Chemical Inhibition of the Glycolate Pathway in Soybean Leaf Cells

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ABSTRACT

Isolated soybean (Glycine max [L.] Merr.) leaf cells were treated with three inhibitors of the glycolate pathway in order to evaluate the potential of such inhibitors for increasing photosynthetic efficiency. Preincubation of cells under acid conditions in α-hydroxypridinemethanesulfonic acid increased 14CO2 incorporation into glycologate, but severely inhibited photosynthesis. Isonicotinic acid hydrazide (INH) increased the incorporation of 14CO2 into glycologate and reduced label in serine, glycerate, and starch. Butyl 2-hydroxy-3-butynoate (BHB) completely and irreversibly inhibited glycologate oxidase and increased the accumulation of 14C into glycologate. Concomitant with glycologate accumulation was the reduction of label in serine, glycologate, and starch, and the elimination of label in glycologate. The inhibitors INH and BHB did not eliminate serine synthesis, suggesting that some serine is synthesized by an alternate pathway. The per cent incorporation of 14CO2 into glycologate by BHB-treated cells or glycologate by INH-treated cells was determined by the O2/CO2 ratio present during assay. Photosynthesis rate was not affected by INH or BHB in the absence of O2, but these compounds increased the O2 inhibition of photosynthesis. This finding suggests that the function of the photorespiratory pathway is to recycle glycologate carbon back into the Calvin cycle, so if glycologate metabolism is inhibited, the Calvin cycle intermediates become depleted and photosynthesis is decreased. Thus, chemicals which inhibit glycologate metabolism do not reduce photorespiration and increase photosynthetic efficiency, but rather exacerbate the problem of photorespiration.

Photorespiration is a light-induced uptake of O2 and release of CO2 that occurs in plants which fix atmospheric CO2 directly by RuDP-carboxylase. It is stimulated by high O2 and low CO2 concentrations, inhibited in low O2 and high CO2 environments, and causes the characteristic CO2 compensation concentration of about 40 μl CO2/l observed in leaves of C3 plants in 21% O2 at 25 C. Because photorespiration reduces the efficiency of photosynthesis with no demonstrable benefit, some investigators consider it to be an unnecessary and wasteful process (5, 13, 14, 33). Growth of C3 plants in high CO2 or low O2 relative to atmospheric concentrations, conditions which inhibit photorespiration, greatly decreases dry matter accumulation (1, 3). Thus, the elimination of photorespiration by genetic or chemical means is thought to be a promising approach for increasing the productivity of C3 plants. Methods suggested for controlling photorespiration include reducing the synthesis of glycologic acid, a photorespiratory intermediate, by altering the kinetic properties of RuDP carboxylase (13, 14) or by inhibiting an undescribed pathway of synthesis (33). Alternatively, photorespiration may be reduced by applying inhibitors of glycologate metabolism so that glycologate, once formed, cannot be oxidized to CO2 (29).

The substrate of photorespiratory CO2 release is still uncertain, although it is most likely either glycologate (24) or glyoxylate (31). The use of specific inhibitors of glycologate metabolism may help to resolve this question, and provide additional evidence on the sequence of carbon flow through the photorespiratory pathway. The response of cell photosynthesis to these inhibitors will also provide information on their suitability for use as plant growth regulators for increasing photosynthetic efficiency of crop plants.

MATERIALS AND METHODS

α-Hydroxypridinemethanesulfonic acid (Fluka®) was recrystallized twice before use. Isonicotinic acid hydrazide (Sigma) was used without further purification. Butyl 2-hydroxy-3-butyrate was synthesized by the procedure of Verny and Vessiere (25), twice distilled at 102 C, and stored at 5 C under N2. Dilution of inhibitors to the appropriate concentration was done in the assay medium immediately before use.

