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ABSTRACT

Isolated soybean leaf mesophyll cells decarboxylated exogenously added [1-14C]glycine and [1-14C]glycolate in the dark. The rate of CO2 release from glycine was inhibited over 50% by isonicotinic acid hydrazide and about 80% by KCN, two inhibitors of the glycine to serine plus CO2 reaction. The release of CO2 from glycolate was inhibited by less than 50% under the same conditions. This indicates that about 50% of the CO2 released from glycolate occurred at a site other than the glycine to serine reaction. The sensitivity of this alternative site of CO2 release to an inhibitor of glycylate oxidase (methyl-2-hydroxy-3-butyrate) but not an inhibitor of the glutamate-glyoxylate aminotransferase (2,3-epoxypropanoate) indicates that this alternative (isonicotinic acid hydrazide insensitive) site of CO2 release involved glycolate. Catalase inhibited this CO2 release. Under the conditions used it is suggested that about half of the CO2 released from glycolate occurred at the conversion of glycine to serine plus CO2 while the remaining half of the CO2 loss resulted from the direct oxidation of glycolate. The rate of glycine decarboxylation by the glycine to serine reaction was apparently controlled by the amount of NAD in the mitochondria. Mitochondrial electron transport inhibitors, KCN and actinomycin A, inhibited glycine decarboxylation while an uncoupler, 2,4-dinitrophenol, stimulated the reaction. Competition within the mitochondria between the enzymes of dark respiration and glycine decarboxylation for limiting NAD may force substantial amounts of the glycolate formed to be decarboxylated by the direct oxidation of glycolate.

In plant species where the primary photosynthetic carboxylation reaction involves the formation of P-glycerate catalyzed by ribulose bisP carboxylase, glycolate is synthesized in the light at rates which are approximately equal, on a molar basis, to the net rates of photosynthetic CO2 fixation (7, 14, 16). The rate of glycine synthesis can be controlled by the atmospheric concentrations of O2 and CO2 (8, 12) and the intracellular concentration of certain key metabolites including aspartate, glutamate, and glycolate (6, 7, 9, 10).

Glycolate is usually assumed to be metabolized by the pathway outlined by Tolbert (13). In this reaction sequence (Fig. 1), glycolate is oxidized by the enzyme glycolate oxidase to glyoxylate. This reaction in leaves is coupled to the reduction of O2 to H2O2. Glyoxylate is converted to glycine by either a glutamate- or serine-dependent glyoxylate transaminase. In the mitochondria several reactions combine to convert 2 mol of glycine to 1 mol each of serine, CO2, and NH3 (1, 5). In isolated mitochondria there is an absolute requirement of NAD for this conversion to occur. The resulting NADH is oxidized in mitochondrial preparations by the oxidative electron transport chain with the concurrent synthesis of 3 mol of ATP for each atom of oxygen taken up (1, 5). The serine thus formed is deaminated to hydroxypropionate which is in turn converted to P-glycerate and then glycine.

In addition to being transaminated to glycine, glyoxylate may be further oxidized directly. H2O2 produced either from the glycolate oxidase reaction (2, 3, 15) or in the light by a Mehler reaction within the chloroplast (17) can be the oxidant (Fig. 1). Such a reaction may or may not be enzymic. The carboxyl carbon from glyoxylate is released as CO2. The C1 fragment from the a-carbon forms formic acid from the reaction with H2O2. Grodzinski (3) suggested that this C1 unit may supply a substrate for the hydroxymethyltransferase and react with glycine to form serine. The a-carbon could also be oxidized completely to CO2 and contribute to the respiratory CO2 loss.

This paper presents a series of experiments which have taken advantage of the known inhibitors of the reactions in the glycolate pathway to attempt to evaluate if two sites of CO2 release do exist in the glycolate pathway, and the relative contribution of each site. The inhibitors used were MHB2, a suicide analog of glycolate that irreversibly inhibits glycolate oxidase (4); INH, an inhibitor of the glycine to serine plus CO2 reaction (11); 2,3-epoxypropanoate also known as glycide, which inhibits glutamate glyoxylate aminotransferase (6), and catalase, an enzyme which decreases glyoxylate oxidation by destroying H2O2.

The existence of multiple sites of CO2 release in the glycolate pathway is clearly demonstrated. It is also proposed that the competition between dark respiration and glycine decarboxylation for NAD in the mitochondria may provide possibilities for directing the choice of the substrate for photosynthetic CO2 between glycine and glycolate.

