

# Inhibition of Photorespiration and Increase of Net Photosynthesis in Isolated Maize Bundle Sheath Cells Treated with Glutamate or Aspartate

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## ABSTRACT

Net photosynthetic  $^{14}\text{CO}_2$  fixation by isolated maize (*Zea mays*) bundle sheath strands was stimulated 20 to 35% by the inclusion of L-glutamate or L-aspartate in the reaction mixture. Maximal stimulation occurred at a 7.5 mM concentration of either amino acid. Since the photosynthetic rate and the glutamate-dependent stimulation in the rate were equally sensitive to a photosynthetic electron transport inhibitor, 3-(*p*-chlorophenyl)-1,1-dimethylurea, it was concluded that glutamate did not stimulate  $\text{CO}_2$  fixation by supplying needed NADPH (NADH) through glutamate dehydrogenase. Treatment of the bundle sheath strands with glutamate inhibited glycolate synthesis by 59%. Photorespiration in this tissue, measured as the  $\text{O}_2$  inhibition of  $\text{CO}_2$  fixation (the Warburg effect), was inhibited by treatment with glutamate. The stimulation in net photosynthetic  $\text{CO}_2$  fixation probably results from the decrease in photorespiratory  $\text{CO}_2$  loss. This metabolic regulation of the rate of glycolate synthesis and photorespiration observed with isolated bundle sheath strands could account for the inability to detect rapid photorespiration in the mature intact maize leaf.

In mature maize leaves  $\text{CO}_2$  fixation occurs in the mesophyll cells which have  $\text{C}_4$  photosynthesis and the bundle sheath cells which have  $\text{C}_3$  photosynthesis. Isolated maize bundle sheath strands exhibit the photorespiratory properties that are typical of all  $\text{C}_3$  plants (11, 19). Glycolate is synthesized and metabolized in the isolated tissue at measurable rates (2, 4) and net photosynthetic  $\text{CO}_2$  fixation is reversibly inhibited (3) by elevated  $\text{O}_2$  concentrations (the Warburg effect). In the intact leaf, photorespiration is either severely limited or nonexistent. Glycolate synthesis occurs in maize at less than 10% of the rate in tobacco and may involve a different mechanism (20). As a result, net photosynthetic  $\text{CO}_2$  fixation in corn is not inhibited by atmospheric levels of  $\text{O}_2$  (6).

Since maize bundle sheath strands have the full complement of enzymes needed to synthesize and metabolize glycolate, yet photorespiration does not occur in intact corn leaves, the enzymes involved may be inhibited *in situ*. High  $\text{CO}_2$  levels and low  $\text{O}_2$  levels have been suggested as possible mechanisms for inhibiting glycolate synthesis by the bundle sheath tissue in the intact corn leaf (8).

We have reported earlier that certain metabolites normally found in plant tissue, including glutamate and aspartate, could severely inhibit the rate of glycolate synthesis in tobacco leaf discs (16) and suggested that this mechanism of regulation could help account for the low photorespiration in  $\text{C}_4$  species. This paper extends these findings to isolated maize bundle sheath strands and demonstrates that glycolate synthesis in this tissue is inhibited by

these amino acids at concentrations similar to those found in the bundle sheath cells (7) as well as by low  $\text{O}_2$  and high  $\text{CO}_2$  levels.

## MATERIALS AND METHODS

Maize plants were grown in a greenhouse for 3 weeks and bundle sheath strands were isolated by a procedure adapted from Hatch and Kagawa (9). Primary and secondary corn leaves (9 g, midribs removed) were held below the surface of 50 ml of the cold grinding solution (20 mM HEPES-KOH [pH 6.7], 0.3 M sorbitol, 2 mM  $\text{MgCl}_2$ , 2 mM  $\text{KH}_2\text{PO}_4$ , and 2 mM sodium isoascorbate) and sliced transversely into 1- to 2-mm sections with a razor blade. The tissue was then homogenized in 30 ml of the grinding solution for 15 sec in a Monel head of a Waring Blendor at full line voltage. The slurry was quickly filtered through four layers of cheesecloth and the corn bundle sheath tissue placed in an enzyme mixture containing 0.2 g of cellulysin (Calbiochem), 0.2 g of hemicellulase (Sigma), and 0.2 g of pectinase (Sigma) in 20 ml of the grinding solution. After 30 min at room temperature the bundle sheath tissue was removed by filtration through four layers of cheesecloth and blended in 30 ml of the grinding solution for 5 sec. The bundle sheath tissue was then filtered once more through four layers of cheesecloth and rinsed thoroughly with 100 ml of the assay solution and resuspended in 20 ml of the same solution.

