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GLYCINE AND GLYOXYLATE DECARBOXYLATION IN *NICOTIANA RUSTICA* ROOTS*

CHARLES W. PRATHER† and EDWARD C. SISLER

Department of Biochemistry, North Carolina State University, Raleigh, NC 27607, U.S.A.

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Abstract—Glycine was decarboxylated only by intact mitochondria to yield carbon dioxide, formaldehyde, and ammonia, probably present as pyridoxamine phosphate. The formaldehyde could become incorporated into serine, via N^5–N^{10} methylene–FH_4, and a requirement was demonstrated for pyridoxal phosphate. Similarly, glyoxylate with pyridoxamine phosphate was also decarboxylated to formaldehyde and carbon dioxide. Glyoxylate could be decarboxylated by at least two additional pathways. One consisted of oxidative decarboxylation yielding formate and carbon dioxide, and requiring thiamine pyrophosphate, manganese ions, and oxygen. The other consisted of glyoxylate condensation with 2-oxoglutarate, yielding carbon dioxide and an intermediate which, upon decarboxylation, appeared to be hydroxylevulinic acid.

INTRODUCTION

BIOLOGICAL methylation plays an important role in the synthesis of many natural compounds, including nicotine. The origin of the *N*-methyl group of nicotine has been the subject of numerous investigations. In addition to methyl donors, the β-carbon of serine[1] and the α-carbon of glycolic acid[2] have been shown to contribute to the *N*-methyl group of nicotine.

Glycolate metabolism via the glycolate cycle[3] involves its oxidation to glyoxylate as a first step, and subsequent transamination to glycine is well known.[4–6] Glycine has been shown to give rise to one-carbon units through a cleavage reaction[7–16] and, therefore, it was of interest to study glycine and glyoxylate decarboxylation reactions in *Nicotiana rustica* as a possible one-carbon source for nicotine production.

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† This work represents part of the requirement for the degree of Doctor of Philosophy at North Carolina State University. Present address: Spruance Research Laboratories, E. I. duPont de Nemours & Co., Inc., P.O. Box 27001, Richmond, VA 23261, U.S.A.

The following abbreviations are used throughout this paper: DMSO = dimethylsulfoxide, DTT = dithiothreitol, EDTA = ethylenediamine-tetraacetic acid, FH_4 = tetrahydrofolic acid, 2,4-DNP = 2,4-dinitrophenylhydrazone, TCA = trichloroacetic acid, 4-AAP = aminoantipyrine.

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RESULTS

Products of Glycine Decarboxylation

The products of glycine decarboxylation by isolated mitochondria appeared to be principally carbon dioxide, formaldehyde, and formate, as shown in Table 1. In the presence of tetrahydrofolate, serine was also produced in large amounts. The formate appeared to be the product of formaldehyde oxidation and not an immediate product of glycine decarboxylation.

Particulate Location of Glycine Decarboxylation Activity

Glycine was decarboxylated by intact *Nicotiana rustica* roots and ten per cent DMSO addition resulted in a 4-fold increase in activity which probably reflected increased cell membrane permeability.[18] Although repeated attempts to locate glycine decarboxylation activity in the soluble fraction of cell-free homogenates were unsuccessful, activity was found in the particulate fraction.

TABLE 1. PRODUCTS OBTAINED FROM UNIFORMLY LABELED GLYCINE IN THE PRESENCE OF MITOCHONDRIA

Product	Total cpm × 10^{-3} Reaction mixture + 5 μM FH_4	+ 10 μM Aminopterin
Carbon dioxide	133	168
Formaldehyde	14	53
Formate	46	80
Glyoxylate	15	16
Serine	244	20
Glycine remaining	1460	1896

The 2·35-ml reaction mixture at pH 7·5 (250 μM Tris-Cl) was aerobically incubated at 30° for 150 min and included 0·1 μM glycine-U-^{14}C (1·52 μCi), pyridoxal phosphate, 0·4 μM and 5 mg mitochondrial protein.

