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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WILLIAM D. Hitz and CECIL R. STEWART
Department of Botany and Plant Pathology, Iowa State University, Ames, Iowa 50011

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% O2 and from 0 to 500 micromolars per liter CO2.

The RuBP level remained constant as CO2 concentration was decreased in atmospheres containing 20 or 60% O2, but increased as CO2 concentration was decreased in atmospheres containing 1% O2. PGA levels decreased at CO2 concentrations near or below the CO2 compensation point under all O2 concentrations. The glycolate pool at 300 micromolars per liter CO2 increased slightly with increasing O2 concentration, but remained nearly constant at very low CO2. The serine pool showed no measurable change over the range of CO2 or O2 concentrations tested. The glycine pool did not change significantly with varying CO2 concentration but increased linearly with increasing O2 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% O2, however, indicates that RuBP level does not limit photosynthesis or photorespiration any more at 50 micromolars per liter CO2 than at 450 micromolars per liter.

The kinetics of RuBP2 carboxylase/oxygenase at saturating RuBP concentrations predict increased rates of oxygenation as CO2 concentrations decrease and increased rates of carboxylation as O2 concentrations decrease (20). The O2 competition with CO2 on RuBP carboxylase has been used to explain the direct inhibition of CO2 fixation by O2 (20, 26). Although the competitive effect of CO2 on the oxygenation reaction and, therefore, on glycolate production is well known at CO2 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 photosynthesis rate (23) all remain nearly constant with decreasing CO2 concentrations below 400 µl/l.

It has been suggested (16, 32) that a relatively constant glycolate synthesis rate and, therefore, photorespiration at CO2 concentrations below normal air levels, may be due to reaction-limiting levels of RuBP at low CO2 concentrations. This low, rate-limiting level of RuBP in the chloroplast could be due to increased rates of glycolate synthesis and export relative to CO2 fixation at low CO2 in the presence of O2. Photosynthesizing chloroplasts have been shown to deplete intermediates of the Calvin cycle at low CO2 concentrations (16, 32). In these studies it was also shown, however, that under conditions of low CO2 in O2, 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 O2 at low CO2 concentration. The specific radioactivity of evolved CO2 (23) and of some glycolate pathway intermediates (24) remains less than that of the fed 14CO2 at air levels of O2 and CO2 concentrations below 400 µl/l. If all photosynthetic CO2 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 CO2 loss (23). Loss of 14C from sucrose- and starch-containing fractions during illumination of C3 leaves in CO2-free O2 has also been shown (21), and the specific activity of CO2 evolved into CO2-free air after periods of 14CO2 fixation declines more rapidly than the rate of CO2 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 CO2 concentration.

To determine whether or not the loss of photorespiratory CO2 at CO2 concentrations near or below the CO2 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 O2 concentrations and CO2 concentrations varying from 0 to 500 µl/l. Net CO2 exchange rate was measured prior to sampling in each atmosphere and each intermediate was assayed directly to avoid problems of 14C 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-hydroxyphenyl)acetic 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 µE 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.
Gas Exchange. Net CO₂ 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 CO₂ in N₂. Gas mixtures were made from pressurized gas cylinders of appropriate upscale and downscale standards and the inlet gas mixture was determined with an O₂ 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 x 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 E-nolpyruvate carboxylase contamination, as shown by the absence of acid-stable ¹⁴C after incubation of the enzyme solution with 20 mM E-nolpyruvate and 5 mM NaH¹⁴CO₃ (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 state of net CO₂ exchange was attained (15-20 min). Leaves were killed 5 min after attaining a maximum rate of net CO₂ exchange in a given O₂/CO₂ 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 low pressure at the cuvette was added to the chamber, 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 N₂.

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-C1 column (0.7 x 3 cm). The column was washed successively with water, 0.02 N HCl, then RuBP and PGA were eluted from the column with 0.5 N HCl. The eluate was immediately frozen and lyophilized.

Samples for the analysis of glycylcic acid, glycine, and serine were extracted with 4 ml 0.5 N HClO₄, sonicated, and centrifuged as were the RuBP-PGA samples. The residue was reextracted with a second portion of 0.5 N HClO₄, and combined extracts neutralized with 1 N KHCO₃ at 0 C. The precipitated KClO₄ was removed by centrifugation and the supernatant was passed through a Dowex 50-H column (0.7 x 3 cm). The column was washed with water, then amino acids were eluted with 2 N NH₄OH. The column wash fraction, which contained the glycylate, was made basic with NH₃·H₂O, then both the wash fraction and the 2 N NH₄OH 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 MgCl₂, and analyzed by the ¹⁴CO₂ method of Ellyard and Gibbs (8).

Acid-stable ¹⁴C 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 PBB0, 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 ¹⁴C per nmol RuBP added to the standard assays agreed with the specific radioactivity of the NaH¹⁴CO₃, 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 NH₄OH 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 NH₄OH. The neutralized samples were passed through a Dowex 1-C1 column (0.7 x 2 cm) to remove acidic amino acids (includes aspartate, glutamate, glutamine, and asparagine originally in the leaf). The column through-puics 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 [¹⁴C]glycine and [¹⁴C]serine (5 mCi/ mmol). The amino acids were separated by two-dimensional TLC using the solvent systems of Hawtho 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 H₂SO₄ 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 HClO₄, extracted leaf residue. The neutralized, lyophilized residue from the through-puics 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

CO₂ Exchange Rates. Net CO₂ exchange rates at 300 μl/l CO₂ averaged 35.5 nmol CO₂ s⁻¹ mg Chl⁻¹ at 1% O₂, 25.0 nmol CO₂ s⁻¹ mg Chl⁻¹ at 20% O₂, and 6.5 nmol CO₂ s⁻¹ mg Chl⁻¹ at 60% O₂. The CO₂ compensation point increased with increasing O₂ in the atmosphere from 2 μl/l at 1% O₂ to 60 and 170 μl/l at 20 and 60% O₂, respectively.

