15). Toward this goal, we de-

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termined the rate of incorporation of ^{18}O into glycolate, glycine, and serine pools of wheat seedlings, maize seedlings, and 3-month-old maize. Photorespiratory rates were determined by an examination of the ^{18}O-labeling kinetics of the three glycolate pathway intermediates, the pool sizes of these intermediates, and nonlinear regression.

MATERIALS AND METHODS

Seeds of *Triticum aestivum* L. (var Hart) and *Zea mays* L. (hybrid PA 8810) were sown in plastic pots filled with vermiculite and germinated in a greenhouse. Upon germination they were watered with a half-strength, complete nutrient solution (35). Wheat and maize seedlings used for either ^{18}O-labeling purposes or photosynthetic CO_2 uptake measurements were maintained in their plastic pots in the greenhouse until they were 9 d old. For cultivation of the 3-month-old maize, 5-d-old seedlings were transplanted to large, individual clay pots filled with greenhouse soil mix and maintained in the greenhouse for the 3 months (May through August). These plants were periodically fertilized with a commercial N-P-K (15-16-17) fertilizer.

When the wheat and maize seedlings were 9 d old, the second leaf was removed for ^{18}O-labeling. The 3-month-old plants had their sixth to eighth leaf selected for the experiments. The leaf tips were cut from the plants, recut under water, and the cut end was placed in a small amount of water-soaked sand within a water-jacketed chamber. Total leaf area used for each experiment was approximately 2 cm^2 and total volume of the chamber was 50 mL. The temperature of the chamber was maintained at 25°C by the use of a circulating water bath. The chamber was sealed with a silicone rubber stopper in which there was both a gas injection and a gas exit port.

The chamber was swept with argon for 30 s. To start ^{18}O-labeling, the argon within the chamber was quickly replaced with a mixture of O_2, at concentrations of 2%, 21%, and 40%, enriched with $^{18}O_2$ (10–99.9% of the total O_2 [Bio-Rad, Richmond, CA]), CO_2, at concentrations of either 0.035% or 1.5%, with the balance argon. The light was turned on (photo flux density = 400 $\mu E/m^2/s$). Prior to this point all operations were performed under normal fluorescent room light. Argon served as the reference gas and was employed as a means to check for leaks in the chamber. The gas composition in the chamber was checked periodically by withdrawing 0.02 mL with a syringe from the chamber, immediately bringing the syringe to the modified injection port of an LKB 9000 mass spectrometer where the first 0.01 mL of air was purged and the remaining 0.01 mL was injected into the mass spectrometer. Accuracy of the mass spectrometric CO_2 determination was checked at each level of O_2 to correct for the reaction of O_2 with traces of carbon present on the mass spectrometer filament. Changes in percent CO_2, percent total O_2, and percent relative isotopic enrichment of oxygen were calculated from changes in recorded peak heights of the CO_2, $^{16}O_2$, and $^{18}O_2$ peaks. Leaf tissues were exposed continuously to $^{18}O_2$ for up to 10 s. The gases within the chamber were not renewed or circulated during this time.

The chamber was opened and the leaf segments were plunged into liquid N_2. This operation took about 1 to 3 s to perform at the end of the labeling period. The leaf segments were stored in liquid N_2 until the photorespiratory intermediates (glycolate, glycine, and serine) were extracted.

The extraction, separation, and silylation of the photorespiratory intermediates followed a modified procedure of Berry *et al.* (5). The modifications consisted of adjusting the pH of the glycolate fraction to 7.0 with 1.0 N NaOH prior to lyophilization and allowing the silylized amino acid fraction to sit overnight to complete the silylation of glycine (4). The pH of the glycolate fraction was raised to 7.0 because glycolate was reported to be volatile under acidic conditions (2). The dry fraction was dissolved in 5 mL of water and passed through a cation exchange column (Bio-Rad AG 50W-X8, 100–200 mesh, H^+ form, 0.8 × 6.0 cm) to remove Na^+. This fraction was then lyophilized. Testing this last procedural step with known quantities of glycolate indicated that the last step represented a major cause for the loss of glycolate. Yet this step had to be done since Na^+ interfered with the silylation of glycolate. Once these intermediates are derivatized they are stable for several months as long as they are stored in a desiccator (4). The final yields of the three compounds at the end of the isolation and silylation procedure, as determined using ^{14}C-labeled glycolate, glycine, and serine cochromatography were 52 ± 5%, 49 ± 3%, and 67 ± 3%, respectively.

Aliquots of the derivatized extracts were injected into an LKB 9000 gas chromatograph/mass spectrometer. The gas column used was 3% SP-2250 on 80/100 Supelcoport (Supelco, Bellefonte, PA). The flow rate of the helium carrier gas was 16 mL/min. For both fractions the temperature program of the gas chromatograph oven was isothermal at 100°C for 5 min followed by a linear increase of 10°C/min. TMS[4]-glycolate eluted at 145°C, while TMS-glycine and TMS-serine eluted at 190 and 200°C, respectively. The ion source temperature of the mass spectrometer was 250°C and the ionizing voltage was 70 eV.

The ionic fragments used to determine molecular percent ^{18}O-label were those of Berry *et al.* (5). The principal mass/charge of the ionic fragments used for molecular percent ^{18}O-label determinations were 177 and 205 for glycolate, 248 and 276 for glycine, and 218, 278, and 306 for serine. The method of Troxler *et al.* (32) was used to correct for the natural abundance of C, N, O, and Si isotopes in derivatized glycolate, glycine, and serine. The corrected mass spectra were used to determine molecular percent ^{18}O-label using the following equation adapted from Berry *et al.* (5):

$$\text{Molecular \% } ^{18}\text{O-label} = (2/N)(P_{m+2})/(P_m + P_{m+2}) \times 100$$

where N = number of carboxyl oxygen(s) present in major or m ionic fragment, P_m = height of a major ionic or m fragment peak containing at least one carboxyl oxygen, and P_{m+2} = corrected height of the peak which has two mass/charge units greater than the m fragment. This peak is generated from a photorespiratory intermediate which has been singly labeled with ^{18}O.

Since the molecular percent $^{18}O_2$ exposed to the leaves varied, the enrichments of the intermediates were expressed

[4] Abbreviations: TMS, trimethylsilyl; Rubisco, ribulose-1,5-biphosphate carboxylase/oxygenase.

as percentages of the $^{18}O_2$ applied to the leaves using the equations of Jolivet-Tournier and Gerster (15).

Pool sizes of the intermediates were determined by measuring the area under the gas chromatograph tracings and comparing it to the area under the tracings of known amounts of the respective compounds. The pool sizes reported in this paper were adjusted for loss that occurred during isolation.

The metabolic rates were determined by analyzing the relationship between ^{18}O-enrichment and time for each photorespiratory metabolite pool (9). This was performed using the Marquart method of nonlinear regression (26) and derived rate equations. The rate equations for glycolate, glycine, and serine were all derived from the equation relating the instantaneous change in concentration of an isotopically labeled metabolite over time as a function of metabolic rate of formation and degradation and the isotopic enrichment of the precursor and the metabolite (24, 27, 37). The equation is:

$$dB^*/dt = r_1E_A - r_2E_B$$

where B^* = concentration of isotopically labeled metabolite (the product), r_1 = rate of product synthesis, r_2 = rate of product conversion to other metabolites (under steady state conditions $r_1 = r_2$), $E_A = A^*/A$, the isotopic enrichment of the precursor at time t, where A^* is the concentration of isotopically labeled precursor and A is total precursor concentration; and $E_B = B^*/B$, the isotopic enrichment of product at time t, where B is the total product concentration.