Bundle sheath strands prepared by this method were essentially free of mesophyll contamination as determined by light microscopy and showed little P-enolpyruvate carboxylase activity, an enzyme found in the mesophyll. The tissue was stored at 0 C and used within 30 min.

Photosynthesis and glycolate synthesis were measured in 10-ml Fernbach flasks (Bellco Corp.) containing a final volume of 2 ml of the assay solution which was composed of 8 mM HEPES-KOH (pH 7.3), 0.25 M sorbitol, 1.6 mM  $\text{MgCl}_2$ , 1.6 mM  $\text{K}_2\text{HPO}_4$ , and 5 mM ribose-5-P. Bundle sheath tissue containing 40 to 70  $\mu\text{g}$  of Chl was placed in the flask in an illuminated ( $340 \mu\text{E m}^{-2} \text{sec}^{-1}$ , 400-700 nm) water bath (30 C) and swirled at 120 cycles/sec. After 5 min 20  $\mu\text{l}$  of 0.1 M  $\text{NaH}^{14}\text{CO}_3$  (1  $\mu\text{Ci}/\mu\text{mol}$ ) for  $\text{CO}_2$  fixation experiments or 0.2 ml of 0.1 M  $\alpha$ -hydroxy-2-pyridine methanesulfonic acid (pH 7.3), to inhibit glycolate oxidase and thus measure glycolate synthesis, was added. Following an additional 15-min period in the light with either  $\text{NaH}^{14}\text{CO}_3$  or the sulfonate, the flasks were removed to a hot plate and 1.0 ml of ethanol added. After the solution boiled (about 15 sec), 5 ml of acetone was added to each flask. The Chl contained in the bundle sheaths was determined after centrifuging the strands from the solution.

Incorporation of  $^{14}\text{CO}_2$  was measured by liquid scintillation counting of samples (0.5-1.0 ml) which were first acidified with

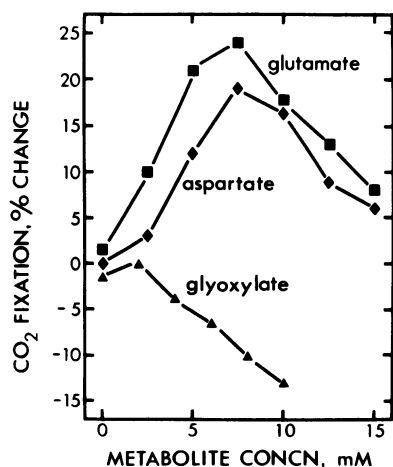


FIG. 1. Effect of glutamate, aspartate, and glyoxylate on the net rate of photosynthetic  $^{14}\text{CO}_2$  fixation by bundle sheath strands isolated from corn. Bundle sheath tissue was isolated and photosynthesis measured as described under "Materials and Methods." Each reaction vessel contained 1 mM  $\text{NaH}^{14}\text{CO}_3$  and 5 mM ribose-5-P. Atmosphere in the flasks was 21%  $\text{O}_2/79\%$   $\text{N}_2$ . Control rates were: 4.6  $\mu\text{mol}$  of  $\text{CO}_2/\text{mg}$  of Chl·hr for the glutamate experiment (■—■); 5.4 for the aspartate experiment (◆—◆); and 7.2 for the glyoxylate experiment (▲—▲).

