# The Effect of Glyoxylate on Photosynthesis and Photorespiration by Isolated Soybean Mesophyll Cells

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## ABSTRACT

Incubating isolated soybean leaf mesophyll cells with glyoxylate increased the rates of $CO_2$ fixation by as much as 150%. In order to cause this stimulation, the glyoxylate must be presented to the cells before the $NaHCO_3$. Significant stimulation was observed 15 seconds after beginning the glyoxylate treatment. The glyoxylate-dependent stimulation was increased by high $O_2$ concentrations and decreased by high $CO_2$ concentrations. Glyoxylate treatment resulted in a 71% inhibition in the rate of $CO_2$ incorporation into glycolate and glycine. Glyoxylate may be stimulating net photosynthesis solely by decreasing photorespiration or it may be increasing the amount of $CO_2$ fixed by both decreasing photorespiration and increasing gross photosynthesis. Ribulose bisphosphate carboxylase, when preactivated and assayed in situ, was unaffected by the glyoxylate treatment.

In all plants where the primary carboxylation is catalyzed by $RuBP^2$ carboxylase, large amounts of glycolic acid are synthesized in the light. As this glycolate is metabolized through the glycolate pathway, 25-100% of the carbon contained in the glycolate is lost as $CO_2$ by the process of photorespiration (12). Could this carbon be conserved, large increases in dry matter accumulation should be possible (25). Blocking the metabolism of glycolate may cause transient increases in net photosynthesis (16, 23) but soon results in the inhibition of photosynthesis (9, 18, 19).

A more promising approach is to alter the rate of glycolate synthesis and thereby decrease photorespiration. Photorespiration is regulated by $O_2$ and $CO_2$ concentrations in the atmosphere. At atmospheric $CO_2$ concentrations, glycolate synthesis increased with increasing $O_2$ concentrations up to about 60% $O_2$. At 21% $O_2$, glycolate synthesis increased as the $CO_2$ level was raised from zero to approximately atmospheric levels and decreased as the $CO_2$ level was elevated further (11). Reducing glycolate synthesis by lowering the partial pressure of $O_2$, or inhibiting glycolate synthesis and stimulating gross photosynthesis by increasing the $CO_2$ concentration results in an increased rate of dry matter accumulation in $C_3$ plants (3, 25).

Glycolate synthesis, and thereby photorespiration in tobacco, can be altered by changing the intracellular concentration of certain key metabolites including glutamate, aspartate (15), and glyoxylate (10, 11, 14). Glyoxylate treatment also inhibits photo-

respiration in isolated spinach cells (5). In this paper, glyoxalate is shown to stimulate net photosynthetic $CO_2$ fixation by isolated soybean leaf mesophyll cells. The stimulation is affected by the concentration of $O_2$ in solution and decreased by high $CO_2$ concentrations. Addition of glyoxylate also results in an inhibition of glycolate and glycine synthesis and a reduction in the $O_2$ sensitivity of net photosynthesis.

## MATERIALS AND METHODS

Mesophyll cells were isolated from 6- to 8-week-old soybean leaves by a mechanical grinding technique (13). Detailed analyses of the activities of isolated soybean leaf cells have been presented (13, 20). Photosynthesis was assayed in stoppered 10-ml Fernbach flasks in a reaction mixture that contained 0.3 M sorbitol, 50 mM Tris-HCl (pH 7.8), 2 mM $NaNO_3$, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM $K_2HPO_4$, and 2 mM DTT (added fresh daily). The isolated soybean leaf cells, containing 5-30 μg Chl and suspended in the same solution, were generally added to previously equilibrated flasks in a 25 C waterbath and illuminated for 15 s to 20 min in the absence of added $NaHCO_3$. Next the $NaH^{14}CO_3$ was added and the illumination continued for an additional 5 min before the reaction was stopped by adding 0.1 ml 3 N HCl. The amount of acid-stable radioactivity was determined by liquid scintillation counting of 0.1- or 0.2-ml aliquots of the acidified reaction mixture.