The equation for [^{18}O]glycolate formation was derived as follows:

$$dP^*/dt = rE_o - rE_p$$

where P^* = concentration of isotopically enriched glycolate, E_o = molecular ^{18}O-enrichment of oxygen, r = rate of glycolate synthesis, and $E_p = P^*/P$, the ^{18}O-enrichment of glycolate, where P is the total glycolate pool.

In this relationship, oxygen was assigned the role of the direct precursor of glycolate. Such as assignment can be made even though phosphoglycolate is the probable direct precursor, since the concentration of phosphoglycolate is small (28) and therefore would not significantly influence the ^{18}O-labeling of glycolate (27). It was assumed that the glycolate pool was in steady state and the rate of glycolate synthesis and the molecular ^{18}O-enrichment of oxygen was constant. A further assumption was that the isotopic enrichment of O_2 surrounding the leaf tissue was a good approximation of the isotopic enrichment of O_2 within the chloroplast (25). Setting $E_o = 1$, then:

$$dP^*/dt + r(P^*/P) = r$$

Multiplying both sides by the integrating factor $e^{rt/P}$, then:

$$(dP^*/dt + rP^*/P)e^{rt/P} = re^{rt/P}.$$

Integrating both sides with respect to time, then:

$$P^*e^{rt/P} = Pe^{rt/P} + C.$$

The constant, C, is determined by arranging the time scale so that $P^* = 0$ when $t = 0$:

$$C = -P = -P(e^{rt/P})(e^{-rt/P}).$$

Therefore,

$$E_p = P^*/P = 1 - e^{-rt/P}. \quad (1)$$

The equation for [^{18}O]glycine synthesis was derived in a similar fashion. Several assumptions were made to derive the equation. One assumption was that the rate of photorespiratory glycine synthesis equals the rate of glycolate formation. A second assumption was that the flow of carbon from glycolate to glycine is unidirectional (28). A third assumption was that the glyoxylate pool is small (7, 28, 30) and therefore would not significantly affect the labeling kinetics of glycine (27). A final assumption was that steady state conditions hold for the pool of glycine. Therefore,

$$dG^*/dt = rE_p - rE_g$$

where $E_g = G^*/G$, the ^{18}O-enrichment of glycine, where G^* is the concentration of [^{18}O]glycine and G is the total glycine pool

$$dG^*/dt + rG^*/G = rE_p.$$

Multiplying both sides by the integrating factor $E^{rt/G}$, substituting equation 1 for E_p, and integrating both sides with respect to time yields:

$$G^*e^{rt/G} = G[e^{rt/G} - P(e^{rt/G})(e^{rt/P})/(P - G)] + C.$$

Since, when $t = 0$, $G^* = 0$, then

$$C = [PG/(P\text{-}G)\text{-}G](e^{rt/G})(e^{rt/G})$$

Therefore,

$$E_g = G^*/G = 1 + P[(e^{-rt/G}) - (e^{-rt/P})]/(P - G) - e^{-rt/G} \text{ when } P \neq G. \quad (2)$$

The equation for [^{18}O]serine synthesis was derived assuming that its rate of formation is one-half of glycolate and glycine synthesis (20) and that the serine pool is in steady state:

$$dS^*/dt = rE_g/2 - rE_s/2$$

where $E_s = S^*/S$, the ^{18}O-enrichment of serine, where S^* is the concentration of [^{18}O]serine and S is total serine pool

$$dS^*/dt + rS^*/2S = rE_g/2.$$

Substituting Eq. 2 for E_g, multiplying both sides by $e^{rt/2S}$, integrating both sides with respect to t, and rearranging terms yield:

$$S^*e^{rt/2S} = Se^{rt/2S}[1 + (G^2e^{-rt/G})/(P - G)(G - 2S) - (P^2e^{-rt/P})/(P - G)(P - 2S)] + C.$$

As before, C is determined by setting $t = 0$ when $S^* = 0$

$$C = S[P^2/(P - G)(P - 2S) - G^2/(P - G)(G - 2S) - 1](e^{rt/2S})(e^{-rt/2S}).$$

Substituting and rearranging terms yields:

$$E_s = S^*/S = 1 + G^2[(e^{-rt/G}) - (e^{-rt/2S})]/(P - G)(G - 2S) + P^2[(e^{-rt/2S}) - (e^{-rt/P})]/(P - G)(P - 2S) - e^{-rt/2S}$$

when

$$P \neq G,\ G \neq 2S, \text{ and } P \neq 2S. \quad (3)$$

It should be emphasized that in Eqs. 1 through 3, photorespiratory rates are expressed in terms of the rate of glycolate synthesis.

Changes in O_2 and CO_2 concentrations and the isotopic composition of O_2 at the leaf surface would not be apparent immediately at the point where the gases were removed from the chamber for mass spectrometer analysis since the gases within the chamber were not circulated. Thus, photosynthetic rates were determined by an alternate method. Leaf tips were cut from the plants, recut under water, and placed in a chamber similar to the one used for ^{18}O-labeling, except the volume of the chamber was 100 mL. Total leaf area was about 2 cm^2. Temperature of the water bath was maintained at 25°C. The chamber was sealed by a silicone rubber stopper, the light turned off, and the air inside the chamber was quickly purged by N_2. The N_2 was replaced by various O_2 and CO_2 concentrations with the balance N_2. A small fan inside the chamber was then turned on. A sample of gas (5–10 μL) was removed from the chamber and checked for its CO_2 initial concentration. The light was then turned on (400 $\mu E/m^2/$ min), and small samples of gas (5–10 μL) were removed to determine decreases in CO_2 concentration. All CO_2 analyses were done using a Hewlett-Packard gas chromatograph, model 5840A, set up to determine CO_2 levels. The column was a PoraPak Q stainless steel column (Supelco, Bellefonte, PA). The temperature of the injection port was 75°C, while the column oven was maintained at 50°C. N_2 was the carrier gas. After passing through the column, CO_2 was converted to methane at a temperature of 350°C using a nickel catalyst. Methane levels were measured using a flame ionization detector at a temperature of 150°C.

RESULTS

The effects of O_2 and CO_2 concentrations on the ^{18}O-labeling kinetics of glycolate in wheat seedlings are illustrated in Figure 1. Under an atmosphere of 2% O_2 and 0.035% CO_2, approximately 10% of the glycolate became labeled with ^{18}O. Maximum enrichment was reached within 1 min and once achieved this value remained relatively constant for the duration of the labeling period (10 min). At the higher O_2 concentrations, 21 and 40%, the level of [^{18}O]glycolate reached its apparent maximum between 45 and 60 s. The maximum enrichment of glycolate was higher at these O_2 concentrations than at 2% O_2, being near 70 and 100%, respectively. Once maximum enrichment was reached at 21% O_2 and 40% O_2, it decreased, reaching a value of about 30 and 50%, respectively, at the end of 10 min. Increasing the CO_2 level to 1.5% completely suppressed the synthesis of [^{18}O] glycolate at 21% O_2.

Maize seedlings behaved in a similar fashion to wheat seedlings with respect to effects of O_2 and CO_2 concentration on the ^{18}O-labeling kinetics of glycolate (Fig. 2). Approximately 10% of the glycolate within the maize seedlings was labeled with ^{18}O under an atmosphere of 2% O_2 and 0.035% CO_2 and it remained at this level throughout the rest of the labeling period. At the higher O_2 concentrations, 21 and 40 %, the level of [^{18}O]glycolate reached its apparent maximum between 45 and 60 s. The maximum enrichment of glycolate was near 50% under 21% O_2 and near 80% under 40% O_2.

