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# Effect of Glyoxylate on the Sensitivity of Net Photosynthesis to Oxygen (the Warburg Effect) in Tobacco

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## ABSTRACT

**The addition of glyoxylate to tobacco (*Nicotiana tabacum*) leaf discs inhibited glycolate synthesis and photorespiration and increased net photosynthetic $^{14}CO_2$ fixation. This inhibition of photorespiration was investigated further by studying the effect of glyoxylate on the stimulation of photosynthesis that occurs when the atmospheric $O_2$ level was decreased from 21 to 3% (the Warburg effect). The Warburg effect is usually ascribed to the increased glycolate synthesis and metabolism that occurs at higher $O_2$ concentrations. Photosynthesis in control discs increased from 59.1 to 94.7 micromoles of $CO_2$ per gram fresh weight per hour (a 60% increase) when the $O_2$ level was lowered from 21 to 3%, while the rate for discs floated on 15 millimolar glyoxylate increased only from 82.0 to 99.7 micromoles of $CO_2$ per gram fresh weight per hour (a 22% increase). The decrease in the $O_2$ sensitivity of photosynthesis in the presence of glyoxylate was explained by changes in the rate of glycolate synthesis under the same conditions.**

**The rate of metabolism of the added glyoxylate by tobacco leaf discs was about 1.35 micromoles per gram fresh weight per hour and was not dependent on the $O_2$ concentration in the atmosphere. This rate of metabolism is about 10% the amount of stimulation in the rate of $CO_2$ fixation caused by the glyoxylate treatment on a molar carbon basis. Glyoxylate (10 millimolar) had no effect on the carboxylase/oxygenase activity of isolated ribulose diphosphate carboxylase. Although the biochemical mechanism by which glyoxylate inhibits glycolate synthesis and photorespiration and thereby decreases the Warburg effect is still uncertain, these results show that cellular metabolites can regulate the extent of the Warburg effect.**

$O_2$ inhibits net photosynthetic $CO_2$ fixation in all plants where the primary carboxylation reaction involves RuDP[1] carboxylase and the Calvin-Benson cycle. This inhibition results from the competitive binding of $O_2$ to the $CO_2$-binding site on the carboxylase with a consequent decrease in the carboxylation rate (1) and also from the stimulation of glycolate synthesis and metabolism caused by the higher $O_2$ levels. Increased glycolate metabolism causes a larger percentage of the newly fixed $CO_2$ to be oxidized back to $CO_2$ thus lowering the net photosynthetic rate still further (7, 15). This inhibition of net photosynthesis at increased $O_2$ levels (or conversely the stimulation of $CO_2$ fixation at lower $O_2$ levels) is often referred to as the Warburg effect.

Glyoxylate, an intermediate in the glycolate pathway normally formed in leaves from the action of glycolate oxidase on glycolate, inhibits glycolate synthesis when supplied to tobacco (*Nicotiana tabacum*) leaf discs (10). This inhibition is accompanied by a decrease in the rate of photorespiration measured as the release of previously fixed $^{14}CO_2$ into a stream of $CO_2$-free air and by a large increase in the net photosynthetic capacity of the tissue (10). The investigation into the ability of glycolate to exert metabolic controls over the rate of glycolate synthesis has been expanded in this study to include the effect of glyoxylate treatment on the increase in the photosynthetic $CO_2$ fixation which results when atmospheric $O_2$ levels are lowered from 21 to 3% (the Warburg effect). This study supports the view that there is a close relationship between photorespiration and the Warburg effect since the glyoxylate, which inhibits photorespiration, also largely abolishes the Warburg effect in leaf discs.

[1] Abbreviation RuDP: ribulose diphosphate.

## MATERIALS AND METHODS

Tobacco (*N. tabacum*) plants of the varieties Connecticut Shade and Havana Seed were grown in soil with automatic subsurface drip watering in a greenhouse with minimum night temperature of 15 C under natural illumination. The α-hydroxy-2-pyridinemethanesulfonic acid and the sodium glyoxylate were from Fluka AG, Buchs, Switzerland (Columbia Organic Chemicals, Columbia, S.C.). The spinach RuDP carboxylase was obtained from Sigma Chemical Co.