All reactions were in equilibrium with air unless otherwise indicated. In some experiments the reaction vessels were equilibrated with $CO_2$-free gases by blowing the gas vigorously against the liquid surface for 10 min before the cells were added. The activity of the RuBP carboxylase in the cells was assayed by first making the membrane more permeable with toluene (7) and then adding the carboxylation substrates RuBP and $NaH^{14}CO_3$. The cells were preilluminated and assayed in the same solution that was used in the photosynthetic studies except that the $MgCl_2$ concentration was increased to 25 mM (1). After the cells were illuminated at 25 C for 5 min, 25-μl samples of the cell mixture were transferred to the reaction flasks. Thirty s later the reaction was terminated by adding 0.1 ml 3 N HCl. The reaction flasks contained 50 μl of a 4:1 (v/v) ethanol to toluene solution, 0.35 mM RuBP, the indicated amount of $NaH^{14}CO_3$, (2-4 μCi/μmol), and the assay solution above with a final volume of 1.0 ml.

Over 97% of the $CO_2$ fixed was dependent on the addition of RuBP and the rate of $CO_2$ fixation was constant for 50 s. After that time, the rate of fixation dropped off substantially. When glyoxylate was included in the reaction, 50 μg of catalase was added to destroy any $H_2O_2$ produced by the toluene-disrupted cells.

## RESULTS AND DISCUSSION

Glyoxylate treatment greatly stimulated the rate of net photosynthetic $CO_2$ fixation by isolated soybean mesophyll cells. Gly-

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[2] Abbreviations: RuBP: ribulose bisphosphate; INH: isonicotinic acid hydrazide; MHB: methyl D,L-2-hydroxy-3-butynoate; HPMS: α-hydroxy-2-pyridinemethanesulfonic acid.

oxylate treatment did not increase the rate of dark $CO_2$ fixation (unpublished results). The photosynthetic rate increased in a sigmoidal fashion with increasing glyoxylate concentration to a maximum at 40–50 mM (Fig. 1). In the experiment shown, increasing the glyoxylate concentration in the media surrounding the cells from 0 to 20 mM stimulated $CO_2$ fixation 5.2 μmol/mg Chl· h (from 32.5 to 37.7 μmol/mg Chl·h). Increasing the glyoxylate concentration an additional 20 mM from 20 to 40 mM resulted in an additional increase of 22.6 μmol/mg Chl·h (from 37.7 to 60.3 μmol/mg Chl·h). High glyoxylate concentrations were probably needed for maximum stimulation because of the limited permeability of the anion at the pH (7.8) of the assay. At concentrations above 50 mM, glyoxylate inhibited $CO_2$ fixation.

In order for glyoxylate to stimulate net $CO_2$ fixation, it must have been presented to the cells before the $^{14}CO_2$. When the glyoxylate was added either together with or after the $CO_2$, photosynthesis was not increased (Fig. 2). If the cells were treated with glyoxylate for as little as 15 s before the $CO_2$ was added, the rate of net $CO_2$ fixation was stimulated by the glyoxylate treatment (Fig. 2). As has been observed with soybean cells earlier (13) the rate of $CO_2$ fixation following a preillumination period increased with the time of the period up to about 5–10 min. If 30 mM glyoxylate was provided during that preillumination period, the rate of $CO_2$ fixation in the subsequent assay period was increased. The per cent stimulation caused by the glyoxylate treatment generally increased with the preillumination time up to about 10 min. With tobacco leaf discs, the stimulation continues for at least 3 h (14).

In order for glyoxylate to stimulate photosynthesis greatly in isolated soybean cells, the cells need not be illuminated during the period of glyoxylate treatment before $CO_2$ fixation was measured (Fig. 3). In this experiment, cells preincubated for 5 min with 40 mM glyoxylate in the light and the dark were stimulated 55 and 44%, respectively. Preincubation in the light increased the photosynthetic rate as noted above.

Glyoxylate markedly stimulated net $CO_2$ fixation only at $CO_2$ concentrations below saturation (Fig. 4). At a $NaHCO_3$ concentration of 0.2 mM in an atmosphere of 21% $O_2$/79% $N_2$, 30 mM glyoxylate stimulated $CO_2$ fixation 83% from 24.0 to 43.8 μmol/ mg Chl·h. When the $NaHCO_3$ concentration was increased to 5