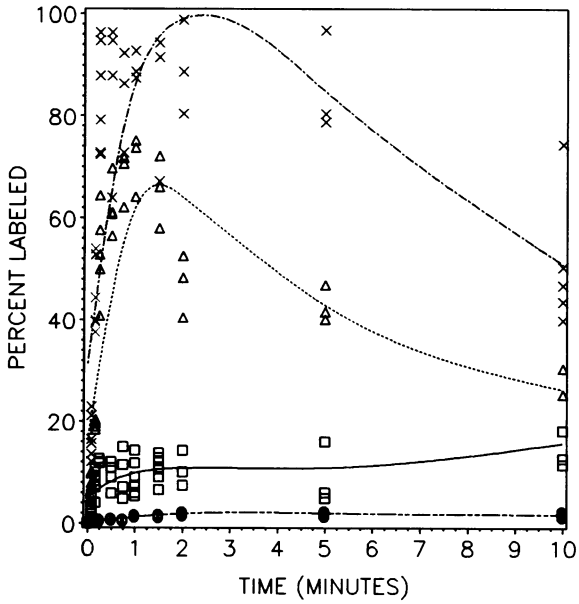

Figure 1. Effects of O_2 and CO_2 concentration on the ^{18}O-labeling kinetics of glycolate in wheat. Y-coordinate is expressed as relative molecular percent ^{18}O-enriched. Initial O_2 and CO_2 concentrations were 2% O_2 and 0.035% CO_2 (□——□), 21% O_2 and 0.035% CO_2 (△---△), 40% O_2 and 0.035% CO_2 (×—·—×), and 21% O_2 and 1.5% CO_2 (○— - - —○).

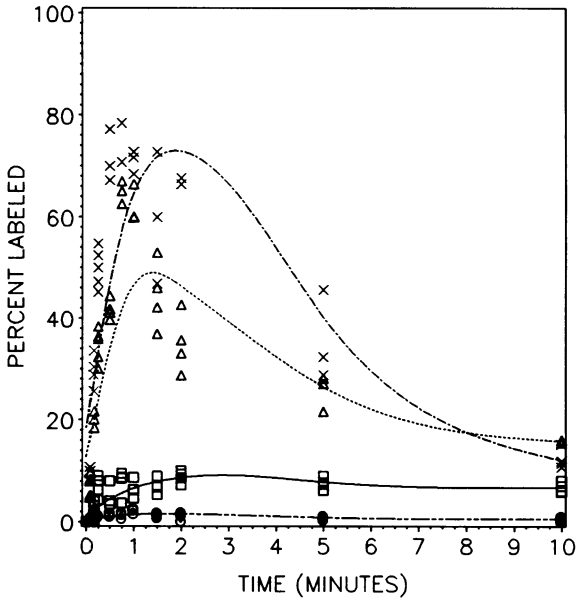

Figure 2. Effects of O_2 and CO_2 concentration on the ^{18}O-labeling kinetics of glycolate in maize seedlings. Y-coordinate is expressed as relative molecular percent ^{18}O-enriched. Initial O_2 and CO_2 concentrations were 2% O_2 and 0.035% CO_2 (□——□), 21% O_2 and 0.035% CO_2 (△- - -△), 40% O_2 and 0.035% CO_2 (×—·—×), and 21% O_2 and 1.5% CO_2 (○— - - —○).

Once maximum enrichment was reached at these higher O_2 concentrations, it declined steadily to values near 20% after 10 min. Increasing the CO_2 level to 1.5% completely suppressed [^{18}O]glycolate synthesis at 21% O_2.

Older maize plants exhibited glycolate labeling patterns unlike those of either wheat or maize seedlings. Under an atmosphere of 2% O_2 and 0.035% CO_2, glycolate became less than 1% ^{18}O-labeled (Fig. 3). Glycolate in the more mature maize did become significantly labeled at the higher O_2 concentrations, 21 and 40%, with the relative ^{18}O-enrichment reaching about 30 and 40%, respectively. Once glycolate reached its greatest relative enrichment, the level of [^{18}O] glycolate rapidly dropped. Like the seedlings, glycolate synthesis at 21% O_2 was completely suppressed by 1.5% CO_2 (data not shown). None of the groups of plants exhibited significant ^{18}O-enrichment of glycolate when exposed to $^{18}O_2$ in the dark (data not shown).

The effects of oxygen concentration on the kinetics of [^{18}O] glycine in wheat are illustrated in Figure 4. As with glycolate, the isotopic labeling of glycine was sensitive to O_2. The maximum enrichment of glycine with ^{18}O was about 8, 60, and 80% for 2, 21, and 40% O_2, respectively. Once the maximum relative enrichment was reached in glycine it remained fairly constant for the rest of the labeling period under 2 and 21% O_2, while the maximum enrichment dropped significantly under 40% O_2. [^{18}O]Glycolate appeared sooner and reached its apparent maximum amount prior to [^{18}O] glycine under all O_2 concentrations. This is consistent with the notion that glycolate is a photorespiratory precursor of this amino acid.

[^{18}O]Glycine synthesis in maize seedlings is comparable to [^{18}O]glycine synthesis in wheat seedlings in many respects (Fig. 5). The level of relative maximum percent ^{18}O-enrichment was sensitive to the O_2 concentration, being the greatest under 40% O_2 followed by 21 and 2% O_2, respectively. [^{18}O] Glycine appeared later and reached its apparent maximum after [^{18}O]glycolate. However, the maximum relative enrichment of glycine was lower in maize seedlings than in wheat seedlings.

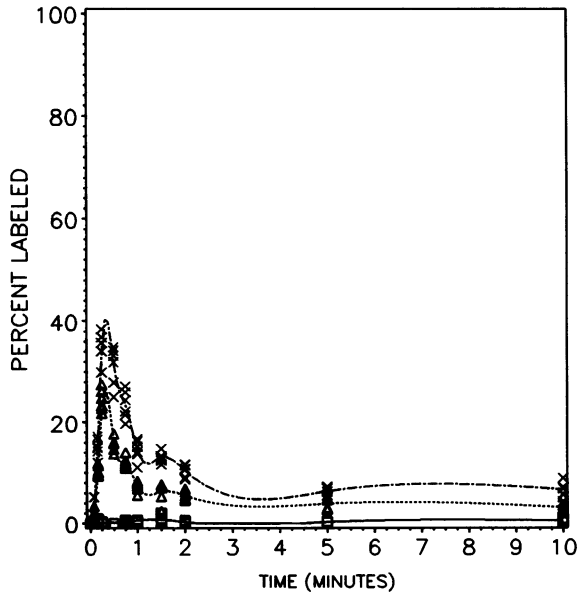

Figure 3. Effects of O_2 concentration on the ^{18}O-labeling kinetics of glycolate in 3-month-old maize. Y-coordinate is expressed as relative molecular percent ^{18}O-enriched. Initial O_2 and CO_2 concentrations were 2% O_2 and 0.035% CO_2 (□——□), 21% O_2 and 0.035% CO_2 (△- - -△), and 21% O_2 and 40% O_2 and 0.035% CO_2 (×—·—×).

