Plant Physiol. (1984) 74, 705–710
0032-0889/84/74/0705/06/$01.00/0

Effects of Glycolate Pathway Intermediates on Glycine Decarboxylation and Serine Synthesis in Pea (*Pisum sativum* L.)[1]

Received for publication September 8, 1983 and in revised form November 10, 1983

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ABSTRACT

Glycine decarboxylation and serine synthesis were studied in pea (*Pisum sativum* L.) leaf discs, in metabolically active intact chloroplasts, and in mitochondria isolated both partially by differential centrifugation (*i.e.* 'crude') and by further purification on a Percoll gradient. Glycolate, glyoxylate, and formate reduced glycine decarboxylase activity ($^{14}CO_2$ and NH_3 release) in the crude green-colored mitochondrial fractions, and in the leaf discs without markedly altering serine synthesis from [1-^{14}C] glycine. Glycolate acted because it was converted to glyoxylate which behaves as a noncompetitive inhibitor (K_i = 5.1 ± 0.5 millimolar) on the mitochondrial glycine decarboxylation reaction in both crude and Percoll-purified mitochondria. In contrast, formate facilitates glycine to serine conversion by a route which does not involve glycine breakdown in the crude mitochondrial fraction and leaf discs. Formate does not alter the conversion of two molecules of glycine to one CO_2, one NH_3, and one serine molecule in the Percoll-purified mitochondria. In chloroplasts which were unable to break glycine down to CO_2 and NH_3, serine was labeled equally from [^{14}C]formate and [1-^{14}C]glycine. The maximum rate of serine synthesis observed in chloroplasts is similar to that in isolated metabolically active mitochondria. Formate does not appear to be able to substitute for the one-carbon unit produced during mitochondrial glycine breakdown but can facilitate serine synthesis from glycine in a chloroplast reaction which is probably a secondary one *in vivo*.

The metabolism of glycolate in photosynthetic organisms is an important biochemical process because the CO_2 evolution resulting from the oxidation of the carboxyl carbon group of a glycolate pathway intermediate is thought to account for photorespiratory CO_2 production (30) and NH_3 recycling (15). There is still controversy regarding the immediate precursor of the photorespired CO_2. Many workers argue that glycine is the primary intermediate of the glycolate pathway which is broken down to release CO_2 (11, 26), while other authors have pointed out that glyoxylate is a potential source of CO_2 *in vivo*, but not NH_3, particularly when the rate of glycine synthesis in peroxisomes is limited by the availability of amino group donors (24, 30).

The conversion of glycine to serine in plants (4, 11, 29) probably involves several enzymes as is the case in mammalian and bacterial cells. The exact number and nature of the enzymes involved in the glycine to serine conversion in photosynthetic cells are not known, but the breakdown of glycine by the mitochondrial enzyme glycine decarboxylase is thought to be the first key step (11). The reaction is such that one molecule of glycine is degraded to form CO_2, NH_3, and a one-carbon fragment although the one-carbon unit has never been demonstrated as a product of glycine breakdown (Equation 1).

$$\text{Glycine} + \text{THF}^2 + \text{NAD} \rightarrow \text{CO}_2 + \text{NH}_3 + \text{CH}_2 - \text{THF} + \text{NADH} \quad (1)$$

$$\text{Glycine} + \text{CH}_2 - \text{THF} \rightarrow \text{serine} + \text{THF} \quad (2)$$

A second glycine molecule combines with the one-carbon unit to form serine in a reaction catalyzed by L-serine hydroxymethyltransferase (Equation 2). The overall reaction is also associated with the reduction of NAD which may be coupled to O_2 uptake and ATP synthesis via the electron transport chain and/or linked to NAD-NADH cycling via the malate-OAA shuttle presumed to be located in the mitochondria and peroxisomes (6, 26, 29).

Glyoxylate can be broken down to produce CO_2 and formate by either a light-stimulated reaction or as a result of a reaction with H_2O_2 which is not completely prevented by peroxisomal catalase (7, 30). Although enzymes catalyzing formate oxidation exist in leaf tissue (18), the formate carbon is not readily lost as CO_2, but is metabolized further, possibly via serine synthesis (3, 7, 9, 10, 28).

The inhibition of glycine decarboxylation and serine synthesis by glyoxylate in mitochondria-rich fractions has been reported elsewhere (9, 19). We report here that this inhibition is noncompetitive and further that formate can reduce glycine breakdown without reducing serine synthesis. This paper indicates that a secondary route utilizing one molecule of glycine and one of formate to produce serine can proceed in chloroplasts and represents an alternative route for serine production other than the 'tightly linked' mitochondrial reaction (26, 29). Preliminary reports of these experiments have been presented (22, 23).