FIG. 2. Effect of time of preillumination with glyoxylate on the subsequent rate of $CO_2$ fixation. Cells were illuminated with or without 30 mM glyoxylate as indicated for 0–20 min before the addition of 0.5 mM $NaH^{14}CO_3$. After $CO_2$ fixation had proceeded for 60 s, the cells were acidified and the acid-stable radioactivity determined. (O): rate without glyoxylate; (●): rate with glyoxylate; (▲): per cent stimulation caused by glyoxylate.
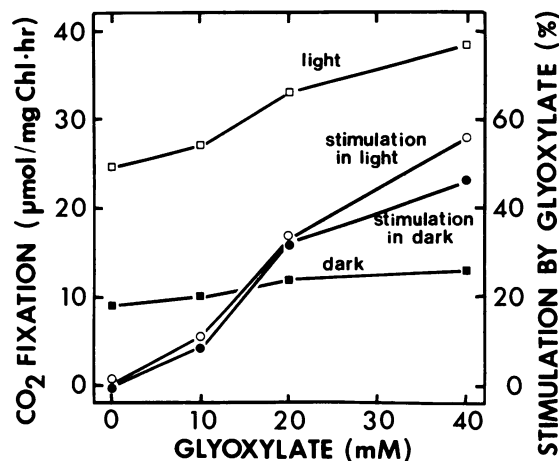


FIG. 3. Effect of light during the preincubation period on the stimulation of net photosynthesis by glyoxylate. Cells were supplied with glyoxylate at the indicated concentration either in the light or the dark. After 5 min, all flasks were illuminated, 0.5 mM $NaH^{14}CO_3$ was added immediately, and $CO_2$ fixation allowed to proceed for an additional 5 min before the reaction was stopped and the acid-stable radioactivity determined. (□): rate in light, (■): rate in dark, (O): stimulation in light, (●): stimulation in dark.
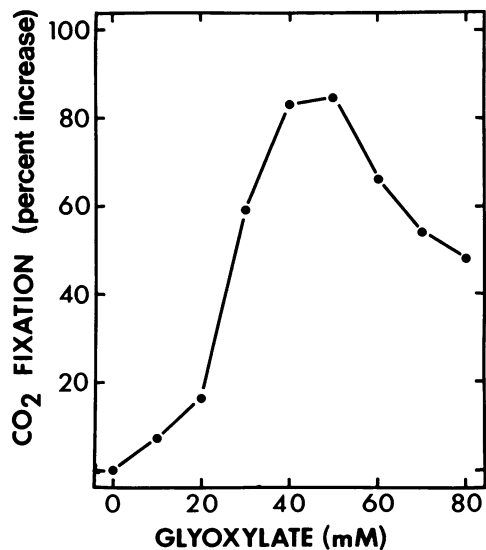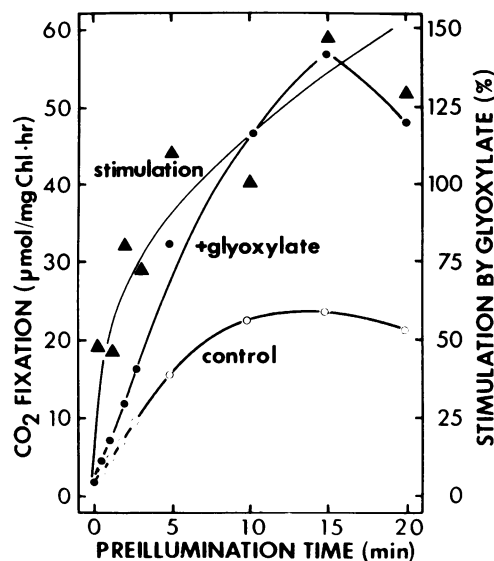


FIG. 1. Effect of glyoxylate on net $CO_2$ fixation by isolated soybean mesophyll cells. Cells were preilluminated for 5 min with the indicated glyoxylate concentration before the addition of 0.5 mM $NaH^{14}CO_3$. After a 5-min fixation period, the reaction was stopped with 0.1 ml 3 N HCl and the acid stable radioactivity determined. Rate of $CO_2$ fixation without glyoxylate was 32.5 μmol/mg Chl·h.

mM, glyoxylate treatment only stimulated net photosynthesis by 27% from 164.6 to 209.3 μmol/mg Chl·h (Fig. 4). Extrapolation of these data indicated no stimulation at saturating $CO_2$ concentrations (Fig. 4).