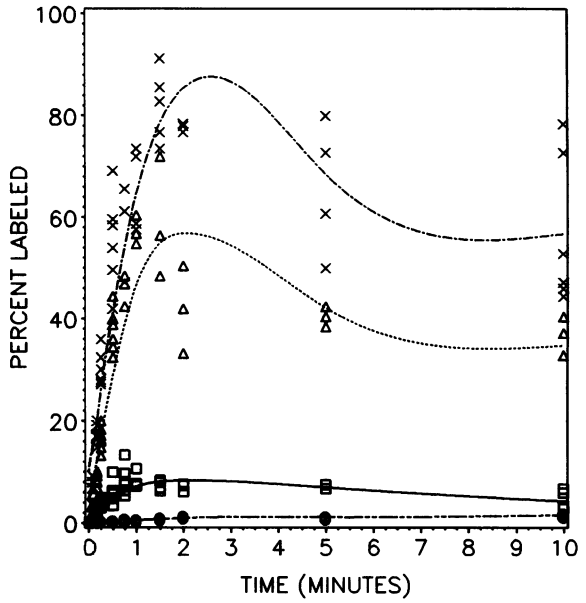

Figure 4. Effects of O_2 concentration on the ^{18}O-labeling kinetics of glycine in wheat. Y-coordinate is expressed as relative molecular percent ^{18}O-enriched. Initial O_2 and CO_2 concentrations were 2% O_2 and 0.035% CO_2 (□——□), 21% O_2 and 0.035% CO_2 (△---△), and 40% O_2 and 0.035% CO_2 (×—·—×).

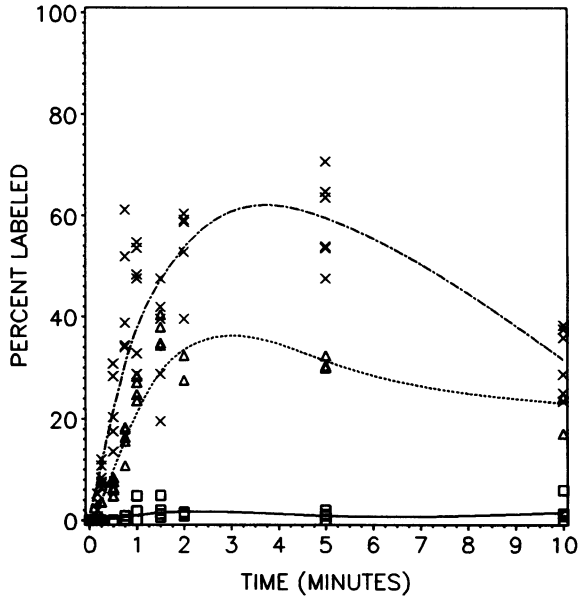

Figure 5. Effects of O_2 concentration on the ^{18}O-labeling kinetics of glycine in maize seedlings. Y-coordinate is expressed as relative molecular percent ^{18}O-enriched. Initial O_2 and CO_2 concentrations were 2% O_2 and 0.035% CO_2 (□——□), 21% O_2 and 0.035% CO_2 (△---△), and 40% O_2 and 0.035% CO_2 (×—·—×).

Three-month-old maize exhibited the lowest percent of isotopic labeling of glycine of all of the plants (Fig. 6). There was no significant synthesis of [^{18}O]glycine in mature maize in the light under a 2% O_2 and 0.035% CO_2 atmosphere. There was synthesis under atmospheres of 21 and 40% O_2,

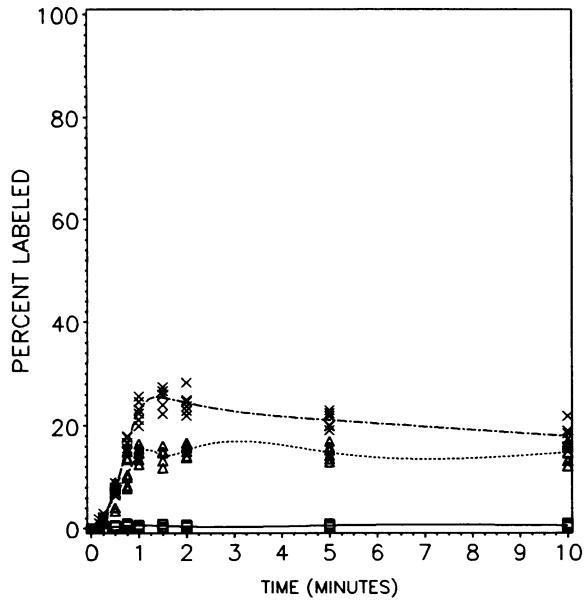

Figure 6. Effects of O_2 concentration on the ^{18}O-labeling kinetics of glycine in 3-month-old maize. Y-coordinate is expressed as relative molecular percent ^{18}O-enriched. Initial O_2 and CO_2 concentrations were 2% O_2 and 0.035% CO_2 (□——□), 21% O_2 and 0.035% CO_2 (△---△), and 40% O_2 and 0.035% CO_2 (×—·—×).

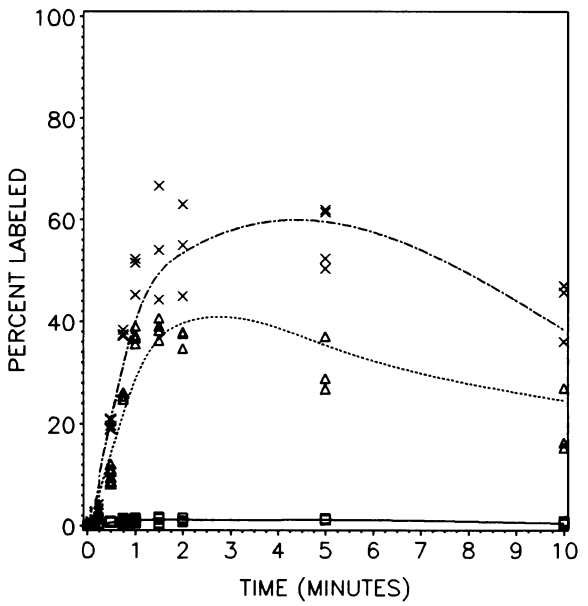

Figure 7. Effects of O_2 concentration on the ^{18}O-labeling kinetics of serine in wheat. Y-coordinate is expressed as relative molecular percent ^{18}O-enriched. Initial O_2 and CO_2 concentrations were 2% O_2 and 0.035% CO_2 (□——□), 21% O_2 and 0.035% CO_2 (△---△), and 40% O_2 and 0.035% CO_2 (×—·—×).

CO_2 concentration 0.035%. The maximum relative enrichment reached about 16 and 26% for the two O_2 concentrations, respectively. Once the maximum relative enrichment was reached in glycine, it remained relatively constant throughout the remainder of the labeling period.

Glycine did not become isotopically labeled in any of the plants when placed in an atmosphere of 21% O_2 and 1.5% CO_2 in the light or in an atmosphere of 21% O_2 and 0.035% CO_2 in the dark (data not shown).

The isotopic labeling of serine in wheat was sensitive to the atmospheric composition (Fig. 7). The maximum ^{18}O-enrichment of serine was highest under 40% O_2 followed by 21% O_2 and 2% O_2. The ^{18}O-enrichment of serine rapidly declined after the maximum enrichment had been reached under a 40% O_2 and 0.035% CO_2 atmosphere. Serine became labeled after glycine.

Isotopic enrichment of the serine pool in maize seedlings exhibited a response to the atmospheric concentrations of O_2 (Fig. 8). The amount of ^{18}O-enrichment was negligible under 2% O_2. The higher levels of O_2 resulted in higher isotopic enrichments. The time course of ^{18}O-enrichment followed the time course of isotopic enrichment of glycine (Fig. 5), indicating a close relationship in the biosynthesis of the two amino acids.