MATERIALS AND METHODS

Reagents. The radioisotopes used in this study, [1-^{14}C]glycine (0.1 mCi/mmol), [3-^{14}C]serine (0.1 mCi/mmol), Na[^{14}C]formate (0.15 mCi/mmol), [U-^{14}C]sorbitol (0.2 mCi/mmol), and ^3H_2O (1.0 mCi/mmol), were obtained from New England Nuclear Corp. Glyoxylate, glycolate, glycine, serine, ADP, OAA, THF, Mops, Hepes, Tricine, dicyclohexylamine, BSA, PVP-40 and

[1] Supported by grants to B. G. from the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Agriculture and Food.

[2] Abbreviations: THF, tetrahydrofolic acid; INH, isonicotinyl hydrazide; Mops, morpholinopropanesulfonic acid; OAA, oxaloacetate; PHMS, pyrid-2-yl-hydroxymethanesulfonate; PPO, 2,5-diphenyloxazole.

Dowex 50W ion exchange media were of the highest grade available from Sigma. Percoll was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The PHMS and INH were obtained from Aldrich Chemical Co. Other reagents of the highest purity available were obtained from Fisher Chemical Co.

Plant Material. Pea plants (*Pisum sativum* L. cv Laxton's Progress) were grown from seeds (Stoke's Seed Co., St. Catharines, Ontario, Canada) for 3 weeks in vermiculite in a controlled environment growth cabinet set for 15-h day (20°C)/9-h night (15°C) periods. Light intensity during the day was 150 $\mu E/m^2 \cdot s$. Leaf discs (6-mm diameter) were prepared from expanded leaflets and $^{14}CO_2$ release from [1-^{14}C]glycine was determined as outlined previously (8).

Isolation of Mitochondria. Leaf mitochondria were isolated from expanded leaflets (30 g) by homogenizing in 250 ml of chilled isolation buffer (300 mM mannitol, 30 mM Mops-KOH [pH 7.5], 0.2% [w/v] BSA, 4 mM cysteine, 1 mM EDTA and 0.6% soluble PVP) using a Polytron equipped with a PT 35 K. probe (Brinkmann Instruments Ltd., Rexdale, Ontario) in a manner similar to that outlined by Arron *et al.* (2). Three 5-s bursts at a dial setting of 5 produced a homogenate which was filtered through 50 μm Nitex cloth (Tetko Inc.) and centrifuged for 5 min at 1,000*g* in a Sorvall R-2B preparative centrifuge. The resulting supernatant was centrifuged for 5 min at 20,000*g*. The pellet was resuspended in 40 ml of wash buffer (300 mM mannitol, 20 mM Mops-KOH [pH 7.4] 1 mM EDTA, 0.2% [w/v] BSA) before centrifuging again for 5 min at 20,000*g*. The 'crude' mitochondria-rich pellet was resuspended in 2.0 ml of wash buffer for use in specified experiments (Table III) or further purified on a Percoll gradient. A continuous Percoll gradient (5 to 50% [v/v] Percoll) consisting of 250 mM sucrose and 20 mM Mops-KOH (pH 7.4) was used for preparation of a Chl-free, metabolically active mitochondrial fraction in a manner similar to that described elsewhere (21). After centrifugation at 10,000*g* for 20 min, the mitochondrial layer was removed with a large bore syringe, resuspended in 30 ml of wash buffer, and pelleted by centrifugation at 20,000*g* for 5 min. The Percoll-purified mitochondria were resuspended in 1.0 ml of wash buffer and maintained at 4°C for use in assays. Protein was determined by the method of Lowry *et al.* (14) after solubilization with 5% deoxycholate. Chl was determined by the method of Arnon (1).

Chloroplast Isolation. Photosynthetically active chloroplasts were isolated as described by Mills and Joy (17). Pea leaflets (20 g) were harvested and placed directly in 80 ml chilled extraction medium (330 mM sorbitol, 50 mM Tricine-KOH (pH 7.9), 2 mM EDTA, 1 mM $MgCl_2$, and 0.1% [w/v] BSA). After homogenization for 5 s with the Polytron, the homogenate was filtered through 100 μm Nitex cloth and 30 ml was layered over 14 ml of Percoll medium (40% [v/v] Percoll, 330 mM sorbitol, 50 mM Tricine-KOH [pH 7.9], and 0.1% BSA [w/v]) in a 50-ml centrifuge tube and centrifuged at 2,500*g* for 2 min. The chloroplast pellet was resuspended in 2 ml of reaction medium (330 mM sorbitol, 50 mM Hepes-KOH [pH 7.6], 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$). Chloroplasts were determined to be about 90% intact by the ferricyanide method (13), and were routinely found to have a bicarbonate-dependent O_2 evolution rate greater than 100 μmol/mg Chl·h.