Glyoxylate stimulated net photosynthetic $CO_2$ fixation by isolated soybean cells most when $O_2$ was present (Fig. 4). Photosynthesis by cells in 21% $O_2$ and 0.2 mM $NaHCO_3$ was stimulated 83% by the glyoxylate treatment. $CO_2$ fixation by cells in $N_2$ and 0.2 mM $NaHCO_3$ was only increased 24% by the glyoxylate treatment (Fig. 4). In another experiment, the glyoxylate effect on net photosynthesis was measured at increasing $O_2$ concentrations.
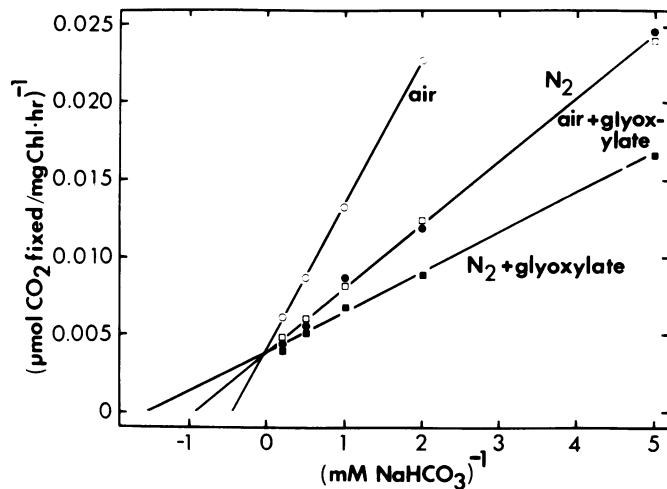
FIG. 4. A double reciprocal plot of the effect of glyoxylate and $N_2$ on $CO_2$ fixation by soybean cells. Cells were preincubated in the light in air or $N_2$ with or without 30 mM glyoxylate as indicated. Each flask contained cells amounting to 5 μg Chl. Cell concentration was kept low to minimize $O_2$ evolution during the preillumination and fixation periods. Cells were preilluminated for 5 min and then allowed to fix $NaH^{14}CO_3$ for an additional 5 min. (O): rate in air no glyoxylate, (●): rate in air with glyoxylate, (□): rate in $N_2$ no glyoxylate, (■): rate in $N_2$ with glyoxylate.
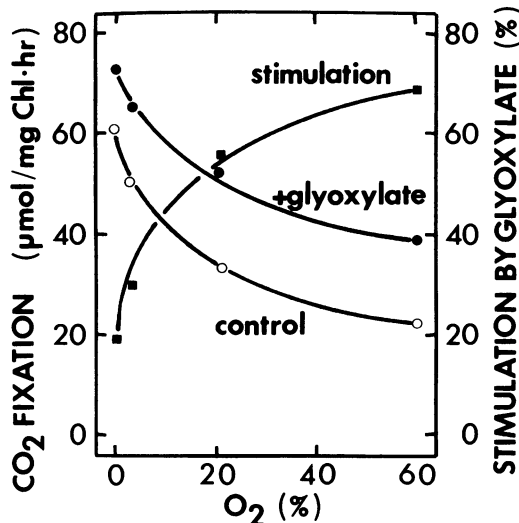


FIG. 5. Effect of $O_2$ concentration on the stimulation of net photosynthesis by glyoxylate. Flasks containing the reaction mixture without cells were equilibrated to the desired $O_2$ level by blowing the indicated gas mixture against the surface of the solution for 10 min. Cells were then added and the flasks illuminated for 5 min before the addition of 0.5 mM $NaH^{14}CO_3$. $CO_2$ fixation period was 5 min. (O): rate without glyoxylate, (●): rate with glyoxylate, (■): stimulation caused by glyoxylate.

The stimulation increased from 19% in $N_2$ to 30% in 3% $O_2$ to 56% in 21% $O_2$ to 69% in 60% $O_2$ (Fig. 5). The $O_2$-dependent stimulation is half-maximal at approximately 20% $O_2$ with 0.5 mM $NaHCO_3$. This was calculated by subtracting the 19% increase that occurs in $N_2$ from all other values. The absolute stimulation caused by the glyoxylate treatment was consistently between 15 and 20 μmol/ mg Chl·h at all $O_2$ concentrations.