Three-month-old maize exhibited the least amount of ^{18}O-enrichment of serine of any of the plants examined (Fig. 9), consistent with the results for the other photorespiratory metabolites. [^{18}O]Serine synthesis was negligible under a 2% O_2 atmosphere. There was only a minor difference in maximum relative enrichment of serine between plants exposed to 21% O_2 and those exposed to 40% O_2. However, the plants exposed to 40% O_2 reached the maximum ^{18}O-enrichment of serine within 60 to 90 s, while the plants exposed to 21% O_2

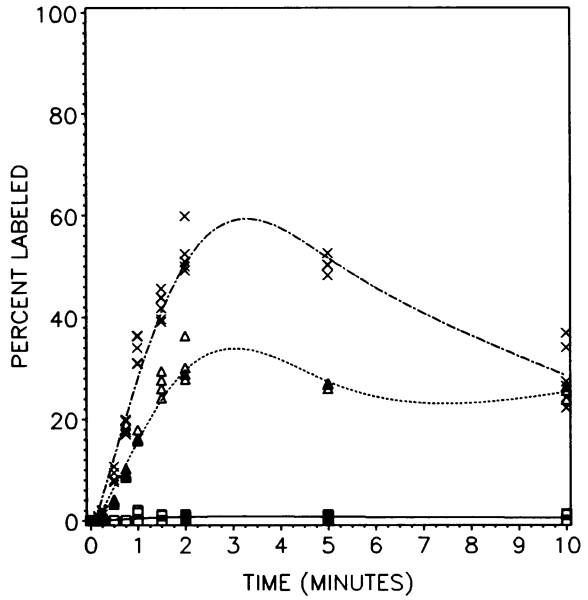

Figure 8. Effects of O_2 concentration on the ^{18}O-labeling kinetics of serine in maize seedlings. Y-coordinate is expressed as relative molecular percent ^{18}O-enriched. Initial O_2 and CO_2 concentrations were 2% O_2 and 0.035% CO_2 (□——□), 21% O_2 and 0.035% CO_2 (△---△), and 40% O_2 and 0.035% CO_2 (×—·—×).

took 120 s to reach peak enrichment. Glycine was labeled prior to serine (Fig. 6), indicating that glycine is a precursor for serine.

Serine was not isotopically labeled when placed in an atmosphere of 21% O_2 and 1.5% CO_2 in the light or when

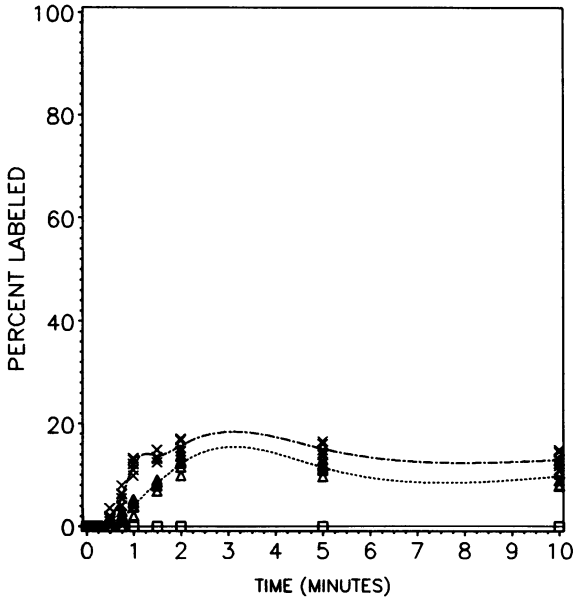

Figure 9. Effects of O_2 concentration on the ^{18}O-labeling kinetics of serine in 3-month-old maize. Y-coordinate is expressed as relative molecular percent ^{18}O-enriched. Initial O_2 and CO_2 concentrations were 2% CO_2 and 0.035% CO_2 (□——□), 21% O_2 and 0.035% CO_2 (△---△), and 40% O_2 and 0.035% CO_2 (×—·—×).

the plants were exposed to 21% O_2 and 0.035% CO_2 in darkness (data not shown).

The pool sizes of the three photorespiratory intermediates in the three groups of plants are given in Table I. The composition of the atmosphere had little effect on the size of the pools. All the pools remained relatively constant from the point prior to the introduction of $^{18}O_2$ until the end of the labeling period, which was as long as 10 min (data not shown). Thus, all of the pools were in apparent steady state for at least the first 10 min of ^{18}O-labeling.

The changes in O_2 concentration, relative molecular percent $^{18}O_2$/total O_2, and CO_2 concentration within the labeling chamber for wheat seedlings are presented in Figures 10, 11, and 12, respectively. Minimal changes occurred in the O_2 concentration and relative molecular percent $^{18}O_2$/total O_2 throughout, although the CO_2 concentration dropped significantly. These changes were similar to what happened during ^{18}O-labeling of mature maize and maize seedlings (data not shown). Data points from 0 to 30 s were used for nonlinear regression determination of glycolate synthesis rate since there were only minimal changes in the CO_2 concentration in the first 30 s of labeling for all groups of plants.

The rates of glycolate synthesis under the various oxygen and carbon dioxide concentrations are listed in Tables II, III, and IV for wheat seedlings, maize seedlings, and 3-month-old maize, respectively. They were determined from the ^{18}O-labeling kinetics of glycolate, glycine, and serine.

There is a general consistency among the rates of glycolate synthesis as determined by the labeling kinetics of the three intermediates for each group of plants, with the exception of wheat seedlings under 21% O_2 and 0.035% CO_2. The rate of glycolate synthesis was highest in wheat seedlings followed first by maize seedlings and then mature maize. For example, the rate in wheat seedlings was 3 times that of maize seedlings and 10 times that of 3-month-old maize under a 21% O_2 and 0.035% CO_2 atmosphere. The rate of glycolate synthesis increased in all groups of plants with increasing O_2 concentration. Photorespiratory rates could not be determined for any of the plants under a 21% O_2 and 1.5% CO_2 atmosphere because of insufficient isotopic labeling of the intermediates.

The net photosynthetic rates for the plants are expressed in Table V. Wheat seedlings exhibited the typical C_3 characteristic of O_2 inhibition of CO_2 uptake (8). An elevated CO_2 atmosphere (1.5%) reversed this O_2 inhibition. By comparison, both the mature maize and maize seedlings exhibited little or no inhibition of photosynthesis by O_2.

Table I. *Pool Sizes in Wheat Seedlings, Maize Seedlings, and 3-Month-Old Maize*
Number of samples is in parentheses.