MATERIALS AND METHODS

[1-14C]Glycine and [1-14C]glycolate were obtained from Amersham Corp. The glycine was purified by high voltage paper electrophoresis and the glycolate by Dowex 1-acetate chromatography (9, 10) before use. Thymol-free catalase (Sigma) was used. The MHB was a generous gift of Dr. P. J. Jewess, and the glycide from Dr. I. Zelitch.

Soybean mesophyll cells were isolated by a mechanical grinding technique from mature leaf tissue (8). The cells had an average photosynthetic capacity of about 200 μmol CO2 fixed/mg Chl-h (35 C, 5 mM NaHCO3, 1,400 μE/m2-s PAR and were contaminated by very few isolated chloroplasts.

The decarboxylation reactions were carried out in 10-ml Warburg flasks with centerwell containing a filter paper wick moistened with 25 μL of 5 N monoethanolamine. The reaction mixture

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2 Abbreviations: MHB: methyl d,l-2-hydroxy-3-butyrate; INH: isonicotinic acid hydrazide; DNP: 2,4-dinitrophenol.
contained a final concentration of 0.33 mM sorbitol, 50 mM Tris-HCl (pH 7.8), 2 mM NaNO₃, 2 mM EDTA, 1 mM MnCl₂, 1 mM MgCl₂, 0.5 mM K-phosphate, and 2 mM DTT (8). After the flasks containing the reaction mixture and any added inhibitors were equilibrated in a 25°C shaking water bath for 2 to 5 min 30 μl of the isolated cell preparation containing 20 to 30 μg Chl was added and the water bath was covered to exclude all light. After a dark preincubation period of 30 min, 100 μl of the radioactive substrate was carefully pipetted into the reaction chamber and the flask sealed with a plastic stopper. After a 30-min dark reaction period, 100 μl of 3 M H₂SO₄ was added to the reaction chamber to kill the cells and release all ¹⁴CO₂ formed. Following an additional 30-min period, the filter paper wicks were removed and the ¹⁴CO₂ trapped was determined by liquid scintillation counting. The toluene-based scintillation cocktail included 100 μl of protocol (Amersham Corp.) to facilitate release of the ¹⁴CO₂ from the filter paper strip. All values were corrected for the amount of ¹⁴CO₂ released from the substrates in the absence of cells.

When the amount of [¹⁴C]glycine and [¹⁴C]glycolate produced was to be determined, the acidified reaction mixture was added to 2 ml of ethanol and placed in a boiling water bath for 5 min. The amino acid fraction was isolated from a Dowex 50 column after the acidic compounds were first removed by passage through a Dowex 1-aceate column (10). Glycine was then isolated by high voltage paper electrophoresis and the amount of radiocarbon determined by liquid scintillation counting (6).

**RESULTS AND DISCUSSION**

Isolated soybean mesophyll cells in the dark released ¹⁴CO₂ from [¹⁴C]glycine or [¹⁴C]glycolate. Rates of CO₂ release were linear for at least 60 min. Because the reactions were carried out at pH 7.8 (with over 99% of either substrate present as the anion) glycine and glycolate decarboxylation was limited by the amount of uptake. The rate of CO₂ release from either substrate was proportional to the substrate concentration up to 0.5 mM and was not saturated by 1.0 mM. Rates of CO₂ release were up to 40 μmol/mg Chl.h at these very high substrate concentrations but averaged much lower at the concentrations (5–10 mM) reported here.

The amount of CO₂ released from either glycine or glycolate was decreased by incubating the cells with INH, an inhibitor of the glycine to serine plus CO₂ reaction, for 30 min before adding the substrate (Fig. 2). INH inhibited the rate of CO₂ release from glycine by over 90%, and that from glycolate by less than 50% (Fig. 2). The differences in sensitivity of these two reactions to INH inhibition indicate that some CO₂ can be released from glycolate at an alternative site which does not involve the conversion of glycine to serine plus CO₂. Since only about half of the CO₂ released from glycolate was sensitive to INH, under conditions where glycine to serine plus CO₂ conversion was blocked, about half of the CO₂ released probably arose from this alternative site.

To corroborate the conclusions from results with INH another inhibitor of the glycine to serine plus CO₂ reaction, KCN, was employed. Cyanide would be expected to inhibit glycine oxidation by preventing the oxidation of NADH to NAD, an essential cofactor for the reaction (1, 5). Like INH, KCN inhibited CO₂ release from both glycine and glycolate (Fig. 3). The oxidation of glycine to CO₂ was inhibited about 75% while the metabolism of glycolate to CO₂ was only inhibited about 25%. This again supports the existence of a substantial source of CO₂ release from glycolate which is not related to the conversion of glycine to serine plus CO₂. Identical results were found when another inhibitor of oxidative electron transport, antimycin A was employed (unpublished observation).