Glycine Accumulation by Mitochondria. The accumulation of [1-^{14}C]glycine by isolated mitochondria was measured using the silicone oil technique of Klingenberg and Pfaff (12). Accumulation was corrected for mitochondrial intermembrane space using [^{14}C]sorbitol and ^3H_2O. Mitochondria (2 mg protein) were allowed to accumulate [1-^{14}C]glycine, [^{14}C]sorbitol, or ^3H_2O for 1 min at room temperature in 1 ml of the standard reaction buffer used for the mitochondrial glycine decarboxylase assay (see below). Samples (200 μl) of the test mixture were layered over 50 μl silicone oil (a 3:1 mixture of Wacker-Chemie AR 200 and AR 20 oils) which in turn had been layered over 50 μl of 2 N HCl. The reaction was terminated by centrifuging for 1 min in a Beckman Microfuge B. The tubes were cut 1 mm below the oil-HCl interface and the pellet resuspended in 3 ml of scintillation fluid and radioactivity determined.

Glycine Decarboxylation and Deamination during Serine Formation. Glycine to serine conversion in both mitochondria and chloroplasts was determined using radiolabeled [1-^{14}C]glycine as substrate. The O_2 consumption, $^{14}CO_2$ release, NH_3 production, and [^{14}C]serine synthesis were determined on the same test mixture by modifying the method described by Arron *et al.* (2) for determining simultaneous O_2 consumption and CO_2 production during mitochondrial glycine breakdown.

Mitochondria (1.0 ± 0.5 mg protein) were incubated in 1.0 ml of reaction mixture consisting of 300 mM mannitol, 10 mM K_2HPO_4 (pH 7.2), 0.2% (w/v) BSA, 10 mM KCl, 5 mM $MgCl_2$, 1 mM ADP, and 10 mM glycine containing 1.0 μCi [1-^{14}C]glycine. Glyoxylate, formate, and formaldehyde were added where indicated. Consumption of O_2 was measured throughout the reaction with a Hansatech O_2 electrode (Kings Lynn, Norfolk, U. K.) at 25°C. Release of $^{14}CO_2$ from [1-^{14}C]glycine was determined by injecting 100 μl of the test mixture directly into 200 μl of 2 N HCl contained in a 2-ml Eppendorf centrifuge tube which was itself encased in a minivial containing 100 μl of KOH (20%, w/v) and sealed with a rubber serum cap. The minivial and contents were routinely left overnight to allow complete absorption of $^{14}CO_2$ by the KOH. The minivials were then opened, the Eppendorf tube removed, and 3 ml of scintillation fluid was added and its radioactivity determined.

Ammonia was measured at the end of the reaction period using an Orion NH_3 electrode (6) connected to a millivoltmeter (Fisher Scientific Co.). Immediately after removal of the 100-μl sample for $^{14}CO_2$ measurement, the remaining 0.9 ml of the test mixture (from the O_2 electrode chamber) was transferred to a glass vial containing 0.25 ml of 2 N HCl to stop the reaction. To this was added test buffer and 0.1 ml of 10 N NaOH to bring the final volume to 3.0 ml and to adjust the pH to alkaline conditions for NH_3 determination. A standard curve was prepared by using known amounts of NH_4Cl.

The key product, [^{14}C]serine, synthesized during glycine breakdown was estimated in the same test mixture used for O_2, $^{14}CO_2$, and NH_3 determination described above. After the NH_3 assay, the samples were passed through 2-ml Dowex 50W columns, washed with 40 ml of H_2O, and then 10 ml of 2 N NH_4OH. The eluate from the NH_4OH washing was collected and taken to dryness before being resuspended in 100 μl of 50% ethanol. These samples were chromatographed on thin layer silica gel plates (Eastman Kodak Chromogram 13179) using 1-butanol:acetone:dicyclohexylamine:H_2O (v/v, 40:40:8:8) and autoradiographs were prepared using X-Omat film (Kodak) for 7 d. Spots located on the silica gel plate were removed and counted in minivials filled with 4 ml of scintillation fluid. The amount of serine was calculated from the percentage of the counts in serine relative to the amont of [1-^{14}C]glycine added. The same procedure was used to determine [^{14}C]serine synthesis in test solutions in which [^{14}C]formate was supplied instead of [1-^{14}C] glycine.