Group	Pool Sizes		
	Wheat seedlings	Maize seedlings	Maize plants
		$\mu mol/dm^2$	
2% O_2, 0.035% CO_2			
Glycolate	2.07 ± 0.38 (25)	1.10 ± 0.14 (23)	0.41 ± 0.17 (27)
Glycine	3.04 ± 0.28 (23)	2.62 ± 0.28 (29)	1.86 ± 0.40 (27)
Serine	2.48 ± 0.48 (21)	1.12 ± 0.28 (23)	0.97 ± 0.37 (24)
21% O_2, 0.035% CO_2			
Glycolate	2.21 ± 0.27 (22)	1.24 ± 0.29 (24)	0.59 ± 0.28 (27)
Glycine	2.90 ± 0.55 (23)	2.35 ± 0.41 (24)	1.93 ± 0.30 (24)
Serine	2.62 ± 0.47 (22)	0.97 ± 0.27 (21)	1.24 ± 0.31 (24)
40% O_2, 0.035% CO_2			
Glycolate	2.35 ± 0.41 (24)	1.17 ± 0.17 (21)	0.65 ± 0.22 (21)
Glycine	2.70 ± 0.43 (25)	2.62 ± 0.43 (22)	2.01 ± 0.23 (23)
Serine	2.43 ± 0.26 (24)	1.01 ± 0.33 (24)	1.13 ± 0.33 (26)
21% O_2, 1.5% CO_2			
Glycolate	2.01 ± 0.28 (23)	1.08 ± 0.22 (23)	0.49 ± 0.18 (27)
Glycine	3.16 ± 0.69 (22)	2.21 ± 0.42 (21)	1.79 ± 0.42 (23)
Serine	2.48 ± 0.55 (19)	0.83 ± 0.39 (20)	0.88 ± 0.38 (21)

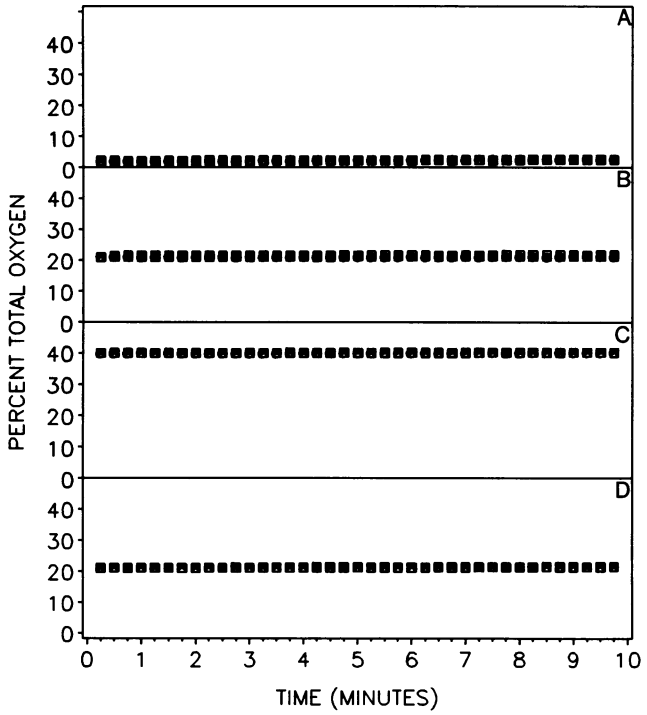

Figure 10. Time course of the change in total O_2 concentration in the leaf tissue chamber during ^{18}O-labeling of wheat seedlings. Each set of symbols represents data from a single 10 min experiment. Initial O_2 and CO_2 concentrations were, respectively: A, 2 and 0.035%; B, 21 and 0.035%; C, 40 and 0.035%; D, 21 and 1.5%.

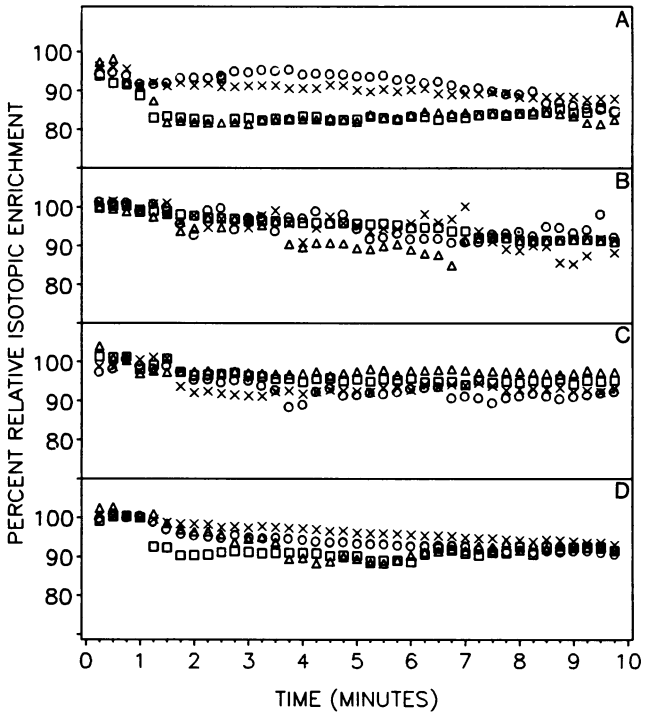

Figure 11. Time course of the change in percent relative $^{18}O_2$ enrichment in the leaf tissue chamber during ^{18}O-labeling of wheat seedlings. Each set of symbols represent data from a single 10 min experiment. Initial O_2 and CO_2 concentrations were, respectively: A, 2 and 0.035%; B, 21 and 0.035%; C, 40 and 0.035%; D, 21 and 1.5%.

DISCUSSION

The order of appearance of the ^{18}O-label was glycolate, then glycine, and then serine in all of the plants. This is in agreement with previous findings for the flow of carbon during photorespiration (28). It supports the hypothesis that C_4 plants have an active photorespiratory pathway, even though photorespiration is not manifested externally (6, 10).

Very few of the metabolite pools in any of the plants reached 100% relative ^{18}O-enrichment. For example, in only one instance did the glycolate pool become completely isotopically enriched. That occurred when wheat seedlings were exposed to 40% O_2 and 0.035% CO_2 (Fig. 1). Failure to reach full enrichment could be due to alternate means of glycolate synthesis, isotopic dilution at the site of ribulose bisphosphate carboxylase/oxygenase (Rubisco) during the transfer of the photosynthetic tissue from the labeling chamber to liquid N_2, or isotopic dilution during the actual experiment. The first possibility is ruled out. All well documented mechanisms of glycolate synthesis in photosynthetic tissue involve the incorporation of oxygen (5, 31). Again, glycolate from wheat seedlings exposed to 40% O_2 did reach full enrichment. It seems unlikely that a nonoxygen incorporating type of glycolate synthesis would operate when wheat was exposed to 2% and 21% O_2 but not 40% O_2.

The second possible cause of incomplete ^{18}O-enrichment of glycolate, isotopic dilution during the transfer of the tissue from the labeling chamber to the liquid N_2, may have had some effect on the ^{18}O-labeling kinetics. Wheat seedlings exposed to 21% O_2 and 0.035% CO_2 had a rate of glycolate synthesis of about 4.2 μmol/dm^2/min as determined from the kinetics of ^{18}O-incorporation into the glycolate pool (Table II). This value is less than 60% of the rates determined from the ^{18}O-labeling kinetics of glycine and serine. This series of experiments was the first to be done in this study. The time between the removal of the tissue from the labeling chamber to liquid N_2 tended to be longer compared to later experiments. The tissue was exposed longer to atmospheric $^{16}O_2$, and glycolate isolated from this tissue would have a lower percent [^{18}O]glycolate than originally present at the end of the labeling period. Berry *et al.* (5 and JA Berry, personal communication) reported similar findings and reached a similar conclusion. Isotopic dilution of $^{18}O_2$ during the transfer of the photosynthetic tissue is the most likely cause of the Jolivet-Tournier and Gerster report (15) that the maximum ^{18}O-enrichment in glycolate in maize was lower than the isotopic enrichment of either glycine or serine.

The glycine and serine pool, being metabolically downstream from glycolate and of a larger size (Table I), would be less affected by a long $^{16}O_2$ exposure time. Rate determination would tend to be higher from the two amino acids than from glycolate. These rates would be a better estimate of the true rate. Further discussions of photorespiratory rates in wheat seedlings in a 21% O_2 and 0.035% CO_2 atmosphere omit the data obtained through the ^{18}O-labeling kinetics of glycolate for this reason. The transfer times in later experiments with wheat and maize were shorter, consisting of only 1 s or less.