Isolation of soybean (Glycine max [L.] Merr. cv. Wayne) leaf mesophyll cells, assay of photosynthesis, and separation of labeled products were done as previously described (21). The temperature during assay was 25 C and illumination was 12 klux. In the presence of inhibitors, photosynthesis and the accumulation of glycologate and glycine were linear during the assay period. CO2 compensation concentration values were estimated from the bicarbonate response curve of photosynthesis (20) and also measured directly with an IR gas analyzer in a closed system.

RESULTS

HPMS. α-Hydroxypridinemethanesulfonic acid has been used successfully to inhibit glycologate oxidase in higher plant tissue (4, 28), causing an accumulation of glycologate. Incubation of soybean leaf cells in 10 μm HPMS at pH 7.8 had little effect on glycologate accumulation or on the rate of photosynthesis (data not shown). HPMS is negatively charged at neutral pH

1 Present address: Department of Agronomy, University of Wisconsin, Madison, Wisconsin 53706
2 Abbreviations: RuDP: ribulose 1,5-diphosphate; HPMS: α-hydroxypridinemethanesulfonic acid; INH: isonicotinic acid hydrazide; BHB: butyl 2-hydroxy-3-butyrate; Γ: CO2 compensation concentration.
3 Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.
and presumably did not penetrate into the cell. Preincubation of cells in HPMS under acid conditions (pH 3.8) allowed HPMS to penetrate the cell membrane and inhibit glycolate oxidase, resulting in glycolate accumulation. Cells photosynthesizing in 100% O₂, 2 mm bicarbonate, and 4 mm HPMS accumulated about 50% of the total [14C] fixed as glycolate, but photosynthesis was inhibited by 95% compared to the control without HPMS (Fig. 1). HPMS also severely inhibited photosynthesis in leaves of tobacco (19) and soybean (22) and in isolated spinach chloroplasts (12). In the latter case, inhibition of photosynthesis by HPMS was described as being nonspecific. Photosynthesis in soybean cells was inhibited to the same extent in 2% or 100% O₂, and inhibition was noncompetitive with respect to bicarbonate concentration (data not shown).

**INH.** Isonicotinic acid hydrazide inhibited the conversion of glycine to serine in *Chlorella* (16, 17) and in leaves of higher plants (2, 11). Incubation of soybean cells in INH stimulated incorporation of [14C] into glycine, and decreased incorporation into serine and insoluble material (starch) (Fig. 2) and glycerate (not shown). Incorporation of label into glycine was maximal at 4 mm INH. INH did not stimulate glycolate accumulation, as was observed in *Chlorella* (16, 17). Cell photosynthesis in 100% O₂, 0.75 mm bicarbonate and 4 mm INH was inhibited about 40%. Addition of 2 mm NH₄⁺ did not increase incorporation of [14C] into glycine, but did stimulate photosynthesis by 30% (data not shown). Generally, the incorporation of [14C] into products of photosynthesis other than glycine, serine, and insolubles was not altered by INH.

Incorporation of [14C] into glycine and serine in the presence of INH was also determined as a function of bicarbonate concentration under 2% and 21% O₂. At 21% O₂ the percentage incorporation of [14C] into glycine was about 40% at 0.5 mm bicarbonate, and decreased to the level of the 2% O₂ control at 10 mm bicarbonate (Fig. 3). The absolute rate of [14C]glycine synthesis reached a maximum at 1 mm bicarbonate and then decreased with increasing bicarbonate concentration, approaching the rate of synthesis in the 2% O₂ control (Fig. 3). The percentage incorporation of [14C] into serine and the total [14C]serine pool size were reduced by INH, particularly at low bicarbonate levels and under 21% O₂ (Fig. 3). The incorporation of label into serine was similar in 2% and 21% O₂ with INH and in 2% O₂ without INH. At 2 mm bicarbonate in 21% O₂ without INH, serine accumulation was about twice as great as in 21% O₂ with INH, but was reduced to the 2% O₂ level at 10 mm bicarbonate. Because serine synthesis in the presence of INH is independent of O₂ concentration and is not reduced by high bicarbonate concentration, it appears that about 50% of the serine synthesized under 21% O₂ and 2 mm bicarbonate came from sources other than glycine. This also suggests that INH completely inhibited glycine conversion to serine.