Estimation of Serine Hydroxymethyltransferase Activity. Glycine to serine conversion was also studied by estimating the synthesis of one carbon fragments during the reverse reaction (*i.e.* serine to glycine) catalyzed by L-serine hydroxymethyltransferase (EC 2.1.2.1) according to Taylor and Weissbach (25). The reaction mixture contained mitochondria (100 μg), 20 mM K_2HPO_4 (pH 7.4), 1 mM mercaptoethanol, 1 mM EDTA, 0.1 mM pyridoxal phosphate, 2 mM THF, 2 mM DTT, and 5 mM [3-^{14}C] serine (0.5 μCi) in a total volume of 0.5 ml. The reaction was started by the addition of the [^{14}C]serine and terminated after 5

min by the addition of 0.5 ml of a 0.4 mM dimedone in 50% ethanol. Samples were heated in a boiling water bath for 5 min and then cooled in an ice bath for 5 min. The radiolabeled dimedone derivative was extracted with 5.0 ml of toluene. After centrifugation, 3 ml of the top (toluene) phase was analyzed for radioactivity by scintillation counting.

RESULTS AND DISCUSSION

Glycine Decarboxylation and Serine Synthesis in Leaf Discs. When pea leaf discs were incubated in the dark in the presence of [1-^{14}C]glycine, $^{14}CO_2$ was released indicating that the breakdown of glycine during formation of ^{14}C-labeled serine occurred. The serine to CO_2 ratio was approximately 1.8 (Table I), somewhat higher than the value of 1.0 which were observed in isolated mitochondria (Equations 1 and 2; Tables III and IV). Serine and CO_2 production were examined in dark-incubated tissue because under this condition the recycling of carbon in the glycolate pathway via sugar synthesis is inhibited (16) and the possibility of CO_2 refixation during photosynthesis is eliminated (8, 30).

When unlabeled glycolate or glyoxylate, precursors of glycine, were added in the presence of an amino group donor (glutamate) the [^{14}C]serine to $^{14}CO_2$ ratio rose to 3.4 and 3.0, respectively (Table I). The increase in the serine to CO_2 ratio was not due to an increase in serine synthesis *per se*, but rather to a diminution in $^{14}CO_2$ release from the carboxyl position of glycine (Table I). The incorporation of label into serine from [1-^{14}C]glycine was similar in the presence and absence of glycolate or glyoxylate. It would appear that dilution of the label, provided as [1-^{14}C] glycine, due to conversion of unlabeled glycolate and glyoxylate to glycine is not sufficient to explain the results.

Glycolate and glyoxylate are converted to other compounds including oxalate and formate. Whereas adding oxalate had little effect on serine and CO_2 production (Table I), addition of formate mimicked the effect of glycolate and glyoxylate. Formate reduced serine synthesis by less than 20% but, the amount of $^{14}CO_2$ released was diminished by over 50%, giving a final serine to CO_2 ratio of about 3.2 (Table I). These data are consistent with the view that an existing pool of one-carbons (*e.g.* formate) reduces the need for glycine breakdown during serine formation. Because of the obvious importance of the mitochondrial reactions (see Introduction), glycine decarboxylation (Equation 1) and serine synthesis (Equation 2) in isolated pea leaf mitochondria were examined further.

Glycine to Serine Conversion in Isolated Leaf Mitochondria. Pea leaf mitochondria (*i.e.* 'crude') prepared only by differential centrifugation, but in a manner similar to that used by many other groups (5, 11, 19, 29), were used in some of our studies (*e.g.* Tables II and III). However, in addition to these crude organelle isolates, serine synthesis and glycine breakdown (*i.e.* CO_2 release and NH_3 production) were examined in mitochondria which had been purified further on a Percoll gradient.

When [1-^{14}C]glycine was added to either our crude mitochondria (Table III) or Percoll-purified mitochondria, $^{14}CO_2$ release, NH_3 production, and serine synthesis were observed in a ratio of 1:1:1 (Table IV) in agreement with previous studies (6, 11).

In the presence of ADP the rate of O_2 consumption measured polarographically was half that of the CO_2, NH_3, or serine generated (Table IV). As expected, known inhibitors of oxidative glycine decarboxylation, aminoacetonitrile, glycine hydroxamate and isonicotinyl acid hydrazide inhibited the reaction in these preparations (data not shown).

When glycolate was added to crude mitochondria, glycine decarboxylation was inhibited by about 50% (Table II). The inhibition was similar with added glyoxylate (Table II). In agreement with earlier studies (9, 19), the inhibition by glycolate in these preparations is dependent on its conversion to glyoxylate by contaminating glycolate oxidase in these preparations. When 6 mM PHMS was added to block the action of the peroxisomal glycolate oxidase, the inhibition by glycolate was only 15% (Table II).

