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Oxygen and Carbon Dioxide Effects on the Pool Size of Some Photosynthetic and Photorespiratory Intermediates in Soybean (*Glycine max* [L.] Merr.)

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ABSTRACT

The levels of ribulose 1,5-bisphosphate (RuBP), 3-phosphoglyceric acid (PGA), glycolate, glycine, and serine were measured in soybean leaflets during photosynthesis in atmospheres ranging from 1 to 60% O_2 and from 0 to 500 microliters per liter CO_2.

The RuBP level remained constant as CO_2 concentration was decreased in atmospheres containing 20 or 60% O_2, but increased as CO_2 concentration was decreased in atmospheres containing 1% O_2. PGA levels decreased at CO_2 concentrations near or below the CO_2 compensation point under all O_2 concentrations. The glycolate pool at 300 microliters per liter CO_2 increased slightly with increasing O_2 concentration, but remained nearly constant at very low CO_2. The serine pool showed no measurable change over the range of CO_2 or O_2 concentrations tested. The glycine pool did not change significantly with varying CO_2 concentration but increased linearly with increasing O_2 concentration.

Measured RuBP levels indicate an RuBP concentration less than the estimated concentration of RuBP carboxylase/oxygenase active sites. The constant RuBP pool size in 20% O_2, however, indicates that RuBP level does not limit photosynthesis or photorespiration any more at 50 microliters per liter CO_2 than at 450 microliters per liter.

The kinetics of RuBP[2] carboxylase/oxygenase at saturating RuBP concentrations predict increased rates of oxygenation as CO_2 concentrations decrease and increased rates of carboxylation as O_2 concentrations decrease (20). The O_2 competition with CO_2 on RuBP carboxylase has been used to explain the direct inhibition of CO_2 fixation by O_2 (20, 26). Although the competitive effect of CO_2 on the oxygenation reaction and, therefore, on glycolate production is well known at CO_2 concentrations near or above 1,000 μl/l (2, 35), the extent of labeling in glycolate pathway intermediates (27, 34), the rate of carbon flux through the pathway (24), and the photorespiration rate (23) all remain nearly constant with decreasing CO_2 concentrations below 400 μl/l.

It has been suggested (16, 32) that a relatively constant glycolate synthesis rate and, therefore, photorespiration rate at CO_2 concentrations below normal air levels, may be due to reaction-limiting levels of RuBP at low CO_2 concentrations. This low, rate-limiting level of RuBP in the chloroplast could be due to increased rates of glycolate synthesis and export relative to CO_2 fixation at low CO_2 in the presence of O_2. Photosynthesizing chloroplasts have been shown to deplete intermediates of the Calvin cycle at low CO_2 concentrations (16, 32). In these studies it was also shown, however, that under conditions of low CO_2 in O_2, chloroplasts are capable of incorporating carbon from externally added sugar phosphate into Calvin cycle intermediates and further exporting glycolate.

Intact leaves apparently import carbon into the Calvin cycle and subsequently into the glycolate pathway during photosynthesis in O_2 at low CO_2 concentration. The specific radioactivity of evolved CO_2 (23) and of some glycolate pathway intermediates (24) remains less than that of the fed $^{14}CO_2$ at air levels of O_2 and CO_2 concentrations below 400 μl/l. If all photorespiratory CO_2 arises from the glycolate pathway, this result is most simply explained by a flux of stored carbohydrate into the Calvin cycle and glycolate pathway during photorespiratory CO_2 loss (23). Loss of ^{14}C from sucrose- and starch-containing fractions during illumination of C_3 leaves in CO_2-free O_2 has also been shown (21), and the specific activity of CO_2 evolved into CO_2-free air after periods of $^{14}CO_2$ fixation declines more rapidly than the rate of CO_2 evolution (7, 17). Chloroplasts in the intact leaf may thus be supplied with a carbon source to maintain the level of Calvin cycle intermediates even at low CO_2 concentration.