Similar results were obtained when increasing concentrations of
unlabeled glycine were added to cells which were metabolizing [14C]glycolate. As the concentration of unlabeled glycine supplied to the cells increased, the specific radioactivity of the glycine pools derived from intracellular [14C]glycolate would be expected to decrease, thus decreasing the amount of 14CO2 released. Figure 4 shows that at unlabeled glycine concentrations that are 20 to 50 times larger than the concentration of [14C]glycolate, the rate of 14CO2 released was decreased by only 40%. Since over half of the 14CO2 formed from [14C]glycolate was not susceptible to dilution by unlabeled glycine, the results suggest that this CO2 was released in the reaction sequence (Fig. 1) prior to glycine formation. The substrate for the alternative site of CO2 release would be glycolate or glycine. Glycine, an inhibitor of glutamate-glyoxylate aminotransferase, inhibited CO2 release from glycolate by 40% while it decreased glycine formation from glycolate by 75% (Table I). The

![Graph](https://example.com/graph1.png)

**Fig. 3.** Effect of KCN on rate of CO2 release from glycolate and glycine. The isolated soybean mesophyll cells were incubated with KCN at the concentration indicated at 25 C. After 30 min, [1-14C]glycolate or [1-14C]glycine (10 mM) was added and the rate of 14CO2 release measured for an additional 30 min.

![Graph](https://example.com/graph2.png)

**Fig. 4.** Effect of increasing concentrations of unlabeled glycine on rate of 14CO2 evolution from [1-14C]glycolate. After the cells were allowed to equilibrate in the reaction mixture for a few minutes at 25 C, 5 mM [1-14C]glycolate and the indicated concentration of unlabeled glycine were added at the same time. The reaction was terminated after 30 min and the 14CO2 released was determined.

higher relative sensitivity of glycine synthesis as compared to CO2 release again supports the idea that a major source of CO2 release from glycolate occurs before glycine formation in the glycolate pathway (Fig. 1). CO2 release from glycolate was highly sensitive (96% inhibited) to the glycolate oxidase inhibitor MHB. This shows that glycine must be oxidized to glyoxylate before any CO2 is released. The source of this alternative (INH-insensitive) site of CO2 release apparently was from glyoxylate since CO2 release from glycolate was sensitive to MHB but not glycine (Fig. 1). Further evidence on this point comes from the finding that this alternate site of CO2 release was inhibited by catalase. As mentioned in the introduction, H2O2, possibly produced by the glycolate oxidase reaction, reacts rapidly with glyoxylate to produce CO2. When excess catalase was supplied to the assay media around the cells, H2O2 was destroyed and thereby the direct oxidation of glyoxylate was prevented (Table I). Catalase decreased the rate of CO2 release from glycolate by about 50%. Catalase probably did not enter the cells. H2O2 is readily diffusable, however, and can be detected in the solution around illuminated leaf tissue. The reduction of H2O2 most likely occurred outside the cells.

The addition of catalase and the resultant inhibition of the direct oxidation of glyoxylate resulted in increased glycine formation from glycolate (Table I). Similar increases in the size of the glycine pool were found when the glycine to serine plus CO2 reaction was blocked with INH. The addition of saturating amounts of both catalase and INH resulted in nearly complete inhibition of CO2 release from glycolate. The additive nature of these two inhibitors indicates that the compounds were effective at two different sites of CO2 release (Table I). This is consistent with the scheme in Figure 1.

None of the inhibitors used decreased the rate of glycolate uptake by the cells or, with the exception of MHB, the activity of glycolate oxidase. Treatment with INH, KCN, and catalase did increase, however, the size of the glycolylate pool by as much as 3-fold (data not shown).

When 25 mM [1-14C]glycolate was fed to INH-treated cells for 30 min under standard conditions 4.55 (± 0.18) μmol CO2/mg Chl was produced and 10.07 (± 0.28) μmol radioactive glycine and glyoxylate were present. If the glycine and glyoxylate pools were completely decarboxylated from glycolate in the absence of INH, they should yield 5.04 μmol CO2/mg Chl. This again indicates that approximately equal amounts of CO2 were released by the glycine to serine plus CO2 reaction and by the direct oxidation of glyoxylate under these conditions. This suggests that for every 1 mol of glycine that was oxidized to CO2 from glycolate, 2 mol of glycine would be processed by the glycine to serine plus CO2 reaction.