Glyoxylate inhibition of glycine decarboxylation was noncompetitive with a K_i of 5.1 ± 0.5 mM while the K_m for glycine decarboxylation was 2.3 ± 0.6 mM in agreement with values reported elsewhere (6, 11, 29). Although the apparent K_i and K_m values of this reaction sequence are of interest in assessing the flow of carbon and nitrogen through the glycolate pathway, they must be viewed with caution since a purified glycine decarboxylase producing CO_2, NH_3, and the predicted one-carbon fragment has never been isolated from any plant tissue.

The products of further glyoxylate metabolism (*e.g.* oxalate, glycine, and formate) must be considered in this discussion. The inhibition of glycine decarboxylation cannot be attributed to further conversion of glyoxylate to oxalate in these preparations since the inhibitor PHMS which blocks oxalate formation by contaminating oxidase, did not alleviate the inhibition of glycine decarboxylation by glyoxylate (Table II). Furthermore, the addition of even 10 mM oxalate did not reduce glycine breakdown in mitochondrial preparations (data not shown) which is consistent with the lack of response to oxalate observed in the leaf disc experiments (Table I). Another possible product of glyoxylate metabolism is glycine. The rate of glycine decarboxylation measured as $^{14}CO_2$ release from [1-^{14}C]glycine would certainly be reduced if unlabeled glycine were being generated in these preparations by a contaminating aminotransferase (28); however, several facts argue against this dilution occurring. No amino group donor (*i.e.* glutamate, alanine, or serine) was added to the mitochondrial test solutions and the glyoxylate blocked NH_3

Table I. *The Effect of Glycolate Pathway Intermediates on Glycine Breakdown and Serine Synthesis in Pea Leaf Discs*

Ten leaf discs (7-mm diameter) were incubated with 10 mM [1-^{14}C] glycine (0.1 mCi/mmol) in the absence (*i.e.* Control) and presence of 10 mM unlabeled glycolate, glyoxylate, oxalate, or formate in the dark at 26°C for 30 min. The reaction was run in the presence of 10 mM glutamate and was terminated by addition of 2 N HCl.

	Glycine Decarboxylation	Serine Synthesis	Serine/CO_2
	nmol $^{14}CO_2$/min	*nmol [^{14}C]serine/min*	
Control	27	50	1.8
+Glycolate	14	48	3.4
+Glyoxylate	15	45	3.0
+Oxalate	24	48	2.0
+Formate	12	40	3.2

Table II. *Effect of Glycolate and Glyoxylate on Glycine Decarboxylation in Crude Pea Leaf Mitochondria-Rich Fractions*

The reaction mixture contained 1.0 ml of mitochondrial assay medium, 0.05 ml of mitochondrial extract, and 10 mM [1-^{14}C]glycine (0.1 mCi/mmol). Additions were as indicated and all assays were carried out in the presence of 10 mM OAA. The measurement of $^{14}CO_2$ was carried out as described in "Materials and Methods." Values in parentheses represent percentage of control rate (−PHMS).

	Glycine Decarboxylation	
	−PHMS	+6 mM PHMS
	nmol $^{14}CO_2$ released/min	
Control (10 mM [1-^{14}C]glycine)	52 (100)	49 (94)
+5 mM glycolate	28 (54)	44 (85)
+5 mM glyoxylate	25 (48)	27 (52)

Table III. *Glycine Accumulation, Serine Hydroxymethyltransferase Activity, Glycine Breakdown, and Serine Synthesis in Crude Mitochondria-Rich Fractions*

Experimental conditions for glycine accumulation were the same as for Figure 1. Serine hydroxymethyltransferase was assayed as described in "Materials and Methods." To 1.0 ml of assay medium was added 0.1 ml of isolated mitochondria and 5 mM [3-^{14}C]serine (0.1 mCi/mmol). Glycine breakdown was measured as the release of $^{14}CO_2$ and NH_3 from [1-^{14}C]glycine with the experimental conditions the same as for Table II except that 2 mM ADP was added instead of OAA. Controls were 10 mM [1-^{14}C]glycine for glycine accumulation, glycine breakdown, and serine production and 5 mM [3-^{14}C]serine for the serine hydroxymethyltransferase assay.