**Assay of Photosynthetic $^{14}CO_2$ Fixation.** Leaves were removed from plants in the greenhouse in the morning after 3 to 4 hr of sunlight and stored in the dark with their bases in water for 30 min. Six 1.6-cm discs were then cut per sample using a modified Latin square technique, strung together on thread and placed in the bottom of large 75-ml Warburg flasks containing 1.2 ml of water without wetting the upper surface of the discs. After 1 hr at 30 C in the light (340 $\mu E\ m^{-2}\ sec^{-1}$, 400–700 nM) the water was replaced with 1.2 ml of either 15 mM K-glyoxylate (pH 4.6) or water for the controls and returned to the light for an additional hr. Following this treatment a stream of gas containing either 21% $O_2$–79% $N_2$ or 3% $O_2$–97% $N_2$ was passed through the flasks and across the upper surface of the leaf discs at a rate of 250 ml/min. After 2 min a peristaltic pump was used to pump the $NaH^{14}CO_3$ solution (13.6 mM at a pumping rate of 0.5 ml/min) into a flask containing 2.0 ml of 2.5 N $H_2SO_4$ which was located in the gas stream prior to entering the Warburg flask. The resulting gas containing 600 $\mu l/l$ $^{14}CO_2$ was passed across the surface of the leaf discs for 5 min (11). The photosynthetic $^{14}CO_2$ fixation was terminated by removing the discs to boiling 20% ethanol. The discs were then homogenized in a TenBroeck homogenizer and the radiocarbon present determined by liquid scintillation counting.

**Assay of Glycolate Synthesis.** Five leaf discs were threaded together and placed in the bottom of 50-ml Erlenmeyer flasks with 1.5 ml of water and illuminated as above at 30 C for 1 hr. The water was removed and replaced with 1.5 ml of either 15 mM K-glyoxylate (pH 4.6) or water. After the flasks had remained open to the air for 45 min the flasks were closed with serum

stoppers and either 21% $O_2$–79% $N_2$ or 3% $O_2$–97% $N_2$ (both contained 600 μl/l $CO_2$) was passed through them at a rate of 250 ml/hr. After an additional 15 min the stoppers were moved aside and the liquid was removed, the discs were rinsed twice with 1.5 ml of water, and then 1.5 ml of 10 mM α-hydroxy-2-pyridinemethanesulfonic acid was added. After 3 min the discs were removed and plunged into boiling 20% ethanol. The discs were then homogenized, the glycolate purified by column chromatography on Dowex 1-acetate, and the glycolate determined colorimetrically (11). A correction was made for the amount of glycolate present in discs treated in an identical manner but without the sulfonate (10).

## RESULTS

**Effect of Glyoxylate on $CO_2$ Fixation by Leaf Discs in 21 and 3% $O_2$.** When the $O_2$ concentration in the atmosphere surrounding tobacco leaf discs was decreased from 21 to 3% the rate of photosynthesis by the tissue increased 60% (from 59.1 to 94.7 μmol $^{14}CO_2$ fixed/g fresh weight·hr [Table I]). Floating the discs on 15 mM glyoxylate for 1 hr resulted in 39% increase in their photosynthetic rate compared with discs on water (from 59.1 to 82.0 μmol $^{14}CO_2$/g fresh weight·hr). In addition, the glyoxylate treatment decreased the stimulation in $CO_2$ uptake observed upon lowering the $O_2$ concentration from 21 to 3% from the 60% increase observed with water-treated discs to only a 22% increase with glyoxylate-treated discs (from 82.0 to 99.7 μmol $^{14}CO_2$/g fresh wt·hr). Thus the Warburg effect, and presumably photorespiration, is three times greater in discs floated on water than in discs treated with glyoxylate.

The control rates of net photosynthetic $CO_2$ fixation are equivalent to about 6 mg/dm$^2$·hr, a rate that is fairly low for an intact tobacco leaf. The 600 μl/l $CO_2$ supplied to the leaf is sufficient to give less than half-maximal rates of $CO_2$ fixation in this system (11). This indicates that with leaf discs floated on water the internal $CO_2$ concentration in equilibrium with 600 μl/l in the atmosphere is somewhat lower than the internal $CO_2$ concentration in normal leaves photosynthesizing in 300 μl/l $CO_2$. Only about one-third of the leaf stomata are on the upper surface of the leaf and therefore exposed to the air stream; this also helps account for the lower rates of net photosynthesis by leaf discs.

The increase in net photosynthetic $CO_2$ fixation, from 59.1 to 82.0 μmol/g fresh weight·hr, caused by the glyoxylate treatment, amounts to an absolute increase in the photosynthetic rate of 22.9 μmol/g fresh weight·hr. When the metabolism of the exogenously added glyoxylate was measured under identical conditions, it was found that glyoxylate was metabolized at 1.3 to 1.4 μmol/g fresh weight·hr by the discs (Table II). The rate of glyoxylate metabolism was not affected by the concentration of $O_2$ in the atmosphere and was constant for 1 hr. These rates of glyoxylate metabolism are about 10% of the glyoxylate-dependent increase in the rate of $CO_2$ fixation on a mole carbon basis. The changes observed in the Warburg effect cannot be explained by changes in the rate of glyoxylate metabolized at different $O_2$ concentrations. The decrease in the Warburg effect caused by glyoxylate treatment can be explained by the difference in the rates of glycolate synthesis under the same conditions.

