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Simultaneous Oxidation of Glycine and Malate by Pea Leaf Mitochondria[1]

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ABSTRACT

Mitochondria isolated from pea leaves (*Pisum sativum* L.) readily oxidized malate and glycine as substrates. The addition of glycine to mitochondria oxidizing malate in state 3 diminished the rate of malate oxidation. When glycine was added to mitochondria oxidizing malate in state 4, however, the rate of malate oxidation was either unaffected or stimulated. The reason both glycine and malate can be metabolized in state 4 appears to be that malate only used part of the electron transport capacity available in these mitochondria in this state. The remaining electron transport capacity was used by glycine, thus allowing both substrates to be oxidized simultaneously. This can be explained by differential use of two NADH dehydrogenases by glycine and malate and an increase in alternate oxidase activity upon glycine addition. These results help explain why photorespiratory glycine oxidation and its associated demand for NAD do not inhibit citric acid cycle function in leaves.

During photosynthetic CO_2 fixation by C-3 plants, a substantial amount of newly formed carbohydrate is oxidized back to CO_2 by the process of photorespiration (29). The photorespiratory CO_2 can arise either from the direct decarboxylation of glyoxylate (9, 19) or during the mitochondrial oxidation of glycine to NH_3, CO_2, and methylene tetrahydrofolate by the enzyme glycine decarboxylase. In the latter case, NAD^+ is the electron acceptor and it in turn is reoxidized by the mitochondrial electron transport chain (5, 17). Current data suggest that in soybean (20, 21) and *Arabidopsis thaliana* (26) the major site of CO_2 release is the glycine decarboxylase reaction. In these species, glyoxylate decarboxylation occurs only when amino donor deprivation results in the accumulation of large amounts of glyoxylate. High rates of formate dehydrogenase activity in spinach, which is distributed in relation to the photosynthetic and photorespiratory activities of the different tissues, may indicate that direct glyoxylate decarboxylation to CO_2 and formate is more important in this species (20).

In mitochondria, the rate of NADH oxidation and, therefore, O_2 reduction is controlled by the availability of ADP. Hence, electron transport is faster in the presence of ADP (state 3) or an uncoupler than it is after the ADP has been phosphorylated to ATP (state 4). Similarly, tricarboxylic acid cycle function and glycine oxidation are restricted by the availability of mitochondrial NAD^+. We were interested in determining if the demand for NAD^+ to satisfy the need for photorespiratory glycine oxidation decreased NAD^+ availability and thereby decreased the rate of tricarboxylic acid cycle function.

Several authors have suggested the total or partial inhibition of the tricarboxylic acid cycle in the light (1, 2, 7, 10). Others have shown that the cycle continued in the light (3, 8, 15). The NADH produced by glycine oxidation can be used by malate dehydrogenase to reduce oxaloacetate to malate (17) and this malate can be linked by a shuttle system to the peroxisomal reduction of hydroxypyruvate to glycerate (12, 28).

Isolated pea leaf mitochondria readily use both glycine and malate as substrates. In the experiments described, we added glycine to mitochondria metabolizing malate as a model reaction for determining the effect of photorespiratory glycine oxidation on tricarboxylic acid cycle function by these plants.

MATERIALS AND METHODS

Mitochondria were isolated from 2- to 4-week-old greenhouse-grown pea (*Pisum sativum* L.) seedlings. Younger plants could not be used because the high lipoxygenase activity precluded the isolation of well coupled mitochondria. After chilling for 1 h, the leaves and stems (approximately 300 g) were ground for 2 s in a 4-L Waring blender in 1 L of grinding medium (0.5 M sorbitol, 50 mM MOPS[2]-NaOH, pH 7.5, 5.0 mM EDTA, and 0.5% BSA). The inclusion of reducing agents, including 5 mM ascorbate, 2 mM DTT, 2 mM thioglycolate, or 10 mM cysteine, resulted in inconsistent coupling ratios and varying amounts of KCN-insensitive respiration and was avoided.