	Glycine Accumulation	L-Serine Hydroxymethyltransferase	Glycine Breakdown		Serine Synthesis	Serine/CO_2
	% control		*nmol NH_3/min/mg protein*	*nmol $^{14}CO_2$/min/mg protein*	*nmol/min/mg protein*	
Control	100	100	80	81	84	1.0
+10 mM glyoxylate	88	61	38	39	63	1.6
+10 mM formate	103	43	42	42	73	1.7
+10 mM formaldehyde	111	20	11	11	12	1.1

Table IV. *Glycine Breakdown and Serine Synthesis in Percoll-Purified Mitochondria and Chloroplasts*

For purified mitochondria, the experimental conditions were the same as for Table III. O_2 consumption and NH_3 and serine production were measured as described in "Materials and Methods." For isolated chloroplasts, the assay mixture contained 1.0 ml of chloroplast reaction medium, 0.1 ml of isolated chloroplasts, and 10 mM [1-^{14}C]glycine (0.1 mCi/mmol). The data in parentheses represent a parallel experiment measuring serine production when 10 mM [^{14}C]formate (0.15 mCi/mmol) was added as labeled substrate and unlabeled glycine was added at concentrations of [a]1, [b]5, and [c]10 mM.

	Formate Concentration	O_2	NH_3	CO_2	Serine	Serine/CO_2
	mM	*nmol/min/mg protein*				
Mitochondria						
	0	32	64	58	62	1.1
	1	32	62	65	61 (2)[a]	0.9
	5	27	45	60	56 (2)[b]	0.9
	10	24	40	55	56 (1)[c]	1.0
Chloroplasts						
	0	4	4	4	5	1.2
	1	4	4	3	3 (1)[a]	1.0
	5	3	3	3	19 (7)[b]	6.3
	10	3	4	3	28 (31)[c]	9.3

release to the same extent as $^{14}CO_2$ production (Table III). Formate is another product of glyoxylate metabolism which could affect glycine decarboxylation in these preparations. Addition of formate to crude mitochondria reduced both $^{14}CO_2$ release and NH_3 production from [1-^{14}C]glycine (Table III). However, as discussed in greater detail below, formate does not appear to inhibit mitochondrial glycine breakdown in the same way as glyoxylate. In summary, there is no compelling evidence that the inhibitory effect of glyoxylate on glycine breakdown can be attributed to either a simple dilution of [1-^{14}C]glycine due to conversion of glyoxylate to glycine or by inhibition due to oxalate or formate production from glyoxylate.

Glycine Accumulation. Since isolated mitochondria and not purified enzymes are being studied, observed effects on the glycine to serine conversion could be due to substrate availability (*i.e.* [1-^{14}C]glycine in the organelle. A recent study with mitochondria similar to those in our crude preparations suggested that glycine accumulation is transport mediated (27). In the present study, glycine accumulation over a range of concentrations up to 100 mM glycine was linear in the presence of the glycine decarboxylase inhibitor INH (Fig. 1). This is indicative of passive glycine movement into the mitochondria in agreement with the swelling studies of Day and Wiskich (5) which argue against transport-mediated movement. Mersalyl, a sulfhydryl poison, did not inhibit glycine accumulation in our preparations (data not shown), further supporting the view that glycine transport is passive. The data in Table III show that glyoxylate, formate, and formaldehyde had little effect on glycine accumulation while the further metabolism of glycine to serine was affected. These compounds also inhibited the reverse reaction, the breakdown of serine, catalyzed by L-serine hydroxymethyltransferase (Table III).

Serine Breakdown. Glyoxylate has been reported to inhibit L-serine hydroxymethyltransferase at high concentrations (>10