0.2 ml of 10 N acetic acid and then dried in a 90 C oven. In order to measure glycolate accumulation, the acetone extract and the supernatant from two 5-ml water washes of the homogenized bundle sheath strands were combined and the glycolate fraction isolated by column chromatography on Dowex 1-acetate (16). Glycolate was determined colorimetrically. In all cases where glycolate synthesis was being measured a correction was made for the color yield obtained with an identical sample of the bundle sheath preparation without the sulfonate.

In the experiments where the atmosphere over the reaction was controlled, the flasks were flushed at 150 ml/min with the mixture described for the entire light period through a serum stopper fitted with two hypodermic needles.

## RESULTS AND CONCLUSIONS

Glutamate and aspartate were added to isolated maize bundle sheath strands to see if, as with tobacco leaf discs (16), glycolate synthesis and photorespiration would be inhibited and net  $^{14}\text{CO}_2$  fixation stimulated. Stimulation of the rate of net photosynthetic  $^{14}\text{CO}_2$  fixation up to a maximum of 20 to 35% was indeed observed for either metabolite (Fig. 1). Both amino acids showed maximal stimulation at about 7.5 mM with less stimulation or even inhibition of the photosynthetic rate at higher concentrations. These similarities suggest that the two amino acids may affect net photosynthesis in the bundle sheath tissue by the same mechanism. This similarity in mode of action was also noted for the effects of glutamate and aspartate on glycolate synthesis and net photosynthesis in tobacco leaf discs (16). Glyoxylate, a metabolite that inhibits glycolate synthesis and stimulates net photosynthesis in tobacco leaf discs (14, 15), did not increase the net photosynthetic rate of isolate corn bundle sheaths (Fig. 1). In order to avoid inhibiting photorespiration in these tissues, rate-limiting concentrations of  $\text{NaH}^{14}\text{CO}_3$  were employed. When saturating concentrations of  $\text{NaH}^{14}\text{CO}_3$  (10 mM) was used, the photosynthetic rate ranged between 30 and 40  $\mu\text{mol}/\text{mg}$  of Chl·hr.

Malate was reported to stimulate net photosynthetic  $^{14}\text{CO}_2$  fixation in isolated maize bundle sheath tissue about 3-fold (9). The reason for at least the majority of this much larger stimulation was believed to be the production of NADPH through the action

of NADP-malic enzyme. Due to the faulty PSII activity (and therefore presumably NADPH production) of isolated maize bundle sheath chloroplasts (1, 12, 13), any reaction which supplies NADPH to the tissue should stimulate photosynthetic  $\text{CO}_2$  fixation. Although glutamate dehydrogenase is normally assumed to favor glutamate production, the reaction is reversible and could also lead to NADPH (NADH) formation.

In order to assess the possibility that an increase in NADPH (NADH) produced by glutamate dehydrogenase was responsible for the stimulation in  $^{14}\text{CO}_2$  fixation observed with added glutamate, the sensitivities of both the basal photosynthetic rate and the glutamate-dependent stimulation in this rate to increasing concentrations of a photosynthetic electron transport inhibitor, CMU,<sup>1</sup> were compared (17). The two activities (both the basal rate and the stimulation) were inhibited in a similar manner with increasing CMU concentrations (Fig. 2). This contrasts with the stimulation in  $\text{CO}_2$  fixation by isolated maize bundle sheath tissue caused by malate, a compound whose dehydrogenation is linked to NADPH production, which is much less sensitive than the basal  $\text{CO}_2$  fixation rate of the tissue to photosynthetic electron transport inhibitors (18). Since only NADPH production by photosynthetic electron transport would be expected to be sensitive to CMU, this experiment probably indicates that glutamate (unlike malate) is not stimulating net photosynthetic  $\text{CO}_2$  fixation by supplying NADPH through the activity of glutamate dehydrogenase. This is further supported by the observation of Rathnam and Edwards (17) with another NADP-malic enzyme plant, *Digitaria sanguinalis*, that aspartate could not supply reducing equivalents for  $\text{CO}_2$  fixation.