The homogenate was filtered through two layers of Miracloth (Calbiochem) and the filtrate was centrifuged at 5,000*g* for 2 min. The mitochondria were then collected at 20,000*g* for 5 min. This pellet was resuspended in cold medium containing 0.5 M sorbitol, 20 mM MOPS-NaOH (pH 7.2), 0.1% BSA, and 2 mM glycine and centrifuged at 1,000*g* for 10 min and then 7,000*g* for 10 min. The final pellet was resuspended in the same medium and either used directly or further purified on a Percoll (Pharmacia Fine Chemicals) gradient. In the latter case, 1 ml mitochondrial preparation was carefully layered atop a step gradient composed of (bottom to top) 2 ml each 40%, 25%, and 15% Percoll in 0.5 M sorbitol, 20 mM MOPS (pH 7.2), and 0.1% BSA. After centrifugation at 6,800*g* for 30 min, the mitochondria were removed from the interface between the 25% and 40% layers. The Chl remained dispersed within the lower portion of the 15% and the upper portion of the 25% steps.

O_2 uptake was measured using a Hansatech O_2 electrode (King's Lynn, U. K.) and $^{14}CO_2$ release from [U-^{14}C]malate (Amersham) or [1-^{14}C]glycine (Research Products International) was determined as described earlier (20, 22). The reaction medium (1 ml) contained 0.3 M sorbitol, 20 mM MOPS-NaOH (pH 7.2), 5 mM sodium phosphate, and 0.1% BSA. All biochemicals were from Sigma Chemical Co.

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[2] Abbreviation: MOPS, 3-(*N*-morpholino)propanesulfonic acid.

RESULTS AND DISCUSSION

Mitochondria isolated from pea seedling leaves metabolized malate and glycine with rates that were equivalent to those noted earlier with spinach leaf preparations (5, 17, 20). The P/O ratios were 2.2 to 2.6 for both substrates whether determined by measuring P esterification or the O_2 electrode technique of Estabrook (6). The respiratory control ratios (state 3/state 4) were consistently higher for malate (4 to 9) than for glycine (2.5 to 5.0). The Percoll gradient-purified mitochondria showed no detectable lipoxygenase activity.

The release of $^{14}CO_2$ from [U-^{14}C]malate showed a reproducible lag of 3 to 5 min, but then proceeded at a linear rate for the next 30 min (Fig. 1). This lag was not apparent in the O_2 electrode traces and indicates that malate dehydrogenase activity precedes malic enzyme activity in these mitochondria. CO_2 release from glycine could be measured immediately as could glycine-dependent O_2 uptake. To maintain the ability to oxidize glycine (up to 9 h), mitochondria had to be stored in a low concentration (2 mM) of glycine. Mitochondria stored on ice without glycine lost the capacity to metabolize glycine with a half-time of decay of about 1 h.

When unlabeled glycine was added to mitochondria that were actively metabolizing [U-^{14}C]malate in state 3, the rate of $^{14}CO_2$ release from the malate was decreased (Fig. 2). In this experiment, 15 mM glycine decreased the rate of CO_2 release from 40 mM malate from 156 to 98 nmol/mg protein·min. Half-maximal inhibition occurred between 2 mM and 4 mM glycine. At 15 mM [1-^{14}C]glycine in the presence of 40 mM malate, the rate of CO_2 release from glycine (state 3) was 92 nmol/mg protein·min. It is interesting to note that the total rate of CO_2 release from 15 mM glycine and 40 mM malate was higher than the rate in saturating concentrations of malate alone.

If the mitochondria metabolizing [U-^{14}C]malate were allowed to consume all the added ADP (state 4) before the glycine was added, the results were quite different. The addition of 15 mM glycine either had no effect on or, as in this experiment, increased the rate of CO_2 release from [U-^{14}C]malate (Fig. 2). These data strongly suggested that pea leaf mitochondria in state 4 that were metabolizing saturating concentrations of malate still had reserve electron transport capacity for oxidizing the additional NADH produced by glycine oxidation.

This phenomenon was studied in more detail with an O_2 electrode. The addition of 20 mM glycine to mitochondria metabolizing malate in state 3 had little effect on the rate of O_2 uptake