To determine whether or not the loss of photorespiratory CO_2 at CO_2 concentrations near or below the CO_2 compensation point is sufficient to reduce the level of Calvin cycle and glycolate pathway intermediates, we have measured the steady-state photosynthesis pool sizes of RuBP, PGA, glycolate, glycine, and serine. Pool size measurements were made at three O_2 concentrations and CO_2 concentrations varying from 0 to 500 μl/l. Net CO_2 exchange rate was measured prior to sampling in each atmosphere and each intermediate was assayed directly to avoid problems of ^{14}C labeling to known specific radioactivity.

MATERIALS AND METHODS

Plant Materials. Three soybeans (*Glycine max* [L.] Merr. c.v. Amsoy 71) were grown in 50-cm pots with nonsterile soil. After expansion of the first trifoliolate, pots were watered every 2nd day with 200 ml double strength, modified Hoagland solution (14). Micronutrients were added according to Evans *et al.* (9). Iron was supplied as the chelate of ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (Sigma) at the rate of 2 μmol Fe/liter nutrient solution.

Growth chamber conditions were 27 C days and 21 C nights, at 70–85% RH. Daylength was 16 h and photon flux density was 60 nE cm^{-2} s^{-1} (400–700 nm) from fluorescent and incandescent lights.

The center leaflet of the fourth trifoliolate, numbering from the primary leaves upward, was used during the 4th week after planting.

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[2] Abbreviations: RuBP: ribulose 1,5-bisphosphate; PGA: 3-phosphoglyceric acid; PBBO: 2-(4′-biphenyl)-6-phenyl-benzoxazole; BBS 3: Bio-Solv solubilizer (Beckman Instruments); Butyl PBD: 2-(4′-tertiary butylphenyl)-5-(4′-biphenylyl)-1,3,4-oxadiazole.

Gas Exchange. Net CO_2 exchange rates were measured with an open gas exchange system. A Beckman model IR-215A IR gas analyzer was used in the differential mode by calibration against appropriate upscale and downscale standards of CO_2 in N_2. Gas mixtures were made from pressurized gas cylinders of N_2, O_2, and 0.12% (v/v) CO_2 in N_2. O_2 content of the inlet gas mixture was determined with an O_2 electrode (Yellow Springs Instruments, Yellow Springs, Ohio) immersed in the inlet gas stream humidifier flask.

Photon flux density in the leaf chamber was 120 nE cm^{-2} s^{-1}, the gas flow rate was 0.033 liter s^{-1}, and the lower leaf surface temperature as measured with an iron-constantan thermocouple was 29 C.

The leaf cuvette enclosed the whole center leaflet on the trifoliolate after removal of the outside two leaflets. The petiole was passed through a gap in the Tygon O-ring which sealed the two halves of the cuvette. The area around the petiole was sealed with silicone stopcock grease before the two halves of the chamber were clamped together.

Preparation of Enzymes. RuBP carboxylase was partially purified from spinach by the method of Racker (30). Remaining ammonium sulfate in the RuBP-containing fraction was removed by passage through a Sephadex G-25 column (1.5 × 45 cm), which was equilibrated with 5 mM Tris-HCl (pH 8.0). The protein-containing fraction was made 2 mM in 2-mercaptoethanol, lyophilized, and stored desiccated at −20 C. The preparation was free of P-enolpyruvate carboxylase contamination, as shown by the absence of acid-stable ^{14}C after incubation of the enzyme solution with 20 mM P-enolpyruvate and 5 mM $NaH^{14}CO_3$ (pH 8.2).

Glycolic acid oxidase was prepared from pea leaves by the method of Kerr and Groves (15), except that the purification was not carried through the gel filtration step. The glycolic acid oxidase preparation was shown to be free of contaminating RuBP carboxylase in the standard RuBP assay described below.

Glyceraldehyde 3-P dehydrogenase and PGA P-kinase were purchased as the mixed enzymes from Sigma.