Steady-state Photosynthesis Level of RuBP. At 20% O₂, the RuBP level during steady-state photosynthesis did not change as

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CO₂ concentration changed over the CO₂ 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% O₂ were somewhat lower than those obtained at 20% O₂ (9 nmol mg Chl⁻¹ at 300 µl/l CO₂) and decreased slightly at low CO₂ concentrations. RuBP level in leaves photosynthesizing in atmospheres containing 1% O₂ and from 300 to 500 µl/l CO₂, was equal to the level in leaves at 20% O₂. The steady-state RuBP level in leaves from atmospheres containing 1% O₂ and less than 300 µl/l CO₂ increased to 60 nmol mg Chl⁻¹ as CO₂ concentration was decreased to 50 µl/l.

**Steady-state Photosynthesis Level of PGA.** At 300 µl/l CO₂, PGA level was 125-140 nmol mg Chl⁻¹ and was affected only slightly by O₂ concentration (Fig. 2). In the presence of either 20 or 60% O₂, the steady-state level of PGA during photosynthesis decreased as the CO₂ concentration was lowered to the compensation point and below in the case of 60% O₂. The PGA level in 1% O₂ also decreased as the CO₂ concentration was decreased to 50 µl/l, although to a much lesser extent than in the presence of higher O₂ concentrations.

**Steady-state Photosynthesis Level of Glycolic acid.** Glycolic acid levels in nmol mg Chl⁻¹ as a function of CO₂ concentration at the three O₂ levels tested are shown in Figure 3. At 20 and 60% O₂, the glycolic acid pool size was slightly less in CO₂-free atmospheres than in the presence of CO₂. No consistent CO₂ interaction was observed in 1% O₂, although a significant glycolate pool size 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% O₂.

**Steady-state Photosynthesis Levels of Glycine and Serine.** The effect of CO₂ concentration on glycine and serine levels during photosynthesis at 1, 20, and 60% O₂ are shown in Figures 4 and 5, respectively. Neither pool size showed interaction with CO₂ concentration over the CO₂ concentration range tested at 1 or 60% O₂. An increase in the pool size of both glycine and serine with decreasing CO₂ concentration was indicated by the regression lines of glycine or serine versus CO₂ concentration at 20% O₂.

The glycine pool size during steady-state photosynthesis increased with increasing O₂ concentration at all CO₂ concentrations tested. The serine pool size was independent of O₂ concentration. The pool sizes of glycine and serine in CO₂-free air at 1, 20, and 60% O₂, along with the rate of net CO₂ evolution into CO₂-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 ¹⁴C O₂-fed sunflower leaves. A major part of this variability is apparently due to plant to plant differences and is inherent in single leaf determinations.
The activity of the glycine decarboxylase-serine hydroxymethyl transferase system has been estimated at a minimum of twice that necessary for the release of photosynthetic CO₂ at 21% O₂ (36). The rate of photosynthetic CO₂ 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 CO₂ evolution into CO₂-free air increase with increasing O₂ concentration (Fig. 6). The glycine pool remaining in 1% O₂, when very little CO₂ evolution into CO₂-free air can be measured, is apparently unavailable for decarboxylation. Above this residual pool (25–30% of the pool in 21% O₂), the glycine pool is directly related to the rate of CO₂ evolution into CO₂-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 CO₂ release into CO₂-free air holds in the presence of CO₂, only a very slight increase in carbon flow through the glycolate pathway is indicated as the CO₂ concentration approaches zero. This interpretation and the [14C]glycine levels measured during photosynthesis at high and low CO₂ concentrations by others (29, 34), agree with the observation (23) that little or no increase in the evolution of photosynthetic CO₂ occurs with decreasing CO₂ 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 μm stroma volume mg Chl⁻¹ (12). The measured RuBP level of 15 nmol mg Chl⁻¹ in 20% O₂, 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% O₂ (Fig. 1) does not support the suggestions (16, 32) that the RuBP pool in intact leaves at very low CO₂ concentrations might limit glycolate synthesis more than at atmospheric CO₂ concentration.

At low O₂ and decreasing CO₂, 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 CO₂ concentration in 21% O₂.

The decreasing PGA pool at CO₂ concentrations at or below the CO₂ compensation concentration indicates that the loss of photosynthetic CO₂ 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 glycylate pool with increasing O₂ at atmospheric CO₂ concentration may be explained by the O₂ dependence of both

**DISCUSSION**

The steady-state photosynthesis level of glycine versus CO₂ concentration at three O₂ concentrations. Points represent single leaves from individual plants. Two experiments with two replicates at each CO₂ concentration were done. One experiment was done using CO₂ 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.

The small increase in the glycylate pool with increasing O₂ at atmospheric CO₂ concentration may be explained by the O₂ dependence of both
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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