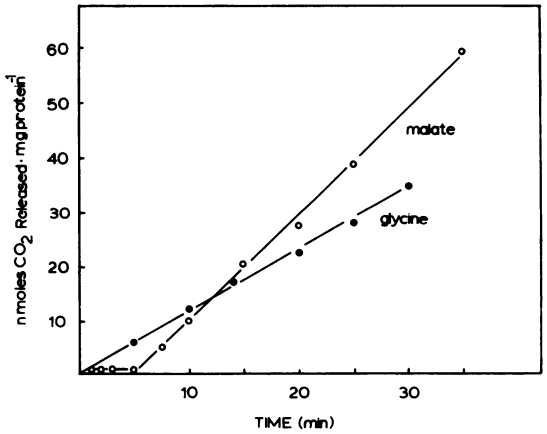

FIG. 1. The release of $^{14}CO_2$ from 20 mM [U-^{14}C]malate or 10 mM [1-^{14}C]glycine by pea leaf mitochondria. Mitochondria in state 3 were incubated with either glycine or malate. At the indicated time, the reaction was stopped by adding 0.2 N H_2SO_4 and the $^{14}CO_2$ released was trapped in ethanolamine and counted (20).

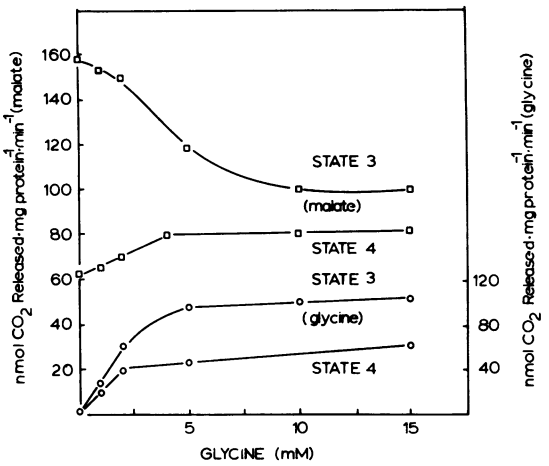

FIG. 2. Effect of increasing glycine concentrations on CO_2 release from malate and glycine by pea leaf mitochondria. Mitochondria were brought into state 3 or state 4 by the addition of 1 mM or 25 μM ADP, respectively. In the top two curves, 20 mM [U-^{14}C]malate and unlabeled glycine at the concentration indicated were added. For the two bottom curves, [1-^{14}C] glycine and 40 mM unlabeled malate were added. After 15 min, the reaction was stopped by acidification and any $^{14}CO_2$ released was trapped and counted as in Figure 1.

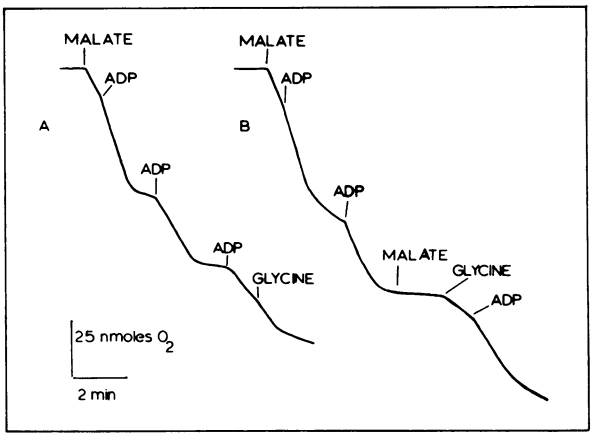

FIG. 3. The effect of glycine addition on the rate of O_2 uptake by pea leaf mitochondria metabolizing 20 mM malate in state 3 (A) and state 4 (B). The amount of ADP added was 100 nmol and glycine was 10 mM.

(Fig. 3A). If, however, the mitochondria were allowed to enter state 4 before the glycine was added, the glycine caused a large increase in the rate of O_2 uptake (Fig. 3B). The increased state 4 rates approached, but never exceeded, the state 3 malate rates. The addition of another 20 mM malate instead of glycine at this point had no effect. The mitochondria, therefore, were not substrate limited. The addition of uncouplers, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and 2,4-dinitrophenol, or inhibitors of mitochondrial glycine oxidation, isonicotinic acid hydrazide (24), amino acetonitrile (27), and glycine hydroxamate (14), prevented glycine from stimulating the state 4 rate of malate oxidation. The glycine-stimulated state 4 rate was constant for at least 15 min.

One possible explanation for these results could be that oxaloacetate and pyruvate were being formed from malate and accumulating in either the matrix of the mitochondria or in the assay medium. These compounds could then be reduced by the NADH formed upon glycine oxidation. Analysis of the mitochondria and supernatant fractions, however, did not reveal significant amounts of either oxaloacetate or pyruvate.