Metabolite Extraction. Leaves were allowed to photosynthesize under the desired atmosphere until a steady rate of net CO_2 exchange was attained (15–20 min). Leaves were killed 5 min after attaining a maximum rate of net CO_2 exchange in a given O_2/CO_2 atmosphere by first cutting the petiole, then removing the clamps holding the lower half of the cuvette and opening the chamber very slightly. The detached leaf was held with forceps, the lower half of the leaf cuvette was allowed to drop away and the leaf was very rapidly plunged into liquified Freon-12 which was held just above its freezing point (−156 C) by partial immersion in liquid N_2.

Frozen leaves were freeze-dried, then ground to a fine powder with mortar and pestle with a small amount of ground glass. Chl was determined (1) on a small amount of the ground powder and the remainder retained for metabolite extraction.

Samples for the analysis of RuBP and PGA were extracted with 4 ml 1 N formic acid. The mixture was sonicated for 30 s at 0 C, centrifuged, and the supernatant was added to a Dowex 1-Cl column (0.7 × 3 cm). The column was washed successively with water, 0.02 N HCl, then RuBP and PGA were eluted from the column with 5 ml 0.5 N HCl. The eluate was immediately frozen and lyophilized.

Samples for the analysis of glycolic acid, glycine, and serine were extracted with 4 ml 0.5 N $HClO_4$, sonicated, and centrifuged as were the RuBP-PGA samples. The residue was reextracted with a second portion of 0.5 N $HClO_4$ and combined extracts neutralized with 1 N $KHCO_3$ at 0 C. The precipitated $KClO_4$ was removed by centrifugation and the supernatant was passed through a Dowex 50-H column (0.7 × 3 cm). The column was washed with water, then amino acids were eluted with 2 N NH_4OH. The column wash fraction, which contained the glycolate, was made basic with NH_4OH, then both the wash fraction and the 2 N NH_4OH eluate were frozen and lyophilized.

Metabolite Analysis. The lyophilized 0.5 N HCl eluate from Dowex 1-Cl was dissolved in 100 mM Tris-HCl (pH 7.8), which was also 10 mM $MgCl_2$, and analyzed by the $^{14}CO_2$ method of Ellyard and Gibbs (8).

Acid-stable ^{14}C was determined by liquid scintillation spectrometry after dissolving the reaction residue remaining in scintillation vials in 0.1 ml water, adding 2 ml ethylene glycol monomethyl ether and 8 ml scintillation fluid (100 ml BBS 3, 0.5 g PBBO, and 8 g Butyl-PBD in 1 liter toluene).

A standard curve was run with each set of analyses. RuBP was standardized by the coupled enzyme method of Racker (30). The dibarium salt of RuBP was converted to the Tris salt by passing it through Dowex 50-H and neutralizing with Tris.

The RuBP assay conditions were adjusted so that RuBP standards (0–25 nmol RuBP per assay) were completely converted to acid-stable products within 30 min. Acid-stable ^{14}C per nmol RuBP added to the standard assays agreed with the specific radioactivity of the $NaH^{14}CO_3$, and aliquots of leaf extracts contained less than 20 nmol RuBP per assay. Recovery of known quantities of RuBP added to extracts of leaves which were frozen after 10 min of darkness gave recoveries of 72–76% through the extraction and analysis procedure. Data presented are not corrected for yield.

PGA was analyzed from an aliquot of the same column fraction which contained RuBP as described by Lowry and Passonneau (22).

Glycine and serine were analyzed in the lyophilized 2 N NH_4OH fractions from the Dowex 50-H columns. The amides in the residue were hydrolyzed with 2 N HCl at 100 C for 2 h. After evaporating to dryness twice, the residue was dissolved and neutralized with dilute NH_4OH. The neutralized samples were passed through a Dowex 1-Cl column (0.7 × 2 cm) to remove acidic amino acids (includes aspartate, glutamate, glutamine, and asparagine originally in the leaf). The column through-puts were frozen and lyophilized then dissolved in 0.1 ml water, and an aliquot (0.025 ml) was spotted on a thin layer cellulose plate (0.5 mm) along with 1 nmol each of [^{14}C]glycine and [^{14}C]serine (5 mCi/mmol). The amino acids were separated by two-dimensional TLC using the solvent systems of Haworth and Heathcote (10) and developing the plates twice in the second solvent system.