**BHB.** 2-Hydroxy-3-butoynoate is an irreversible inhibitor of bacterial lactate oxidase (26) and higher plant glycolate oxidase (7). The butyl ester of 2-hydroxy-3-butoynoic acid was found to penetrate readily soybean leaf cells at assay pH and irreversibly inhibit glycolate oxidase. Figure 4 shows the effect of increasing BHB concentration on the per cent of [14C] incorporated into glycolate, glycine, and insolubles. Maximum per cent incorporation of [14C] into glycolate occurred when cells were preincubated in 0.5 mm BHB. Since BHB is an irreversible inhibitor of glycolic oxidase, it was not necessary to add it to the assay medium. Concomitant with glycolate accumulation was the almost complete inhibition of glycine synthesis. This inhibition is particularly evident in the presence of INH and indicates that not only is glycine a product of glycolate metabolism, but that all of the labeled glycine is derived from glycolate. It does not appear that glycine is synthesized from serine. The percentage of [14C] fixed into glycolate in the presence of BHB was always about twice the amount of glycine synthesized in the presence of INH. INH had no effect on the percentage incorporation of [14C] into glycolate by BHB-treated cells (Fig. 4).

INH had been reported previously to inhibit glycolate synthesis in tobacco leaf discs in the presence of HPMS (32). In the presence of BHB, the percentage of [14C] incorporated into glycolate increased with increasing O₂ concentration to a maximum of 90% at 100% O₂ and 0.5 mm bicarbonate (Fig. 5). Percentage of [14C] incorporated into glycolate decreased with increasing bicarbonate concentration. In 21% O₂, [14C]glycolate synthesis was highest at 2 mm bicarbonate and lowest at 10 mm bicarbonate, which is similar to the pattern of glycine accumulation in the presence of INH. In 2 mm bicarbonate the rate of [14C]glycolate synthesis was decreased above 50% O₂. It was not determined whether the rate of glycolate synthesis was actually decreased or whether the specific radio-

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**Fig. 1.** Effect of HPMS concentration on photosynthesis rate and percentage incorporation of [14C]CO₂ into glycolate. Cells were incubated in the light for 5 min in assay medium without buffer (pH 7.8) in the presence of HPMS (final concentration as indicated) while flushed with 100% O₂. Buffer (50 mm tris·Cl, pH 7.8) was added and assays were immediately initiated with 2 mm bicarbonate and terminated after 15 min. Control (assayed as above but without HPMS) rate of photosynthesis was 11.6 μmol CO₂/mg Chl·hr.

**Fig. 2.** Effect of INH concentration on photosynthesis rate and percentage incorporation of [14C]CO₂ into glycine. Cells were incubated in assay medium (pH 7.8) with INH (final concentrations as indicated) for 1 hr in the dark. Cells were then added in the light to vials containing assay medium and INH and flushed with 100% O₂ for 5 min. Assays were initiated by adding 0.75 mm bicarbonate and terminated after 15 min. Control rate of photosynthesis was 7.6 μmol CO₂/mg Chl·hr.
activity of the glycolate was much reduced at high O2 and low CO2 concentrations. Mahon et al. (10) found that the specific radioactivity of various glycolate pathway intermediates decreased with decreasing CO2 concentration and increasing O2 concentration.