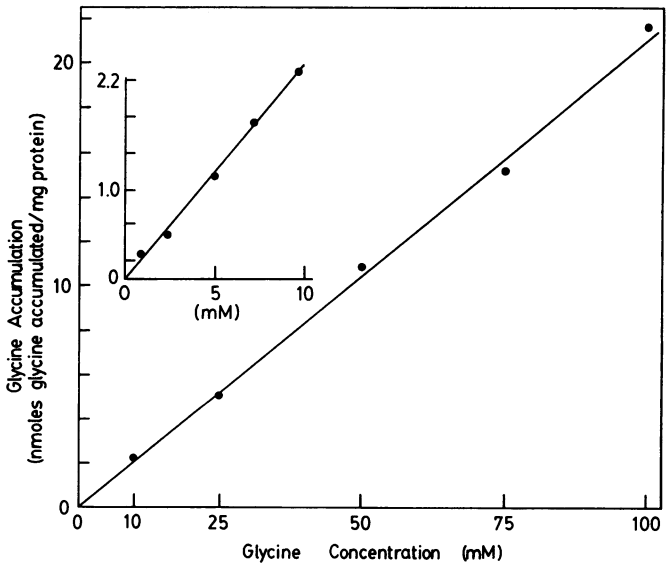

FIG. 1. Glycine accumulation by isolated mitochondria. A 100-μl aliquot of the crude mitochondrial fraction was added to 1.0 ml of assay medium with varying concentrations of glycine containing [1-^{14}C]glycine (0.1 mCi/mmol) in the presence of 10 mM INH. [^{14}C]Sorbitol (0.2 mCi/mmol) and ^3H_2O (1.0 mCi/mmol) were added in separate experiments to determine the extent of the intermembrane and intramitochondrial spaces, respectively. Values reported are the means from two experiments.

mM) but to enhance its activity at low concentrations (19). In our crude mitochondrial preparations, glyoxylate inhibited the reverse reaction catalyzed by serine hydroxymethyltransferase by approximately 40% (Table III). Glyoxylate inhibition of $^{14}CO_2$ and NH_3 release from [1-^{14}C]glycine was approximately 50% in the same preparations. Neither of these observations correlates with the glyoxylate inhibition of serine synthesis which was only 25% in the crude mitochondria (Table II) and 10% in the leaf discs (Table I). The data underscore a basic problem inherent in relying on an estimate of the back reaction as the sole indicator of the more complex glycine to serine conversion occurring during photorespiration.

One-carbon units are important in both the forward reaction (serine synthesis) and in the back reaction (serine breakdown). When formaldehyde was tested, serine to glycine conversion was reduced by 80% (Table III). Formaldehyde also inhibited serine synthesis and glycine decarboxylation to a similar extent (approximately 85%). However even at low concentrations (<1 mM) formaldehyde blocked several mitochondrial processes such as malate- and succinate-dependent O_2 consumption. Together these results imply that formaldehyde has a general inhibitory effect. Formate, an alternate one-carbon fragment, inhibited serine hydroxymethyltransferase activity and glycine decarboxylation by over 50% (Table III). Unlike formaldehyde, however, formate inhibition of serine synthesis was low (<15%) in both isolated mitochondria (Tables II and III) and leaf discs (Table I). The effect of glyoxylate was similar, raising the possibility that the effect of glyoxylate on these systems may be due to its conversion to formate. As reported previously (22), concentrations of glyoxylate and formate which reduced glycine decarboxylation and serine synthesis did not inhibit succinate or malate dependent O_2 consumption in mitochondria.

The Role of Formate in Serine Synthesis. Glyoxylate breakdown leads to formation of formate which is metabolized further via serine synthesis rather than being oxidized directly to CO_2 (7, 9). Pea mitochondrial formate dehydrogenase, for example, oxidized only 2 nmol formate/min/mg protein whereas serine synthesis proceeded at a rate of 60 to 80 nmol/min/mg protein in our preparations. The enzymes required for the incorporation of formate, such as formyl-tetrahydrofolate synthetase, exist in leaf tissue (7, 10) and [^{14}C]formate is incorporated into serine when fed to leaf tissue (3). The data in Tables I and III, specifically the serine to CO_2 ratio, suggest that formate not derived from glycine contributes to serine synthesis. The lowered rate of glycine breakdown measured both as NH_3 and CO_2 production suggests that serine synthesis from two glycolate molecules may not require breakdown of 50% of the associated glycine. The data in Table III indicate that formate and glycine produced serine; however, in spite of the fact that this occurred in both leaf discs and our crude mitochondrial preparations, we argue that the reaction does not actually occur in leaf mitochondria but proceeds in another part of the cell, probably the chloroplast.

Leaf mitochondrial fractions prepared only by differential centrifugation, although metabolically active, are known to contain contaminants including nonmitochondrial enzymes (Table II) and Chl, an obvious chloroplast component. The Percoll-purified mitochondrial fraction was relatively free of contaminating Chl. Therefore, it is significant that [1-^{14}C]glycine fed to this mitochondrial fraction was readily taken up and metabolized resulting in O_2 consumption, NH_3, CO_2, and serine production in the expected ratio of 0.5:1:1:1 (Equations 1 and 2; Table IV). Although glyoxylate inhibited glycine breakdown and serine synthesis in these preparations, the serine to CO_2 ratio remained close to 1. When formate was added at 5 or 10 mM, glycine to serine conversion was not significantly altered and in contrast to the experiments with leaf discs (Table I) and the crude green mitochondria (Table III), the ratio of [^{14}C]serine to $^{14}CO_2$ produced was still close to 1 (Table IV).