During the course of these studies, we discovered that, whereas

arsenite had no effect on malate oxidation (Fig. 4A), it was a very potent inhibitor of glycine decarboxylase activity (Fig. 4B). The inhibition was not reversed when the arsenite was removed by centrifugation and the half-time of inhibition was dependent on the concentration of arsenite but not of glycine (data not presented). The irreversible noncompetitive inhibition probably results from the reaction of arsenite with the lipoamide cofactor (H protein using the scheme of Kikuchi, Ref. 13) of the glycine decarboxylase.

The addition of 2 mM arsenite also prevented glycine from stimulating the state 4 rate of malate oxidation (data not shown).

The effect of glycine on the state 3 to state 4 transition of mitochondria metabolizing malate can be seen in another series of experiments (Fig. 5). The malate concentration was kept at 20 mM while the glycine concentration was increased from 0 mM to 10 mM. Although the P/O ratio remained between 2.5 and 2.6, the respiratory control ratio fell from 4.85 at 0 mM glycine, 20 mM malate to 2.61 with 1 mM glycine, 20 mM malate, and 2.44 with 5 mM glycine, 20 mM malate. In all cases, the state 3 rates were approximately equal and the control ratio decreases resulted from increased state 4 rates.

Two enzymes, NAD^+-malate dehydrogenase and NAD^+ malic enzyme, are involved in the first step of malate oxidation by plant mitochondria (23). Several authors (11, 25) have suggested that the NADH generated by these malic oxidases may provide reducing equivalents to the mitochondrial electron transport chain by two different NADH dehydrogenases (NADH-Q reductases). Recently, Moller and Palmer (16) have identified two different NADH dehydrogenases in inverted Jerusalem artichoke submitochondrial particles. The two components of the electron transport chain had different kinetic parameters and only one was sensitive to rotenone. Rustin *et al.* (25) have suggested that one of these dehydrogenases accepts electrons specifically from the NADH produced by malate dehydrogenase and is rotenone-sensitive while the other oxidizes NADH produced by malic enzyme activity and is rotenone-insensitive. Differential usage of these two dehydrogenases by NADH from glycine oxidation might explain why mitochondria saturated with malate can have their rate of O_2 uptake stimulated by the addition of glycine.

It should be noted that a physical association between malic enzyme, malate dehydrogenase, and the two NADH dehydrogenases need not occur. At low NADH concentrations, the rotenone-sensitive NADH dehydrogenase would be favored due to its lower K_m. At higher NADH concentrations, conditions that restrict the conversion of malate to oxaloacetate by malate dehydrogenase,

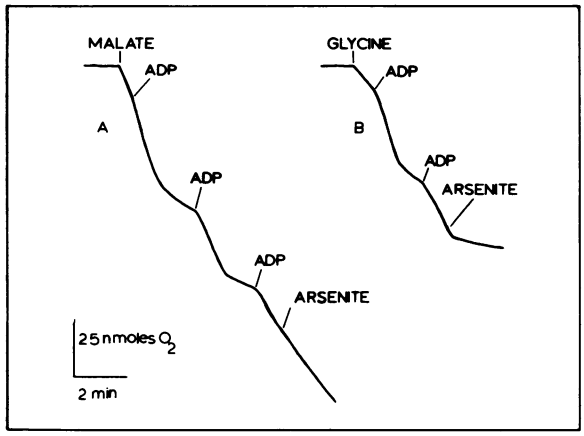

FIG. 4. The inhibition of glycine-dependent O_2 uptake by 2 mM arsenite. The concentrations were malate, 20 mM; glycine, 10 mM; ADP, 100 nmol; and arsenite, 2 mM.