Glutamate (7.5 mM) inhibited glycolate accumulation in the presence of  $\alpha$ -hydroxy-2-pyridinemethanesulfonic acid (Table I). Bundle sheath strands in the standard reaction mixture synthesized glycolate at a rate of 2.07  $\mu\text{mol}/\text{mg}$  of Chl·hr while those in the reaction mixture with 7.5 mM glutamate synthesized glycolate at 0.84  $\mu\text{mol}/\text{mg}$  of Chl·hr. Glutamate treatment inhibited glycolate accumulation in the presence of the sulfonate by 59%. The rate of photosynthetic  $\text{CO}_2$  fixation was two to five times greater than glycolate synthesis in this tissue on a molar basis. This is considerably different from the approximately equal rates seen with tobacco leaf discs (15, 16). The lower relative rates of glycolate synthesis found with bundle sheath strands probably result from the limited uptake of the ionized sulfonate at the pH (7.3) of the assay. These rates of glycolate synthesis are similar to those reported earlier by others (2, 4).

Additional support for the hypothesis that glutamate and aspartate are stimulating photosynthesis in isolated maize bundle sheath strands by inhibiting glycolate synthesis and therefore photorespiration comes from an investigation of the effect of these two amino acids on the  $\text{O}_2$  sensitivity of net photosynthesis (the Warburg effect). The Warburg effect is a measure of photorespi-

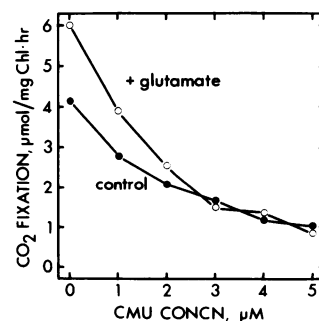


FIG. 2. Effect of CMU on  $\text{CO}_2$  fixation and the glutamate-dependent stimulation of  $\text{CO}_2$  fixation by isolated corn bundle sheath strands. Bundle sheath tissue was isolated and photosynthetic  $^{14}\text{CO}_2$  fixation was measured as described under "Materials and Methods."  $\text{NaH}^{14}\text{CO}_3$  concentration was 1 mM; atmosphere in the flasks was 21%  $\text{O}_2/79\%$   $\text{N}_2$ .

<sup>1</sup> Abbreviation: CMU: 3-(p-chlorophenyl)-1,1-dimethylurea.

Table I. The effect of glutamate on glycolate synthesis in isolated maize bundle sheath tissue.

Glycolate accumulation in the presence of 10 mM  $\alpha$ -hydroxy-2-pyridinethanesulfonic acid was assayed as described in MATERIALS AND METHODS. The atmosphere in the reaction vessels was 21% O<sub>2</sub>:79% N<sub>2</sub>. The glutamate concentration in the tissue was determined by ninhydrin analysis of the glutamate fraction from a Dowex 1-acetate column (14). The samples were treated in the same manner as those used to measure glycolate synthesis except no sulfonate was added and the strands were washed 3 times with 10 ml water before killing. The glutamate concentration supplied initially was 7.5 mM and the results shown are the means of three independent experiments.

Treatment	Glutamate	Rate of
	in Tissue	Glycolate Synthesis
	mM	$\mu\text{mol/mg Chl}\cdot\text{hr}$
H <sub>2</sub> O	0.2	2.07
Glutamate	0.6	0.84

Table II. The effect of glutamate on the O<sub>2</sub> sensitivity of CO<sub>2</sub> fixation by isolated maize bundle sheath strands.

Photosynthetic <sup>14</sup>CO<sub>2</sub> fixation was measured as described in MATERIALS AND METHODS. The final NaH<sup>14</sup>CO<sub>3</sub> concentration was 1 mM. All samples contained 5 mM ribose-5-phosphate, and where indicated the flasks contained 7.5 mM glutamate. The samples were flushed as indicated with either 100% O<sub>2</sub> or 3% O<sub>2</sub>:97% N<sub>2</sub> at a rate of 150 ml/min. The results shown are the means of four independent experiments.