Glyoxylate treatment reduced the $O_2$ sensitivity of $CO_2$ fixation by isolated soybean cells (the Warburg effect) as it did with tobacco leaf tissue (10). At low $NaHCO_3$ concentrations (0.2 mM), untreated cells fixed 42% less $CO_2$ in air than in $N_2$ (24.0 and 41.3 μmol/mg Chl·h, respectively). Under these conditions, treatment

with 30 mM glyoxylate decreased the Warburg effect to 17% from 43.8 μmol/mg Chl·h in air to 51.2 μmol/mg Chl·h in $N_2$ (Fig. 4). Glyoxylate treatment does not effect the maximal velocity of $CO_2$ fixation in air by isolated soybean cells, but does reduce the concentration of $CO_2$ needed for half-maximal activity. The limiting factor for $CO_2$ fixation at saturating $CO_2$ concentrations has not been altered by the glyoxylate treatment. These limitations were probably caused by electron transport or photophosphorylation (8). Glyoxylate increased the availability of $CO_2$ to the carboxylating reaction at limiting $CO_2$ concentrations or inhibited a respiratory process that occurred at low $CO_2$ concentrations but not at high.

The glyoxylate effect on net photosynthesis was blocked at saturating $CO_2$ concentrations and was dependent on high $O_2$ concentrations. These parameters of the glyoxylate effect are identical to those of photorespiration. To confirm the possibility that glyoxylate was affecting photorespiration directly, the effect of glyoxylate on glycolate synthesis by soybean cells was investigated. The incorporation of radiocarbon into glycolate from $^{14}CO_2$ was substantially decreased by the glyoxylate treatment (Table I). This inhibition resulted when glycolate oxidase activity was inhibited by either MHB (6) or HPMS (23). The sulfonate experiments were run at pH 4.5 to allow penetration into the cells. Exposure of the cells to this low pH caused severe inhibition of photosynthesis. Glyoxylate treatment has been shown to inhibit glycolate synthesis in tobacco leaf discs (10, 11, 14). Similarly, glycine synthesis was inhibited by glyoxylate treatment in cells where glycine decarboxylation was blocked by isonicotinic acid hydrazide (17) (Table I).

Glyoxylate reacts rapidly with isonicotinic acid hydrazide in solution (I. Zelitch, personal communication). The inhibition of the glycine to serine plus $CO_2$ reaction was complete following the 30-min preincubation period and the inhibition was not reversed during the subsequent assay period (15). The reaction between glyoxylate and INH, therefore, can not explain the reduction in glycine accumulation caused by glyoxylate. The stimulation in net photosynthesis caused by glyoxylate treatment resulted, at least in part, from the decrease in the rate of photorespiration caused by reduced amounts of glycolate synthesis.

Glyoxylate had been shown earlier to have no effect on the rate of carboxylation or the $O_2$ sensitivity of $CO_2$ fixation by activated

Table I. *Effect of Glyoxylate Treatment on Glycolate and Glycine Synthesis*

Isolated soybean cells were preincubated in the dark for 30 min with 3 mM MHB or 10 mM INH as indicated. Reactions involving 10 mM HPMS were carried out (pH 4.5) and the sulfonate was added with the $NaH^{14}CO_3$. All cells were illuminated for 5 min and then allowed to fix $NaH^{14}CO_3$ for an additional 5 min. The glyoxylate concentration was 30 mM except with the sulfonate experiments where 15 mM was used. Glycolate and glycine were isolated as described earlier (11, 15). The figures in parentheses indicate the per cent of total $CO_2$ fixed that was found in that fraction. This correction was important because the glyoxylate treatment increased $CO_2$ fixation under all conditions.

| Treat-ment | Rate of $CO_2$ Fixation | | Rate of Glycolate Synthesis | | Rate of Glycine Synthesis | |
|---|---|---|---|---|---|---|
| | With-out Glyox-ylate | With Glyox-ylate | Without Glyoxyl-ate | With Glyoxyl-ate | Without Glyoxyl-ate | With Glyoxyl-ate |
| | *μmol/mg Chl·h* | | | | | |
| Control | 23.1 | 32.0 | | | | |
| + INH | 13.5 | 19.2 | | | 5.6 (41) | 2.2 (12) |
| + MHB | 14.7 | 22.8 | 8.7 (59) | 2.9 (13) | | |
| + HPMS | 1.2 | 1.9 | 0.7 (58) | 0.4 (21) | | |

purified RuBP carboxylase. The soybean cell system allowed an analysis of the effect of glyoxylate on activated RuBP carboxylase *in situ*. Cells were preilluminated with or without glyoxylate for 5 min.