Table I. Effect of Glyoxylate on the Stimulation of Net Photosynthesis by Lowered $O_2$ Levels

Net photosynthesis was determined as described in MATERIALS AND METHODS after the discs were floated on a glyoxylate solution or water for 1 hr. The results presented are the means of seven different experiments. The $CO_2$ concentration was 600 μl/l.

| Treatment | Rate of Net Photosynthesis | | Stimulation by Lowering $O_2$ to 3% |
|---|---|---|---|
| | 21% $O_2$ | 3% $O_2$ | |
| | μmol $^{14}CO_2$/g fresh wt·hr | | % |
| Water | 59.1 | 94.7 | 60 |
| Glyoxylate | 82.0 | 99.7 | 22 |
| Glyoxylate Effect | +39% | +5% | |

Table II. Rate of Metabolism of [1-$^{14}$C]Glyoxylate by Tobacco leaf Discs in the Light

Glyoxylate metabolism was measured using the techniques described in MATERIALS AND METHODS for measuring $^{14}CO_2$ fixation except that the 15 mM glyoxylate was labeled with $^{14}C$ in the C-1 position ($10^6$ dpm/μmol) while the $CO_2$ was unlabeled. The gas flowing across the surface of the discs was 600 μl/l $CO_2$ with either 3% or 21% $O_2$ (balance $N_2$). After exiting the reaction flask, the gas was bubbled through 1 M ethanolamine to collect $^{14}CO_2$ released for determination by scintillation counting. After 1 hr the discs were quickly rinsed with water and plunged into boiling 20% ethanol. The killed discs were homogenized, and the glyoxylate and the products of its metabolism isolated by Dowex 1-acetate chromatography (10). The results shown are the average of two experiments.

| Atmosphere | Rate of Glyoxylate Metabolism | $CO_2$ Release 30 min | $CO_2$ Release 60 min |
|---|---|---|---|
| | μmol/g fresh wt·hr | μmol/g fresh wt | |
| 21% $O_2$ 600 μl/l $CO_2$ | 1.36 | 0.0098 | 0.019 |
| 3% $O_2$ 600 μl/l $CO_2$ | 1.35 | 0.0055 | 0.010 |

**Effect of Glyoxylate on Glycolate Synthesis by Tobacco Leaf Discs.** Glycolate accumulation by the tobacco leaf discs in the presence of the sulfonate decreased 54% when the $O_2$ concentration was decreased from 21 to 3%, from 57.2 to 26.4 μmol of glycolate accumulated/g fresh weight·hr (Table III). Discs floated on a glyoxylate solution for 1 hr synthesized 56% less glycolate when the $O_2$ level was decreased (from 33.5 μmol/g fresh weight·hr at 21% to 14.8 at 3% $O_2$).

Glycolate synthesis showed the same sensitivity to glyoxylate treatment in 3 and 21% $O_2$ (Table III). This suggests that the same mechanism of glycolate synthesis functioned at both $O_2$ levels. The fact that only a small increase in the net photosynthetic rate occurred upon glyoxylate treatment in 3% $O_2$ (Table I) indicates that at this $O_2$ concentration, because of the slow rate of glycolate synthesis, the photorespiratory $CO_2$ loss was not a major determinant in the net photosynthetic rate.

The substantial rates of glycolate synthesis observed in 3% $O_2$ in illuminated leaf discs suggest that the $O_2$ concentration at the site of glycolate synthesis within the leaf, presumably in the chloroplast, is considerably higher than the concentration around the leaf disc under these conditions. When the $O_2$ concentration in the atmosphere was lowered to 0%, the rate of glycolate synthesis was less than 10% of that which occurred in 21% $O_2$ (unpublished results).