The sensitivity of CO2 release from glycine and glycolate to cyanide (or antimycin A) suggests that under the conditions used
The NADH formed in the mitochondria from the glycine to serine reaction (1, 5) was oxidized back to NAD by the electron transport chain. This contention was further supported by the observation that DNP, an uncoupler of oxidative phosphorylation, stimulated the rate of CO₂ release from both glycine and glycolate (Fig. 5). With the uncoupler increasing the rate of electron transport, NADH oxidation would increase thereby supplying more substrate (NAD) for the glycine decarboxylation reaction.

CONCLUSIONS

All reactions described were carried out in the dark to avoid the complications presented by photosynthetic refixation of photorespired CO₂ and the production of H₂O₂ from the few isolated chloroplasts in the cell preparations. When CO₂ release from the glycine to serine plus CO₂ reaction was inhibited by INH and KCN (or antimycin A) or when glycine formation was inhibited by glycidate, isolated soybean mesophyll cells were still able to metabolize about 50% as much [¹⁴C]glycine to [¹⁴C]CO₂ as did uninhibited cells. Similarly, a 20- to 50-fold excess of exogenously added unlabeled glycine only reduced the rate of [¹⁴C]CO₂ from [¹⁴C]glycolate by about 50%. These data show that substantial amounts of CO₂ can be released from glycolate by an alternate reaction which does not involve the conversion of glycine to serine plus CO₂. The sensitivity of this alternate site to MHB and catalase suggests that the reaction involves the oxidation of glycolylate by H₂O₂.

The effects caused by INH, KCN, glycidate, and catalase all suggest that under the conditions employed isolated soybean mesophyll cells oxidizing exogenously added glycolate are able to release approximately equal amounts of CO₂ from the direct oxidation of glycolylate and from the conversion of glycine to serine plus CO₂. This is further supported by the observation (12) that rates of glycolylate synthesis are about twice the rates of glycine synthesis in soybean cells. All of these studies involved the use of inhibitors and therefore some perturbations were to be expected in the normal steady-state pool sizes of some intermediates in the glycolate pathway, notably glycine (Table I). Such changes in substrate availability may alter the relative rates of CO₂ release by the two sites. Exact quantification of the rates of CO₂ release by the two sites must await further experiments that avoid the complications that result from using metabolic inhibitors.

The stoichiometry of CO₂ release from glycolylate and glycine is expected to be different. Glycolylate oxidation should yield 1 mol of CO₂ (from the carboxyl carbon) and 1 mol of a C₁ fragment from the methylene carbon per mol of glycolylate metabolized. Grodzinski (3) suggested that this C₁ fragment may react with glycine to form serine or may be further oxidized to CO₂. Depending on which reaction predominated, 50 to 100% of the carbon in glycolylate metabolized by this pathway would be released as CO₂. The stoichiometry of the glycine to serine plus CO₂ reaction releases as CO₂ 25% of the carbon from glycolate that transverses this step. The relative contributions of the two pathways of CO₂ release would control the percentage of the carbon in glycolate released as CO₂.

CO₂ released from the conversion of glycine to serine was probably tightly regulated by the amount of NAD in the mitochondria. When NADH oxidation, and presumably NAD availability, was decreased by KCN treatment, CO₂ release from glycine and glycolate was lessened (Fig. 3). Conversely, when NADH oxidation was increased by the uncoupler DNP, CO₂ release from both glycine and glycolate was stimulated.

Since there is a requirement within the mitochondria for NAD for both glycine decarboxylation and dark respiration (the Krebs tricarboxylic acid cycle), it is interesting to consider the possible interaction of these two pathways. The rate of CO₂ release by photorespiration is approximately three to five times the rate of CO₂ release by dark respiration in C₃ plants (16). The rate of oxidative phosphorylation and electron transport limit the availability of NAD within the mitochondria, as indicated by the ability of DNP to stimulate glycine decarboxylation in the dark (Fig. 5). If the amount of respiration (photorespiration and dark combined) in the light is much greater than the amount in the dark, then glycine decarboxylation and dark respiration must be in competition for sharply limiting supplies of NAD in the light.

Assuming that NADH oxidation and oxidative phosphorylation are tightly and invariably coupled and that no other major mechanisms of NADH oxidation exist in the mitochondria in the light, then the rates of glycine oxidation and dark respiration must be co-regulated through NAD. Rapid flux of metabolites through the tricarboxylic acid cycle should lessen glycine decarboxylation. If dark respiration proceeds at the same time as rapid rates of photorespiratory CO₂ loss, then a substantial portion of the CO₂ must be released by the direct oxidation of glycolylate. Glycolylate metabolized by this reaction releases two to four times as much carbon as CO₂ as does the decarboxylation of glycine. Thus, dark respiration may indirectly control photorespiration, not by regulating glycolate synthesis but rather by altering the efficiency of glycine decarboxylation.

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LITERATURE CITED