The plasmalemma and the outer chloroplast membrane were made permeable with toluene and RuBP and $NaH^{14}CO_3$ were added. The residual photosynthetic rate after the toluene treatment was less than 3% of the untreated rate and cells in the dark supplied with $NaHCO_3$ and RuBP fixed less than 2% as much $CO_2$ without the toluene treatment as with it. The carboxylase was apparently fully activated by this treatment and routinely gave a $K_m$ for $NaHCO_3$ of 1.0–1.2 mM (Fig. 6). This gave a calculated $K_m$ for $CO_2$ of 20–26 $\mu M$ which was essentially the same as the low $K_m$ form of the carboxylase measured with isolated chloroplasts using a similar technique (1).

When the enzyme was assayed in this manner, glyoxylate had no effect on the affinity of the carboxylase for $CO_2$ or $O_2$ or on the rate of the carboxylation reaction. The apparent $K_m$ for $HCO_3^-$ was 1.2 and 1.1 mM in the presence and absence of glyoxylate. The $V_{max}$ was 90.0 $\mu mol/mg$ Chl·h in the control and 87.0 $\mu mol/$ mg Chl·h when glyoxylate was included during the preincubation and assay periods (Fig. 6).

Glyoxylate did not affect purified RuBP carboxylase (10) or the enzyme *in situ*. These results may not exclude the possibility that a metabolite of glyoxylate was affecting the carboxylase. Analysis of the products of [1-$^{14}$C]glyoxylate metabolism in the light for 60 s either with or without added $NaHCO_3$ revealed no difference in the distribution of major metabolites (unpublished results). When the $CO_2$ was excluded from the preincubation step, net $CO_2$ fixation during a subsequent 2-min period in this experiment was 54% greater than when the glyoxylate effect was blocked by adding the $NaHCO_3$ with the glyoxylate. Apparently no metabolite of glyoxylate was causing the glyoxylate effect.
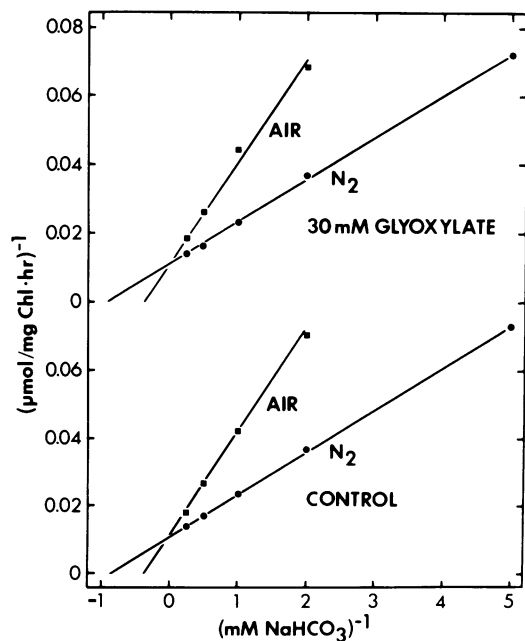


FIG. 6. A double-reciprocal plot of the effect of glyoxylate on the rate and $O_2$ sensitivity of $CO_2$ fixation by soybean RuBP carboxylase *in situ*. Isolated soybean cells were illuminated in a small beaker with or without 30 mM glyoxylate. After 5 min, 25-$\mu l$ samples (5 $\mu g$ Chl) were transferred to darkened reaction flasks which contained, in a final volume of 1 ml, the reaction solution which was altered to contain 30 mM $MgCl_2$, 0.35 mM RuBP, $NaH^{14}CO_3$ at the indicated concentration, 50 $\mu g$ catalase, and 50 $\mu l$ of a 4:1 (v/v) ethanol to toluene solution. After 30 s, 0.1 ml of 3 N HCl was added to stop the reaction and liberate any unfixed $^{14}CO_2$.

## CONCLUSIONS

Glyoxylate treatment increased net $CO_2$ fixation in isolated soybean leaf mesophyll cells as it did in tobacco leaf discs (14). This stimulation was decreased at high $CO_2$ concentrations and increased at high $O_2$ concentrations. Glyoxylate treatment decreased the rate of glycolate and glycine synthesis and reduced the $O_2$ sensitivity of net photosynthesis (the Warburg effect) by the cells. Together these facts provide strong evidence that, as with the tobacco leaf disc system (10, 11, 14), a portion of the stimulation of $CO_2$ fixation observed resulted from the inhibition of photorespiration.