**Kinetics of Photosynthesis and O2 Inhibition.** In the absence of O2, INH and BHB had little effect on photosynthesis rate (Table I), indicating that these compounds are not inhibitors of the photosynthesis cycle. However, in the presence of 21% O2, both INH and BHB increased the magnitude of O2 inhibition even though both compounds inhibited carbon flow through the photorespiratory pathway (Figs. 2 and 4). In 21% O2 at 0.5 mM bicarbonate (equivalent to 340 μl CO2/l), O2 inhibition of photosynthesis increased from 30% in the control to 49% with INH and 56% with BHB. The differential effect of the inhibitors on photosynthesis in the presence and absence of O2 is graphically represented in Figure 6. The double reciprocal plot of photosynthesis and bicarbonate concentration follows the same line in the presence of inhibitors under N2, but shows increased O2 inhibition under 21% O2. The Vmax and Km (CO2) are unchanged by the inhibitors, being about 130 μmol CO2/mg Chl·hr and 30 μM, respectively, but the apparent Km (O2) was decreased from 650 μM O2 in the control to 270 μM O2 with INH and 160 μM O2 with BHB. The CO2 compensation concentration was unchanged in the presence of either inhibitor, whether estimated by extrapolation of the bicarbonate response curve or measured directly with an IR gas analyzer.

**DISCUSSION**

The metabolism of glycine and glycolate appeared to be inhibited completely in isolated soybean cells by the specific photorespiratory enzyme inhibitors INH and BHB. The accumulation of glycine with INH, and glycolate with BHB, is consistent with the sequence of carbon flow through the glycolate pathway as proposed by Tolbert (24). All of the glycine synthesized was derived from glycolate. Glycine was metabolized to serine, but not all of the serine was derived from glycine. Under air levels of CO2 and O2, about half of the serine was synthesized by the glycolate pathway and half by another pathway, perhaps from P-glycerate. Therefore, it is not correct to assume that serine biosynthesis is an in vivo assay for photorespiratory activity (15, 23).

The patterns of glycolate and glycine accumulation as functions of CO2 and O2 concentrations were similar, as expected if glycolate is a precursor of glycine. Increasing O2 concentration increased the absolute rate of synthesis and the percentage incorporation of 14C into these compounds, and increasing bicarbonate concentration reduced the rate of synthesis and percentage incorporation. The percentage incorporation of 14C into these compounds was found to be strongly dependent on the O2/CO2 ratio during photosynthesis. The percentage 14C incorporated into glycine was always about one-half the percentage incorporated into glycolate. The reason for this difference is not known. It is inconsistent with the proposed scheme of peroxisomal metabolism (5, 24) in that it indicates that a constant proportion of glycolate is metabolized to products other than glycine.

HPMS and INH inhibited 14CO2 release from both [14C]glycolate and [14C]glycine when applied to leaf tissue (4, 8, 30). If glycolate or glycine is a substrate for photorespiratory CO2, then inhibition of their metabolism should reduce Γ. The
finding that BHB and INH inhibit glycolate and glycine metabolism, respectively, but do not alter $\Gamma$ is inconsistent with current theories that these compounds are precursors of photosynthetic CO$_2$ (5, 24, 32). INH and HPMS also failed to reduce $\Gamma$ when supplied to intact soybean leaves (22). These observations suggest that the substrate of photosynthetic CO$_2$ precedes glycolate in the pathway, but firm conclusions on this point require additional data.

The average rate of glycolate synthesis by tobacco leaf discs in the presence of HPMS was measured to be 67 $\mu$mol/g fresh wt·hr (32). Soybean cells under atmospheric concentrations of CO$_2$ (0.5 mM bicarbonate) and O$_2$ (21%) accumulated glycolate, in the presence of INH, and glycolate, in BHB, at rates of 14 and 23 $\mu$mol/g fresh wt·hr, respectively, based on 4 mg Chl/g fresh wt. A similar rate of glycolate synthesis has been reported for pea leaf discs (7). Zelitch (28) reported that in 21% O$_2$ and 10 mM HPMS, about 50% of the $^{14}$C fixed by tobacco leaf discs was incorporated into glycolate. In the present study, under air levels of CO$_2$ and O$_2$, BHB-treated soybean cells accumulated about 55% of the $^{14}$C fixed into glycolate (Fig. 5). Warburg and Krippahl (27) reported that Chlorella incorporated about 90% of the carbon fixed into glycolate under 100% O$_2$ and 500 $\mu$l/l CO$_2$. Under similar O$_2$ and CO$_2$ levels, BHB-treated soybean cells incorporated about 90% of the $^{14}$C fixed into glycolate when BHB was present. From these data it appears that the ratio of glycolate synthesis to photosynthesis is a reproducible characteristic of C$_3$ plants, and is dependent on the external CO$_2$ and O$_2$ concentrations. The mutual antagonism between CO$_2$ and O$_2$ with respect to photosynthesis and glycolate synthesis, and the reproducible stoichiometry of this antagonism (Fig. 7), indicate a tightly coupled competition between the two gases for a common substrate. This interaction is best explained by competition between CO$_2$ and O$_2$ for RuDP, mediated by RuDP carboxylase (9, 13, 14).