Spots were located by autoradiography then removed by covering the outlined area with stripping mixture as described by Redgwell *et al.* (31). To each of the removed spots in acid-washed tubes 0.5 ml borate buffer was added (6). The buffer solution was evaporated to dryness under vacuum over NaOH and H_2SO_4 to remove traces of ammonia. Ninhydrin determinations were done on the dried residue by the Moore and Stein (25) method. To the dried residue 0.5 ml water and 2 ml ninhydrin-hydrindantin reagent was added (25).

Glycolic acid was determined in the acid fraction from 0.5 N $HClO_4$ extracted leaf residue. The neutralized, lyophilized residue from the through-puts of the Dowex 50-H columns was redissolved in 1 ml 50 mM Hepes-K buffer (pH 8.3). Glycolic acid was determined by a method similar to that described by Laing (19) except that the Hepes-K buffer was used instead of pyro-P and the total reaction volume was reduced to 1.1 ml.

RESULTS

CO_2 Exchange Rates. Net CO_2 exchange rates at 300 μl/l CO_2 averaged 35.5 nmol CO_2 s^{-1} mg Chl^{-1} at 1% O_2, 25.0 nmol CO_2 s^{-1} mg Chl^{-1} at 20% O_2, and 6.5 nmol CO_2 s^{-1} mg Chl^{-1} at 60% O_2. The CO_2 compensation point increased with increasing O_2 in the atmosphere from 2 μl/l at 1% O_2 to 60 and 170 μl/l at 20 and 60% O_2, respectively.

Steady-state Photosynthesis Level of RuBP. At 20% O_2, the RuBP level during steady-state photosynthesis did not change as

CO_2 concentration changed over the CO_2 concentration range tested, maintaining a level of 15 nmol RuBP mg Chl^{-1} (Fig. 1). RuBP levels of 7 nmol mg Chl^{-1} during photosynthesis by isolated chloroplasts have been reported (3, 16), and 20 nmol mg Chl^{-1} were measured in leaf discs during photosynthesis in air (18).

Levels of RuBP at 60% O_2 were somewhat lower than those obtained at 20% O_2 (9 nmol mg Chl^{-1} at 300 μl/l CO_2) and decreased slightly at low CO_2 concentrations. RuBP level in leaves photosynthesizing in atmospheres containing 1% O_2 and from 300 to 500 μl/l CO_2, was equal to the level in leaves at 20% O_2. The steady-state RuBP level in leaves from atmospheres containing 1% O_2 and less than 300 μl/l CO_2, increased to 60 nmol mg Chl^{-1} as CO_2 concentration was decreased to 50 μl/l.

Steady-state Photosynthesis Level of PGA. At 300 μl/l CO_2, PGA level was 125–140 nmol mg Chl^{-1} and was affected only slightly by O_2 concentration (Fig. 2). In the presence of either 20 or 60% O_2, the steady-state level of PGA during photosynthesis decreased as the CO_2 concentration was lowered to the compensation point and below in the case of 60% O_2. The PGA level in 1% O_2 also decreased as the CO_2 concentration was decreased to 50 μl/l, although to a much lesser extent than in the presence of higher O_2 concentrations.

Steady-state Photosynthesis Level of Glycolic acid. Glycolic acid levels in nmol mg Chl^{-1} as a function of CO_2 concentration at the three O_2 levels tested are shown in Figure 3. At 20 and 60% O_2, the glycolate pool size was slightly less in CO_2-free atmospheres than in the presence of CO_2. No consistent CO_2 interaction was observed in 1% O_2, although a significant glycolate pool was present, as the glycolate pool size after 10 min of darkness in air was less than one-half that measured in the light at 1% O_2.