Glyoxylate treatment (Fig. 2) increased net photosynthesis in isolated soybean leaf cells by as much as 150% (2.5 times the control rate). Photosynthetic $CO_2$ fixation in tobacco was stimulated over 125% (2.25 times the control value) by the glyoxylate treatment (14). Can these large increases in the $CO_2$ fixation rate be ascribed solely to an inhibition of photorespiration? Similarly, was enough photorespiration occurring in $N_2$ to account for the glyoxylate-dependent stimulations in photosynthesis observed (Fig. 4) if the only effect of glyoxylate was inhibition of glycolate synthesis?

Photorespiration has been measured to be at least 50% of net photosynthesis (24). All measurements of photorespiration based on $CO_2$ exchange measurements underestimate the amount of photorespiratory $CO_2$ release because they can not correct for the amount of $CO_2$ that is photosynthetically refixed before being released from the leaf.

Measurements of the rate of glycolate synthesis indicate that glycolate is synthesized in these systems at rates which are approximately equal to or greater than net photosynthesis (11, 15, 21). These measurements also are underestimates in that they assume, for example, that the time between addition of the inhibitor and complete blockage of glycolate oxidation is negligible. Uncertainties in the mechanism of glycolate metabolism prevent precise analysis of the magnitude of photorespiration from estimates of glycolate synthesis, but 25% to 50% of the carbon in glycolate is probably lost under most conditions (12). Photorespiratory $CO_2$ release, therefore, could range from a minimum of 50% to over 100% of net photosynthesis. Glyoxylate treatment might be able to cause the large increases in $CO_2$ fixation observed in 21% $O_2$ 0.5 mM $NaHCO_3$ by inhibiting photorespiration alone, but uncertainties in the measurements involved do not permit an unequivocal conclusion.

At subsaturating $CO_2$ concentrations, glyoxylate stimulated $CO_2$ fixation in $N_2$ (Fig. 4) where very limited glycolate synthesis and photorespiration should occur. The glyoxylate-dependent stimulation in net photosynthesis measured in $N_2$ and 0.5 mM $NaH^{14}CO_3$ was 26% (from 81.5 to 102.5 $\mu mol$ $CO_2$ fixed/mg Chl·h).

Glyoxylate treatment may have had a dual effect on net photosynthetic $CO_2$ fixation. The large effect was an inhibition of glycolate synthesis and photorespiration with a resulting increase in photosynthesis. In addition, glyoxylate may have stimulated gross photosynthesis. This could be the reason for the increase in $CO_2$ fixation in $N_2$. It is possible, however, that photorespiration in $N_2$ was 26% of net photosynthesis and that glyoxylate inhibited this reaction.

Irrespective of whether glyoxylate treatment is just inhibiting photorespiration or both inhibiting photorespiration and stimulating gross photosynthesis, it is not increasing the total capacity for photosynthesis (the rate at saturating $CO_2$) but is decreasing the concentration of $CO_2$ needed for half maximal activity.

Glyoxylate would cause the observed results if it facilitated the uptake of $CO_2$ or bicarbonate into the chloroplast stromal compartment. By increasing the concentration of $CO_2$ at the site of the carboxylating reaction, $CO_2$ fixation at subsaturating $CO_2$ concentrations would be increased. The elevated $CO_2$ concentrations would also decrease glycolate synthesis and therefore photorespir-

ation by the cells. The nonfacilitated nature of $CO_2$ uptake into the chloroplast (22) and the large amounts of $CO_2$ fixed per glyoxylate metabolized (14), however, do not support this mechanism.

A detailed explanation of the mechanism by which glyoxylate inhibits glycolate synthesis and photorespiration and stimulates net photosynthesis must await the results of further study.

Recently, these results have been partially confirmed by Hunt and Ogren (4) who have repeated the glyoxylate-dependent stimulation in net photosynthesis and partial reduction in the Warburg effect with isolated soybean leaf cells. Baumann and Gunther (2) were able to show that the glyoxylate treatment strongly inhibited glycine and serine synthesis in isolated *Chenopodium album* cells. They were not, however, able to see any increase in net photosynthesis under the conditions employed.

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