Inhibition of glycolate metabolism in tobacco leaf discs with HPMS increased the photosynthesis rate, presumably by preventing photorespiratory metabolism (29). This finding was not
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because neither INH nor BHB affected photosynthesis in the absence of O₂. Only under photorespiratory conditions did they inhibit, and the inhibition increased as the conditions for photorespiration became more favorable. INH and BHB inhibition of photosynthesis was greatest at low CO₂ and high O₂ concentrations, and was decreased by increasing the CO₂ concentration or by decreasing the O₂ concentration (Fig. 6). The enhanced O₂ inhibition caused by these compounds likely occurred because they prevented glycine or glycolate carbon from being recycled back into the Calvin cycle, leading to a depletion of Calvin cycle intermediates, particularly RuDP. Such a mechanism of O₂ inhibition has previously been suggested in chloroplast photosynthesis (6, 18). Isolated chloroplasts do not contain enzymes of the glycolate pathway, so as in INH- or BHB-treated cells, glycolate carbon cannot be returned to the Calvin cycle.

INH appeared to block completely the conversion of glycine to serine and BHB completely inhibited glycolate oxidase. However, BHB was a considerably more potent inhibitor of photosynthesis under conditions of photorespiration (Fig. 6 and Table 1), indicating that BHB keeps more carbon out of the Calvin cycle than does INH. This is consistent with the suggestion made earlier in the discussion, based on the observation that glycine accumulation was always less than glycolate accumulation (Fig. 7), that not all glycolate was metabolized to glycine during photorespiration. The data indicate a branch point in the glycolate pathway between glycine and glycolate, and that about one-half of the glycolate is metabolized to glycine and the remainder is recycled back to the Calvin cycle. Current schemes of photorespiration consider that glyoxylate is the only intermediate between glycine and glycine (5, 24, 31). If true, then the branch point must be either at glyoxylate or glycine. The stoichiometry of about 1:1 between the proportion of glycolate carbon metabolized to glycine and that apparently returned to the photosynthesis cycle may be helpful in determining the mechanism of carbon distribution at the branch point.

In leaves and untreated leaf cells, O₂ reduces photosynthesis by competitive inhibition of CO₂ fixation and by stimulation of photorespiratory CO₂ evolution (9, 13, 14, 20). The magnitude of this inhibition, at air levels of O₂ and CO₂, is about 30%. In isolated chloroplasts, and in cells treated with a photorespiration inhibitor, photosynthesis is inhibited by 50 to 80% under the same conditions (6, 18; Fig. 6). Thus, inhibition or removal of the glycolate pathway at atmospheric concentrations of CO₂ and O₂ is severely deleterious to photosynthesis, and it is essential that glycine, once synthesized, be metabolized through the complete photorespiratory pathway if normal photosynthesis rates are to be maintained. This means that increasing photosynthetic productivity by inhibition or alteration of the glycolate pathway is not feasible, and that the search for chemical or genetic control of photorespiration must focus on reducing or preventing the diversion of carbon from the photosynthesis cycle into the glycolate pathway. This might be achieved by alteration of the kinetic characteristics of RuDP carboxylase so that the rate of carboxylation is increased relative to the rate of oxygenation (13, 14).

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