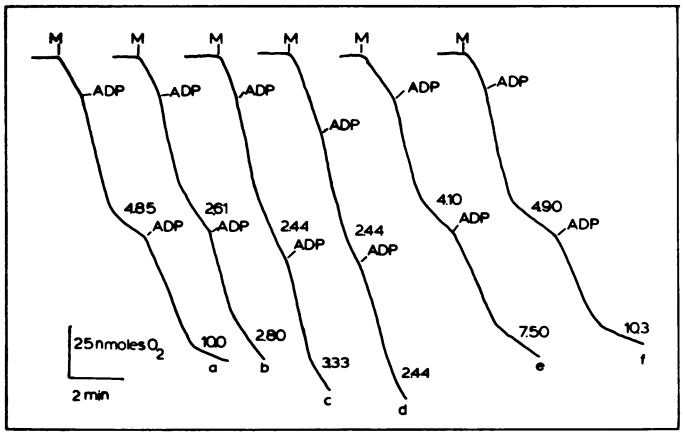

FIG. 5. Respiratory control ratios and P/O ratios for pea leaf mitochondria oxidizing different mole ratios of malate and glycine. The mitochondria (M) were added to reaction mix containing the substrates. The substrates were: a, 20 mM malate; b, 20 mM malate, 1 mM glycine; c, 20 mM malate, 5 mM glycine; d, 20 mM malate, 10 mM glycine; e, 10 mM glycine; f, 30 mM malate. The numbers shown are the respiratory ratios (state 3/state 4) after the addition of 150 nmol ADP.

Table I. *Effect of Rotenone on O_2 Uptake and CO_2 Release by Pea Leaf Mitochondria*

Mitochondria (100 μg protein) were incubated with 1 μCi [U-^{14}C]malate (20 mM) or [1-^{14}C]glycine (10 mM) as indicated for 15 min. The final ADP concentration was 1.0 mM for state 3 and 25 μM for state 4. O_2 uptake was measured polarographically under identical conditions. The rotenone concentration was 10 μM.

	State 3		State 4	
	− Rotenone	+ Rotenone	− Rotenone	+ Rotenone
	nmol mg^{-1} protein min^{-1}			
Glycine				
O_2 uptake	64	44 (−31%)	30	28 (−7%)
CO_2 release	117	86 (−27%)	55	45 (−17%)
Malate				
O_2 uptake	110	24 (−78%)	22	26 (+18%)
CO_2 release	77	65 (−16%)	79	93 (+18%)

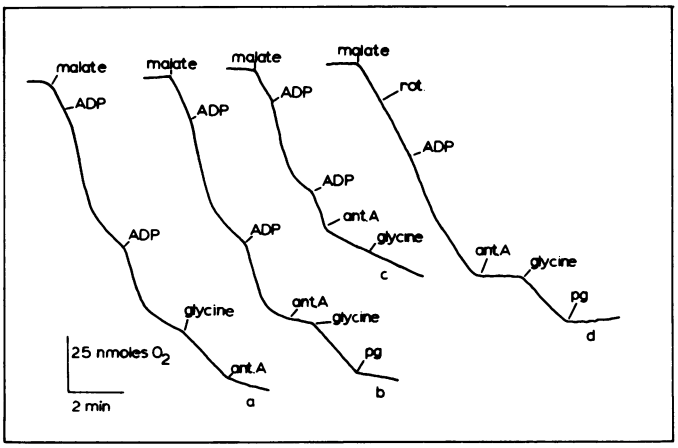

FIG. 6. The effect of glycine addition on alternate oxidase activity by pea leaf mitochondria. The concentration of substrates and inhibitors added were: malate, 20 mM; glycine, 10 mM; ADP, 100 nmol; antimycin A (ant. A), 1 μg/ml; and rotenone (rot.) 10 μM. The curves represent: a, alternate oxidase in state 4 with glycine and malate; b, increase in alternate oxidase activity in malate-dependent state 4 following glycine addition; c, lack of effect of glycine addition on the alternate oxidase activity during malate-dependent state 3; d, effect of rotenone on b. pg, propyl gallate.

the rotenone-insensitive NADH dehydrogenase would become more important. In state 4, the increased NADH levels would inhibit malate dehydrogenase activity. Under these conditions, malic enzyme activity would account for the majority of malate metabolism and the high K_m rotenone-insensitive pathway would be in operation.

In the presence of arsenite, an inhibitor of pyruvate dehydrogenase, the only citric acid cycle reaction that releases $^{14}CO_2$ from [U-^{14}C]malate is malic enzyme. By measuring the inhibitory effect of rotenone on CO_2 release from malate in the presence of arsenite and glycine, it was possible to measure the relative contribution of both NADH dehydrogenases toward total O_2 uptake (Table I).

CO_2 release from malate was much less sensitive to rotenone than malate-dependent O_2 uptake in state 3. Since the rate of CO_2 release was a measure of malic enzyme activity, the NADH dehydrogenase associated with malic enzyme was much less sensitive to rotenone than the NADH dehydrogenase supplied by malate dehydrogenase. The observations agree with the conclusions of Rustin *et al.* (25). In state 4, the rate of CO_2 release exceeded the rate of O_2 uptake, suggesting that malic enzyme activity was not inhibited by the lack of ADP and that the NADH produced by malic enzyme was used to reduce oxalacetate via malate dehydrogenase. Neither O_2 uptake nor CO_2 release was rotenone-sensitive under these conditions.