Treatment	Rate of <sup>14</sup> CO <sub>2</sub> fixation		Stimulation
	100% O <sub>2</sub>	3% O <sub>2</sub>	
	$\mu\text{mol/mg Chl}\cdot\text{hr}$		%
H <sub>2</sub> O	2.3	3.9	70
Glutamate	2.8	4.0	43
Glutamate effect	+22%	+3%	

ration and in tobacco leaf discs added glyoxylate, an inhibitor of glycolate synthesis and photorespiration (15), inhibited the Warburg effect (14). When the O<sub>2</sub> concentration in the atmosphere was lowered from 100% to 3% the photosynthetic rates of untreated bundle sheath strands increased 70% from 2.3 to 3.9  $\mu\text{mol}$  of <sup>14</sup>CO<sub>2</sub> fixed/mg of Chl·hr (Table II). Decreasing the O<sub>2</sub> concentration with bundle sheath strands treated with glutamate resulted in only a 43% increase from 2.8 to 4.0  $\mu\text{mol}$  of <sup>14</sup>CO<sub>2</sub>/mg of Chl·hr. The glutamate treatment lessened the Warburg effect, indicating decreased photorespiration.

## DISCUSSION

Treatment with either glutamate or aspartate inhibited glycolate synthesis in isolated maize bundle sheaths. This inhibition caused a decrease in the amount of photorespiration as measured by the Warburg effect and, as a result, a stimulation in the rate of net photosynthetic CO<sub>2</sub> fixation.

Glyoxylate treatment did not increase the net photosynthetic rate of isolated bundle sheath strands (Fig. 1), although it had profound effects on photosynthesis and photorespiration by tobacco leaves (14, 16). This, combined with the differences in the magnitude and reversibility of the effects seen with glyoxylate and glutamate on leaf discs (15, 16), strongly suggests that these two compounds are inhibiting glycolate synthesis and photorespiration at two different biochemical sites.

Three mechanisms currently exist to explain the *in situ* inhibition of the enzyme(s) responsible for glycolate synthesis by bundle sheath cells in intact mature maize leaves. One hypothesis suggests

that the relative activities for the fixation and transport of CO<sub>2</sub> into the bundle sheath tissues, compared with the rates of fixation within this tissue, result in greatly elevated CO<sub>2</sub> concentrations within the bundle sheath cells. In order to remove the O<sub>2</sub> sensitivity of CO<sub>2</sub> fixation completely it has been estimated that the CO<sub>2</sub> concentration within the bundle sheath chloroplast must be at least 61  $\mu\text{M}$  (5). This compares with the 0.6 mM total CO<sub>2</sub> or 13  $\mu\text{M}$  free CO<sub>2</sub> measured in maize bundle sheath tissue (10). A second possibility is that the decreased O<sub>2</sub> production observed with isolated bundle sheath strands and chloroplasts (1, 12) represents a lesion which occurs in the PSII activity of these cells in intact tissue and may result in decreased O<sub>2</sub> concentration within the tissue. Lower O<sub>2</sub> levels would result in slower glycolate synthesis and therefore a decreased photorespiration (19). Finally, glycolate synthesis and photorespiration in bundle sheath strands may be inhibited by the presence of suitable concentrations of the amino acids, glutamate and aspartate. An analysis of the aspartate concentration in isolated maize bundle sheath tissue before and after a 15-min exposure to 7.5 mM aspartate revealed that this treatment increased the aspartate concentration within the tissue from approximately 0.2 to 0.6 mM. The higher value is similar to the aspartate pool size measured in <sup>14</sup>CO<sub>2</sub> pulse-chase experiments with maize (7). Should the isolated strands function as the tissue does *in situ*, this concentration of aspartate could account for the lack of photorespiration found with mature intact maize leaves. Any of these mechanisms either alone or in combination with the others may explain the slow photorespiration in intact maize leaves. It remains to be determined whether glutamate and aspartate, or some metabolic product(s) of these amino acids, exert the inhibitory effect *in vivo*.

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