The approximately equal rates of glycolate synthesis and net photosynthetic $CO_2$ fixation are in agreement with previously published experimental findings (10, 11, 15) and theoretical considerations (14). As a minimal value, 25% of the carbon atoms in glycolate are lost as $CO_2$ during the glycine decarboxylase reaction

Table III. Effect of Glyoxylate on the Inhibition of Glycolate Synthesis Caused by Decreased $O_2$ Levels

Glycolate accumulation was measured as described in MATERIALS AND METHODS after the leaf discs were floated on a glyoxylate solution or water for 1 hr followed by a 3 min treatment with α-hydroxy-2-pyridinemethanesulfonic acid to block glycolate oxidase activity. The $CO_2$ concentration was 600 μl/l. The results shown are the average of three experiments.

| Treatment | Rate of Glycolate Synthesis | | Inhibition by Lowering $O_2$ to 3% |
|---|---|---|---|
| | 21% $O_2$ | 3% $O_2$ | |
| | μmol/g fresh wt·hr | | % |
| Water | 57.2 | 26.4 | 54 |
| Glyoxylate | 33.5 | 14.8 | 56 |
| Glyoxylate Effect | -41% | -44% | |

(a still larger percentage would result from any additional direct decarboxylation of glyoxylate that occurs [5]). Equal rates for the two reactions would require that for every molecule of net $CO_2$ fixed, 1 molecule of glycolate (2 atoms of carbon) is formed. If 25% of the carbon in glycolate is lost, for each mole of $CO_2$ fixed (net), 0.5 mol of $CO_2$ would be lost by photorespiration. In other words, the photorespiratory rate would by approximately one-half of the net photosynthetic rate.

**Effect of Glyoxylate on $CO_2$ Fixation by Purified Ribulose Diphosphate Carboxylase.** Glyoxylate did not affect the rate of $CO_2$ fixation by purified activated RuDP carboxylase or the percentage inhibition of this activity by 100% $O_2$, a measure of the oxygenase activity of the enzyme. The enzyme assayed as in (9) with or without 10 mM glyoxylate showed an activity of 0.38 and 0.28 μmol/mg protein·min in 21 and 100% $O_2$, respectively. This indicates that the effects observed in leaf discs are not the result of glyoxylate itself modifying the synthesis of P-glycolate (and subsequently glycolate) by RuDP carboxylase/oxygenase. This does not, however, exclude the possibility that some compound derived from glyoxylate (or synthesized because of the presence of glyoxylate) may affect the carboxylase in the leaf tissue and thereby inhibit glycolate synthesis.

## DISCUSSION

Several independent types of evidence indicate that the stimulation in net photosynthetic $CO_2$ fixation that results upon lowering the $O_2$ concentration from 21 to 3% (the Warburg effect) is caused by the decreased rate of glycolate synthesis and therefore photorespiration that occurs at the lower $O_2$ level. The Warburg effect is largest in typical $C_3$ plants which show the most rapid photorespiration, intermediate in *Panicum milioides*, a species with intermediate photorespiratory rates (2), and smaller or nonexistent in $C_4$ plants which show little or no photorespiration (15). The temperature responses of photorespiration and the Warburg effect are similar (6). High concentrations of $CO_2$ that inhibit photorespiration decrease the extent of the Warburg effect in a similar manner, and glyoxylate, a metabolite that inhibits glycolate synthesis (Table II) and photorespiration (10) in leaf discs, also inhibits the Warburg effect (Table I). Chollet (4) was unable to obtain a glyoxylate-dependent stimulation in net photosynthesis and the lessening of the Warburg effect by glyoxylate in tobacco leaf discs. This difference may in part be explained by the very different plant growth and assay conditions that were used.

Glycolate is synthesized at substantial rates by leaf discs being flushed with 3% $O_2$. In the experiments summarized in Table III, the rate of glycolate synthesis in 3% $O_2$ was 46% of the rate at 21% $O_2$. Assuming that this glycolate contributes to photorespiratory $CO_2$ loss, estimates of photorespiration which are based solely on the difference in net photosynthesis between 3 and 21% $O_2$ (3) may only measure about one-half of the actual photorespiration because of the severe underestimation of the gross photosynthetic rate.

The magnitude of the Warburg effect, because it is a measure of photorespiration, has been assumed by many authors to be determined only by the basic enzymology of the carboxylating enzymes in $C_3$ plants (1, 7) and the concentrations of $CO_2$ and $O_2$. Robinson *et al.* (12, 13) have shown with isolated chloroplasts that the Warburg effect can be decreased by supplying sugar phosphates including ribose-5-P and fructose-1,6-diP in addition to changing the $CO_2$ concentrations. These authors do not attribute this difference to changes in glycolate synthesis. The glyoxylate effect on leaf discs reported here appears to be the first demonstration that the Warburg effect of intact leaf tissue can be altered by factors other than the $O_2$ and $CO_2$ levels, and temperatures which may in part be explained by the $O_2$ and $CO_2$ effects (8). This observation confirms that the Warburg effect and the rate of glycolate synthesis and photorespiration can be controlled by altering the intracellular concentration of key metabolites. The exploitation of such an approach may lead some day to increased photosynthesis and yield in important crop species.

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