Steady-state Photosynthesis Levels of Glycine and Serine. The effect of CO_2 concentration on glycine and serine levels during photosynthesis at 1, 20, and 60% O_2 are shown in Figures 4 and 5, respectively. Neither pool size showed interaction with CO_2 concentration over the CO_2 concentration range tested at 1 or 60% O_2. An increase in the pool size of both glycine and serine with decreasing CO_2 concentration was indicated by the regression lines of glycine or serine *versus* CO_2 concentration at 20% O_2.

The glycine pool size during steady-state photosynthesis increased with increasing O_2 concentration at all CO_2 concentrations tested. The serine pool size was independent of O_2 concentration. The pool sizes of glycine and serine in CO_2-free air at 1, 20, and 60% O_2, along with the rate of net CO_2 evolution into CO_2-free air is shown in Figure 6.

The level of both glycine and serine during photosynthesis was variable. Although some of this variation arises from the analytical procedure, the problem of variable pool size was also encountered by Canvin *et al.* (4) in determining the specific radioactivity of glycine and serine in $^{14}CO_2$-fed sunflower leaves. A major part of this variability is apparently due to plant to plant differences and is inherent in single leaf determinations.

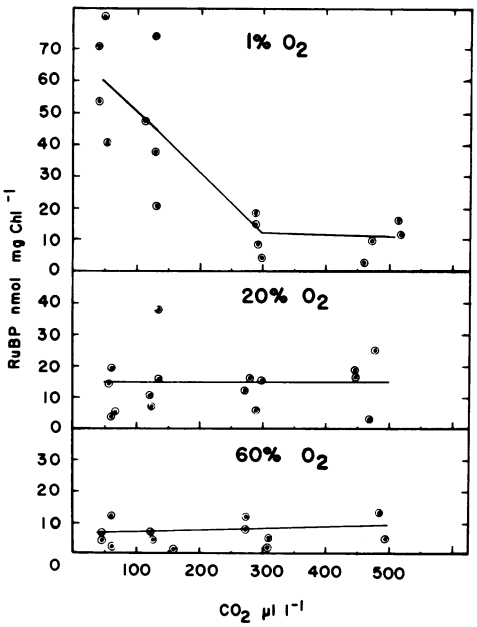

FIG. 1. Steady-state photosynthesis level of RuBP *versus* CO_2 concentration (average in leaf chamber) at three O_2 concentrations. Points represent single leaflets from individual plants in two experiments done in duplicate.

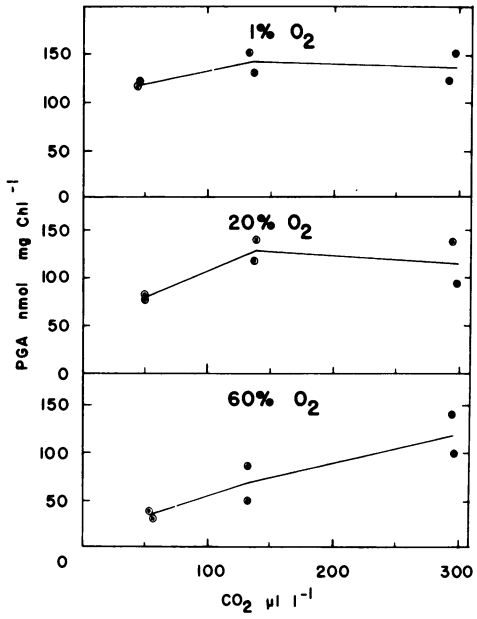

FIG. 2. Steady-state photosynthesis level of PGA *versus* CO_2 concentration at three O_2 concentrations. Experimental conditions were as described in Figure 1, with one experiment done in duplicate.