The rotenone sensitivity of both O_2 uptake and CO_2 release from glycine was approximately equal in state 3 and intermediate in value between the high sensitivity seen for malate dehydrogenase and the low sensitivity for the malic enzyme. This suggested that the NADH produced upon glycine oxidation can be reoxidized by either NADH dehydrogenase (NADH-Q reductase).

Just as the electrons from glycine appear to be able to enter the mitochondrial electron transport chain by either NADH dehydrogenase, they are available to reduce O_2 through either terminal oxidase. After glycine had increased the rate of O_2 uptake by mitochondria metabolizing malate in state 4, all of the additional electron transport was antimycin A-sensitive (Fig. 6a). When the antimycin A was added to mitochondria oxidizing malate in state 4 before the addition of glycine, however, the additional electron transport was mainly via the alternate oxidase (Fig. 6b). When the antimycin A was added in state 3, no glycine stimulation was observed (Fig. 6c). The addition of rotenone decreased both the respiratory control ratio and the P/O ratio for mitochondria oxidizing malate (Fig. 6d). After antimycin A addition, glycine still increased the electron transport rate using the alternate oxidase. These data suggest that the electrons from glycine are able to enter the chain through either the rotenone-sensitive or the rotenone-insensitive NADH dehydrogenases and that they can reduce O_2 through either the Cyt or the alternate pathway. This may partially explain the additional electron transport capacity available to glycine but not to malate.

CONCLUSIONS

Mitochondria isolated from pea leaves rapidly oxidized both glycine and malate with the concomitant reduction of NAD^+ to NADH. The NADH was reoxidized to NAD^+ by the mitochondrial electron transport chain and was coupled to three sites of ATP synthesis. Malate oxidation by these mitochondria in state 3 was inhibited by the addition of glycine due to competition for available NAD^+. In state 4, however, glycine did not decrease the malate oxidation rate. This appeared to have resulted because in state 4 malate oxidation occurs predominantly by the malic enzyme, and the rotenone-insensitive NADH dehydrogenase. This may leave additional NADH dehydrogenase capacity available to NADH produced by glycine decarboxylase.

In addition to finding unused NADH dehydrogenase capacity, glycine may stimulate O_2 uptake by diverting some additional electrons to the alternate oxidase. Because the alternate oxidase is coupled to only one site of ATP synthesis and electron transport in state 4 is limited by the rate of ATP synthesis, deflecting reducing equivalents to the alternate oxidase may have contributed to the increase in O_2 uptake observed.

Partial conformation of this observation comes from the work of Day and Wiskich (4). They have shown that with pea leaf mitochondria the rate of CO_2 and NH_3 release from glycine was unaltered by the addition of malate, succinate, or α-ketoglutarate. Again, this suggested at least partial independence of the electron transport mechanisms connecting glycine and citric acid cycle intermediates to O_2.

No known mechanisms exist for the complete isolation of reducing equivalents from glycine oxidation and citric acid cycle activity. Exogenous oxaloacetate can use the NADH formed by glycine oxidation (4, 12, 28). Similarly, added oxaloacetate could be reduced by NADH formed by malic enzyme activity (18). The mechanism that separated the reducing equivalents from glycine oxidation and citric acid cycle, therefore, was not absolute. Some exchange of reducing equivalents must occur. Indeed, in state 3, glycine and malate were competing for the electron transport capacity of the mitochondria (Fig. 1).

Although the details of the mechanism are not understood, the separation of reducing equivalents generated by glycine decarboxylation and the citric acid cycle would allow for continuous citric acid cycle function under the high electron transport demands placed by photorespiratory glycine oxidation.

Some of the results with rotenone conflict with a recent report by Johnson-Flanagan and Spencer (11). These authors reported that rotenone inhibited pea cotyledon mitochondrial malic enzyme. Table I shows that malic enzyme from pea leaf mitochondria was not strongly inhibited by rotenone. The decreased rate of O_2 uptake (relative to the rate of CO_2 release) suggests that malate dehydrogenase activity was not inhibited either.

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