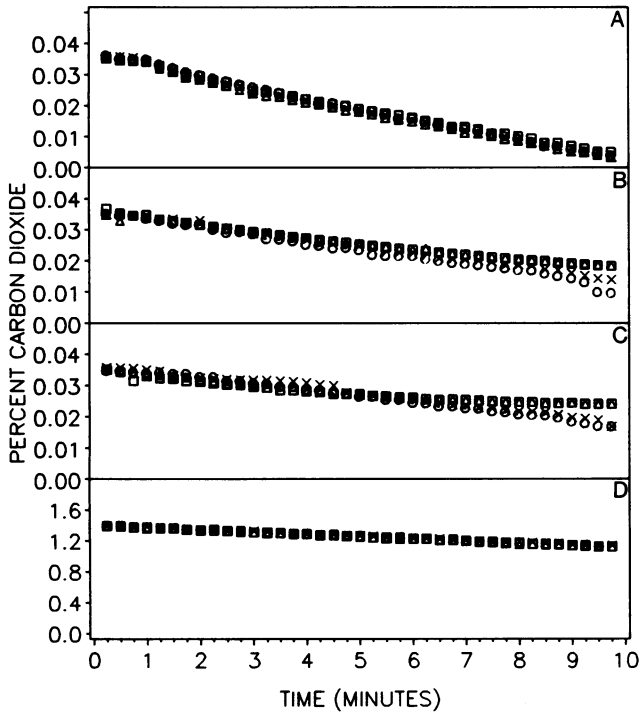

Figure 12. Time course of the decrease in CO_2 concentration in the leaf tissue chamber during ^{18}O-labeling of wheat seedlings. Each set of symbols represent data from a single 10 min experiment. Initial O_2 and CO_2 concentrations were, respectively: A, 2 and 0.035%; B, 21 and 0.035%; C, 40 and 0.035%; D, 21 and 1.5%.

Table II. *Rate of Glycolate Synthesis in Wheat during the First 30 s of Illumination as Calculated by ^{18}O-Labeling Kinetics of Glycolate, Glycine, and Serine*

Group	Rate	Standard Error	r^2
	$\mu mol/dm^2 \cdot min^{-1}$		
2% O_2, 0.035% CO_2 from			
Glycolate	1.02	0.04	0.97
Glycine	1.04	0.10	0.92
Serine	1.51	0.06	0.91
21% O_2, 0.035% CO_2 from			
Glycolate	4.19	0.43	0.89
Glycine	7.04	0.18	0.96
Serine	7.61	0.19	0.93
40% O_2, 0.035% CO_2 from			
Glycolate	10.31	1.02	0.93
Glycine	11.70	0.23	0.98
Serine	10.25	0.23	0.93
21% O_2, 1.5% CO_2 from			
Glycolate	ND[a]		
Glycine	ND		
Serine	ND		

[a] Not detected.

Under these shorter transfer times, there were only minor differences between the rates calculated from glycolate and those calculated from glycine and serine. Further discussion about these plants will be based upon data from all three intermediates.

The third possible cause can create problems in calculating the rate of photorespiration. If, during the experiment, photosynthetically generated $^{16}O_2$ significantly dilutes $^{18}O_2$, then the calculated rate of glycolate synthesis will be an underestimation of the true rate. However, Samish (25) calculated that the extent of the photorespiratory refixation of photosynthetically generated $^{16}O_2$ should cause only a negligible underestimation of the true photorespiratory rate. His calculations were based upon the assumption that the molecular percent $^{18}O_2$ of the air immediately surrounding the leaf remains constant throughout the labeling period. This involves constant renewal of the labeling gases, which was not done in this study because of the high cost of $^{18}O_2$. Thus, localized reduc-

Table III. *Rate of Glycolate Synthesis in Maize Seedlings during the First 30 s of Illumination as Calculated by ^{18}O-Labeling Kinetics of Glycolate, Glycine, and Serine*

Group	Rate	Standard Error	r^2
	$\mu mol/dm^2 \cdot min^{-1}$		
2% O_2, 0.035% CO_2 from			
Glycolate	0.21	0.03	0.66
Glycine	0.13	0.03	0.13
Serine	ND[a]		
21% O_2, 0.035% CO_2 from			
Glycolate	1.65	0.08	0.97
Glycine	1.99	0.04	0.97
Serine	2.69	0.13	0.75
40% O_2, 0.035% CO_2 from			
Glycolate	2.77	0.15	0.97
Glycine	3.21	0.16	0.85
Serine	3.98	0.10	0.91
21% O_2, 1.5% CO_2 from			
Glycolate	ND		
Glycine	ND		
Serine	ND		

[a] Not detected.

Table IV. *Rate of Glycolate Synthesis in 3-Month-Old Maize during the First 30 s of Illumination as Calculated by ^{18}O-Labeling Kinetics of Glycolate, Glycine, and Serine*

Group	Rate	Standard Error	r^2
	$\mu mol/dm^2 \cdot min^{-1}$		
2% O_2, 0.035% CO_2 from			
Glycolate	ND[a]		
Glycine	ND		
Serine	ND		
21% O_2, 0.035% CO_2 from			
Glycolate	0.61	0.04	0.91
Glycine	0.61	0.03	0.86
Serine	0.75	0.04	0.56
40% O_2, 0.035% CO_2 from			
Glycolate	1.27	0.10	0.90
Glycine	1.23	0.02	0.92
Serine	1.51	0.04	0.89
21% O_2, 1.5% CO_2 from			
Glycolate	ND		
Glycine	ND		
Serine	ND		

[a] Not determined.

Table V. *Net Photosynthetic Rate of Wheat Seedlings, Maize Seedlings, and 3-Month-Old Maize*
Number of samples is in parentheses.

Atmosphere	Wheat Seedlings	Maize Seedlings	Maize Plants
	$\mu mol\ CO_2\ consumed/dm^2 \cdot min^{-1}$		
2% O_2, 0.035% CO_2	23.3 ± 2.4 (8)	19.6 ± 1.9 (4)	20.8 ± 2.6 (5)
21% O_2, 0.035% CO_2	13.6 ± 2.0 (6)	19.0 ± 2.5 (4)	20.0 ± 2.8 (5)
40% O_2, 0.035% CO_2	9.1 ± 1.9 (7)	17.1 ± 2.3 (4)	19.0 ± 2.3 (6)
21% O_2, 1.5% CO_2	24.7 ± 2.8 (5)	23.0 ± 1.8 (4)	24.6 ± 3.3 (5)

tion in the molecular percent ^{18}O-enrichment would be expected, especially during prolonged labeling periods. This is the likely reason that in all experiments the molecular percent ^{18}O-enrichment of glycolate fell after having achieved a maximum (Fig. 1, 2, and 3). Berry *et al.* (5) reported a similar finding. In the experiments reported here it was assumed that there was no significant localized reduction in the $^{18}O_2$ enrichment within the initial 30 s. Thus, metabolic rates determined using data from these time points are considered reasonable estimates of the true rates.

Glycine and serine should have reached about 100% relative isotopic enrichment like glycolate (14, 29). Full enrichment was not reached in most cases. We attribute the failure of the two amino acids to reach full isotopic enrichment primarily to the failure of their precursor, glycolate, to become fully labeled. Most likely it was due to localized reduction in the molecular percent $^{18}O_2$.