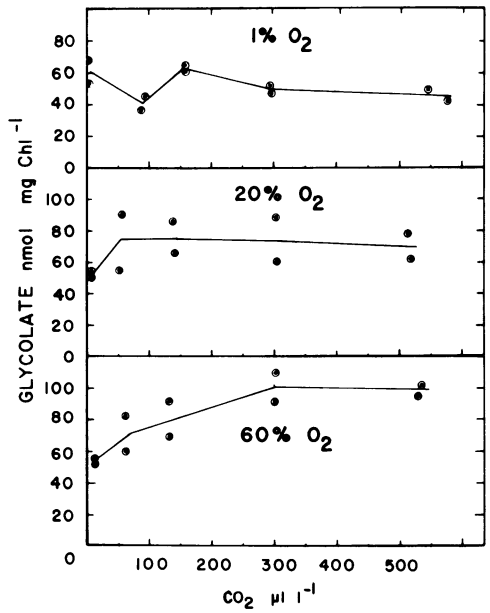

FIG. 3. Steady-state photosynthesis level of glycolate *versus* CO_2 concentration at three O_2 concentrations. Points represent single leaves from individual plants in two replications done in a single experiment.

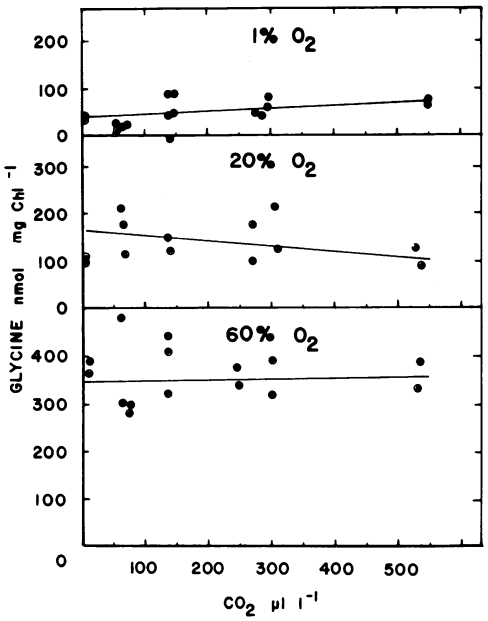

FIG. 4. Steady-state photosynthesis level of glycine *versus* CO_2 concentration at three O_2 concentrations. Points represent single leaves from individual plants. Two experiments with two replicates at each CO_2 concentration were done. One experiment was done using CO_2 concentrations from 0 to 450 µl/l and a second from 50 to 300 µl/l. Lines through data points were fit by linear regression.

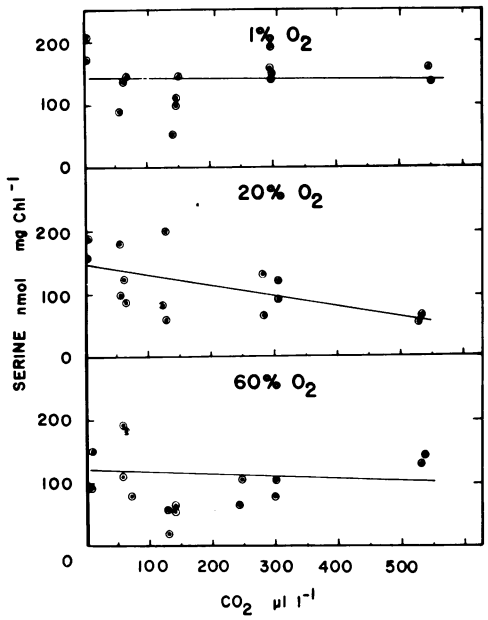

FIG. 5. Steady-state photosynthesis level of serine *versus* CO_2 concentration at three O_2 concentrations. Serine was determined from same gas exchange experiments as was glycine. Lines through data points were fit by linear regression.