It is possible that during Percoll purification the mitochondria may have lost a component associated with formate metabolism. However, attempts to include suspected cofactors, such as tetrahydrofolate and pyridoxal phosphate (4), did not change the amount of serine or CO_2 produced (data not shown). Furthermore when Percoll-purified mitochondria were incubated with [^{14}C]formate and unlabeled glycine (Table IV; data in parentheses) the radioactivity was not recovered in serine indicating that formate and glycine do not readily combine to produce serine in mitochondria. These results tend to underscore the view of Woo and Osmond (29) that the mitochondrial glycine to serine enzyme system is indeed tightly linked.

Isolated chloroplasts, in contrast, which were determined to be over 90% intact and photosynthetically active do not appear to be able to take up and readily convert two glycine molecules to CO_2, NH_3, and serine as do the mitochondria (Table IV). Addition of formate at 5 and 10 mM concentrations increasd the amount of label appearing in serine from [1-^{14}C]glycine. As a result, the serine to CO_2 ratio was much greater than 1, an observation which helps explain the high ratio of serine to CO_2 reported with leaf discs (Table I) and the crude mitochondrial preparation (Table III). When ^{14}C-labeled formate was added with unlabeled glycine to these chloroplast preparations [^{14}C] serine was produced (Table IV; data in parentheses). Furthermore, the amount of radioactivity recovered in serine was similar when comparable amounts (*e.g.* 10 mM) of the two substrates were added in parallel experiments. For example when 10 mM [^{14}C]formate was added with 10 mM unlabeled glycine, 28 nmol [^{14}C]serine/min/mg protein was recovered compared with 31 nmol [^{14}C]serine/min/mg protein when 10 mM [1-^{14}C]glycine was supplied with 10 mM unlabeled formate (Table IV).

CONCLUSIONS

In summary, these observations support earlier suggestions that a secondary route other than that in the mitochondria exists in leaves whereby glycine produced during photorespiration can be converted to serine (7, 9, 20, 23). Several points, however, should be made regarding the source of possible reducing equivalents and formate. In the dark, formate may be utilized by a formate dehydrogenase system to drive the glycine to serine reaction. This could partially explain the lack of serine synthesis from glycine at low levels of formate in the chloroplasts where the reducing equivalents may not be generated. In comparing the results of added formate and glycine in isolated mitochondria and chloroplasts, it is still not clear why formate inhibits glycine decarboxylation and NH_3 release in crude mitochondrial preparations while serine production is largely unaffected. Possibly the presence of formate directs more glycine to the chloroplasts and away from the mitochondria although this seems unlikely at the high concentrations (10 mM) of these substrates. Another explanation might be that upon purification of the mitochondria there was a loss of a component associated with one-carbon insertion which is needed for serine production. A third alternative may be that the crude mitochondrial preparations contained a contaminant which in the presence of formate depressed mitochondrial glycine breakdown. Studies with leaf peroxisomes where glyoxylate is converted to glycine (26) support the view that the bulk (*i.e.* over 90%) of the glyoxylate formed is readily converted to glycine when amino group donors are present and no system such as an excess of H_2O_2 or a reduction in catalase efficiency induced at higher temperatures is operating (8). There is considerable room for debate regarding the amounts of CO_2 and NH_3 actually metabolized *in vivo* (7, 15). It is noteworthy that the secondary route for serine synthesis in the chloroplast is consistent with all ^{18}O, ^{14}C, or ^{15}N labeling patterns published using intact tissue. Recent studies have revealed genetic mutants of

Arabidopsis which have contributed to our understanding of the photorespiratory pathway (24). Of particular interest at present is a mutant which is deficient in mitochondrial serine hydroxymethyltransferase and apparently incapable of producing serine from glycine. The mutation appears to occur on a nuclear allele; therefore, the enzyme(s) required by the chloroplast for serine synthesis may also be lacking.

Although the chloroplast reaction appears to be a 'secondary' route requiring relatively high formate and glycine levels to drive it in the direction of serine, the rates measured on a protein basis are half the mitochondrial rate (Table IV). It is difficult to predict the extent of competition between the mitochondrial route for serine synthesis and the chloroplast route *in vivo*. Corrected on a whole homogenate basis for isolation of the respective organelles (*i.e.* chloroplasts by Chl and mitochondria by fumarase), the amount of serine generated in the chloroplast reaction is comparable to that resulting from the mitochondrial process. Clearly because of the different subcellular location and availability of key substrates, serine synthesis during photorespiration is a more complex problem than if all of the components were associated with a single organelle.

Acknowledgment—The authors would like to thank Ms. Ingrid Boesel for excellent technical assistance.

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