The photorespiratory rates of wheat, maize seedlings, and 3-month-old maize as calculated by averaging the estimated rates from Table II, III, and IV, respectively, are given in Table VI. O_2 and CO_2 had a direct influence on glycolate synthesis rates in all plants. Increasing the O_2 concentration stimulated glycolate synthesis, whereas increasing the CO_2 depressed glycolate synthesis. This is in agreement with gas-exchange studies which have shown that O_2 stimulates photorespiration and high CO_2 depresses it (3, 8, 13).

Photorespiratory rates are typically expressed in terms of the percent photorespiratory CO_2 evolution of net photosynthetic CO_2 uptake (6, 36). Photorespiratory CO_2 evolution should be one-half the glycolate synthesis rates listed in Table V (21). The values for percent photorespiration of net photosynthesis are listed in Table VII.

Wheat seedlings exhibited a strong correlation between percent photorespiration of net photosynthesis and O_2 content. A 10-fold increase in O_2 concentration from 2 to 21% resulted in a 10-fold increase in the proportion of photorespiration of net photosynthesis. A further doubling of the O_2 concentration to 40% brought about a doubling of the relative rate of photorespiration.

The rate of glycolate synthesis in maize was calculated to be 0.66 $\mu mol/dm^2/min$ under normal atmospheric concentrations of O_2 and CO_2 (Table VI). The fact that this rate was influenced by O_2 and CO_2 concentration the way it was is evidence for C_4 photorespiration. However, even though 3-month-old maize photorespires, it does so at a considerably reduced rate compared to wheat. Glycolate production in maize was about 9% that of wheat on a leaf area basis under 21% O_2 and 0.035% CO_2. Maize had a photorespiratory rate of about 6% that of wheat relative to net photosynthesis under this atmosphere. These results are consistent with the proposal that C_4 plants lower their photorespiratory rates by the elevation of the CO_2 concentration at the site of Rubisco (6).

Many researchers have suggested that the developmental stage of a C_4 plant influences its photorespiratory activity (16, 18, 22, 23). Perchorowicz and Gibbs (23) found that the tissue age had a significant effect on the photorespiratory activity of maize. A nonprimary leaf from a young seedling had a photorespiratory metabolism intermediate between a mature maize leaf and a C_3 leaf. Our results support these findings. We found that under a 21% O_2 or 40% O_2 and 0.035% CO_2 atmosphere the rate of glycolate synthesis was 3 times higher in 9-d-old maize than 3-month-old maize (Table VI). The

Table VI. *Average Photorespiratory Rates of Wheat Seedlings, Maize Seedlings, and 3-Month-Old Maize*
Values were calculated from the ^{18}O-labeling kinetics of glycolate, glycine, and serine unless otherwise indicated.

Atmosphere	Average Photorespiratory Rates		
	Wheat seedlings	Maize seedlings	Maize plants
	$\mu mol/dm^2 \cdot min^{-1}$		
2% O_2, 0.035% CO_2	1.19	0.34[a]	ND[b]
21% O_2, 0.035% CO_2	7.33[c]	2.11	0.66
40% O_2, 0.035% CO_2	10.75	3.32	1.34
21% O_2, 1.5% CO_2	ND	ND	ND

[a] Calculated using only glycolate and glycine labeling kinetics. [b] Not determined. [c] Calculated using only glycine and serine labeling kinetics.

Table VII. *Percent Photorespiration of Net Photosynthesis of Wheat Seedlings, Maize Seedlings, and 3-Month-Old Maize Under Various O_2 and CO_2 Concentrations*
Calculations performed as explained in the text.

Atmosphere	Photorespiration of Net Photosynthesis		
	Wheat Seedlings	Maize Seedlings	Maize Plants
	%		
2% O_2, 0.035% CO_2	2.6	0.4	ND[a]
21% O_2, 0.035% CO_2	26.9	5.6	1.6
40% O_2, 0.035% CO_2	59.1	9.7	3.5
21% O_2, 1.5% CO_2	ND	ND	ND

[a] Not determined.

photorespiratory CO_2 evolution of net photosynthesis was 3 to 4 times higher in maize seedlings than in mature maize (Table VII). Nonetheless, photorespiration in maize seedlings was still significantly reduced compared to wheat. Glycolate production in maize seedlings was about 30% of that of wheat (Table VI). The percent photorespiration of net photosynthesis is 5 to 7 times higher in wheat than in maize seedlings (Table VII). Thus, even though the C_4 pathway is in operation in young C_4 leaf tissue, it has apparently not obtained its full activity relative to mature tissue.

It could be argued that the photorespiratory rates presented here may not reflect true steady state conditions, that the tissue within the closed chamber might be from the beginning of the labeling period CO_2-limited. If this is true then photorespiration would increase relative to photosynthesis. Also, the rates could be, in part, related to the dark/light transitions under which the experiments were conducted. However, it should be pointed out that the photorespiratory rate of wheat under 21% O_2 and 0.035% CO_2, 27% of net photosynthesis, is very close to the value determined by Gerbaud and André (12) for wheat under similar O_2 and CO_2 concentrations. They used $^{18}O_2$ to measure photorespiratory O_2 consumption. It can be calculated from their work that photorespiratory CO_2 evolution was about 30% of net photosynthesis. Their chamber was considerably larger than in our study and their experiments were carried out for 10 d. Neither CO_2 limitation nor dark/light transitions should have contributed to their results. Thus, it seems that neither CO_2 limitations nor dark/light transitions were a significant factor in our studies based upon the similarity between their results and our own. Other researchers who have used either postillumination CO_2 bursts, CO_2 efflux into CO_2-free air, or photosynthesis at 21% O_2 *versus* 2% O_2 assays typically measure photorespiratory rates 50% or greater of net photosynthesis (36). However, all of those methods inherently overestimate the true rate of photorespiration (6, 25). Some researchers have estimated photorespiratory rates to be between 10 and 15% of net photosynthesis using the differential uptake of $^{14}CO_2$ and $^{12}CO_2$ to determine gross photosynthesis and net photosynthesis (6, 17, 20). However, this method inherently underestimates photorespiration (25, 36). The photorespiratory rates of a representative C_3 plant as determined by this study falls between those underestimated and overestimated values.

The pool size of the three photorespiratory intermediates were unaffected by changes in either CO_2 or O_2 concentrations during the duration of the 10-min labeling period. This was true of wheat and maize seedlings and 3-month-old maize. It would be expected that at least the glycolate pool would have been influenced by the different O_2 and CO_2 concentrations used in this study if the regulation of the photorespiratory pathway was solely through the synthesis of phosphoglycolate via Rubisco. It may have been that the labeling period was not carried out long enough for changes in pool sizes to become evident. Alternate modes of biosynthesis of the three intermediates may have been a factor or the glycolate pathway may be regulated by other means in addition to glycolate synthesis. These other means could be such mechanisms as regulation of some of the enzymes of the photorespiratory pathway, or regulation of carbon flow between organelles involved in photorespiration.

In summary, the photorespiratory rate in wheat, under normal concentrations of CO_2 and O_2, was 27% of net photosynthesis. This is close to the estimated ratio between photorespiration and photosynthesis based upon *in vitro* affinities of Rubisco for CO_2 and O_2 (19). Furthermore, the rapid ^{18}O-labeling of glycolate, glycine, and serine in maize indicates that C_4 plants actively photorespire. The pathway is similar to C_3 plants, but it occurs at a considerably lower rate. Moreover, C_4 seedlings exhibit an intermediate photorespiratory rate relative to mature C_4 plants and C_3 plants. The method used in this study, ^{18}O-labeling of photorespiratory intermediates, is one of the few methods in which it is possible accurately to quantify photorespiration in intact C_3 and C_4 plant tissue.

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