DISCUSSION

The activity of the glycine decarboxylase-serine hydroxymethyl transferase system has been estimated at a minimum of twice that necessary for the release of photorespiratory CO_2 at 21% O_2 (36). The rate of photorespiratory CO_2 evolution should be dependent upon the glycine concentration at the active site of the decarboxylating enzyme. Consistent with a large decarboxylating capacity, both the glycine pool size and the rate of CO_2 evolution into CO_2-free air increase with increasing O_2 concentration (Fig. 6). The glycine pool remaining in 1% O_2, when very little CO_2 evolution into CO_2-free air can be measured, is apparently unavailable for decarboxylation. Above this residual pool (25–30% of the pool in 21% O_2), the glycine pool is directly related to the rate of CO_2 evolution into CO_2-free air. Similar estimates of the amount of glycine available for decarboxylation have been obtained from soybean leaf cells (33) and sunflower leaves (4).

If the correlation between the glycine pool size and the rate of CO_2 release into CO_2-free air holds in the presence of CO_2, only a very slight increase in carbon flow through the glycolate pathway is indicated as the CO_2 concentration approaches zero. This interpretation and the [^{14}C]glycine levels measured during photosynthesis at high and low CO_2 concentrations by others (29, 34), agree with the observation (23) that little or no increase in the evolution of photorespiratory CO_2 occurs with decreasing CO_2 concentration below 400 µl/l.

RuBP is apparently compartmentalized within the chloroplast (3, 11) so that whole leaf RuBP levels should also represent chloroplast levels. Jensen and Bahr (13) have estimated the chloroplast concentration of RuBP carboxylase/oxygenase active sites to be about 3.5 mM. The RuBP levels measured in this study may be similarly converted to concentrations using a value of 25 µl stroma volume mg Chl^{-1} (12). The measured RuBP level of 15 nmol mg Chl^{-1} in 20% O_2, when corrected for yield, represents a chloroplast concentration of 0.8 mM. Whereas the enzyme is not substrate-saturated and the rates of carboxylation and oxygenation are directly dependent upon RuBP concentration, the constant RuBP pool size observed in 20 and 60% O_2 (Fig. 1) does not support the suggestions (16, 32) that the RuBP pool in intact leaves at very low CO_2 concentrations might limit glycolate synthesis more than at atmospheric CO_2 concentration.

At low O_2 and decreasing CO_2, the rate of both carboxylation and oxygenation caused a large increase in the RuBP pool size. A similar pattern has been observed in isolated spinach leaf cells (5), although the RuBP pool size also showed a slight increase at very low CO_2 concentration in 21% O_2.

The decreasing PGA pool at CO_2 concentrations at or below the CO_2 compensation concentration indicates that the loss of photorespiratory CO_2 does place a drain on at least some intermediates of the photosynthetic carbon reduction cycle even though this carbon loss is not expressed as a net decrease in the RuBP pool size.

Neither the glycolate nor serine pools increase greatly under conditions which cause an increase in the glycine pool and flux through the glycine decarboxylation reaction. The small increase in the glycolate pool with increasing O_2 at atmospheric CO_2 concentration may be explained by the O_2 dependence of both

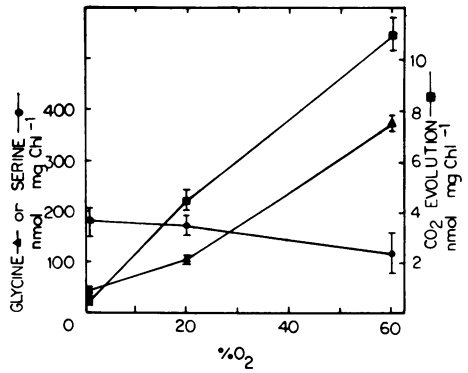

FIG. 6. Pool sizes of glycine and serine *versus* O_2 concentration in CO_2 free air and steady-state rate of CO_2 evolution into CO_2-free air *versus* O_2 concentration. Glycine and serine data were taken from Figures 4 and 5, respectively.

glycolate synthesis (2) and oxidation (15), while the constant serine pool indicates that multiple serine pools exist. Servaites and Ogren (33) also found evidence for a serine pool in soybean leaf cells which is not available for metabolism through the glycolate pathway. The constant pool size under differing O_2 concentrations is also consistent with the suggestion of several authors (28, 29, 33) that more than one route of serine synthesis occurs in photosynthesizing cells.

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