TABLE 2. INACTIVATION OF MITOCHONDRIAL GLYCINE DECARBOXYLATION UPON ATTEMPTS AT SOLUBILIZATION

Experiment	Mitochondrial treatment	Glycine decarboxylation (% of control)
1	2% Digitonin	10
2	Osmotic shock	80
3	0·045% Triton X-100	20
4	Butanol-washed	0
5	Acetone-dried powder	2
6	Sonication (2 min)	6

Results are from combined experiments.

To examine more closely the location of glycine decarboxylation activity within the cell, the peroxisome-rich fraction of *Nicotiana rustica* leaves and the corresponding root preparation was subjected to sucrose density gradient centrifugation. The resulting fractions were assayed for glycine decarboxylation activity, cytochrome oxidase (mitochondrial membrane marker), glyoxylate reductase (peroxisomal marker), and in the case of leaves, chlorophyll content (chloroplast marker).

Results presented in Fig. 1 show that in roots, glycine decarboxylation activity is closely associated with the mitochondria. In the centrifugation experiments with leaf preparations, glycine decarboxylation activity was also closely associated with the mitochondria. In the leaf preparations, peroxisomal and chloroplast fractions were easily identified by glyoxylate reductase and chlorophyll content, but neither fraction contained glycine decarboxylation activity. These results are consistent with other reports.[15–17]

[17] T. KISAKI and N. E. TOLBERT, *Plant and Cell Physiol.* **11**, 247 (1970).
[18] D. P. DELMER and S. E. MILLS, *Plant Physiol.* **44**, 153 (1969).

Mitochondrial glycine decarboxylation activity exhibited a sharp activity optimum at pH 7·5. The same sharp pH optimum was also observed with intact roots and may indicate that their activity was due to the mitochondrial activity within.

Heated mitochondrial suspensions rapidly lost glycine decarboxylation activity. Activity was lost (50%) within 3 min at 47°, while the same amount was lost within 1 min at 53°. Full glycine decarboxylation activity of mitochondrial suspensions (in 0·36 M mannitol) was retained through two freeze-thaw cycles (frozen at −15° for one hour each cycle) and DMSO was not necessary for the preservation of activity.

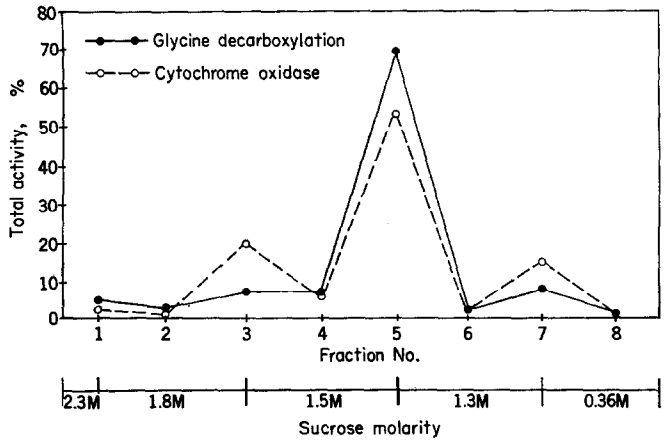

FIG. 1. CORRELATION OF GLYCINE DECARBOXYLATION ACTIVITY WITH ROOT PARTICULATE FRACTIONS OBTAINED BY SUCROSE DENSITY GRADIENT CENTRIFUGATION.
The 100–6000 *g* pellet from *Nicotiana rustica* roots was subjected to sucrose density gradient centrifugation and enzyme activities were measured as described. These results are representative of three additional experiments.

Solubilization of Glycine Decarboxylation Activity from Mitochondria

To more closely study glycine decarboxylation in *Nicotiana rustica*, attempts were made to solubilize the activity from mitochondria. Mitochondrial treatment with detergents, ultrasonic energy, organic solvents and osmotic shock were unsuccessful in solubilizing the enzyme activity from mitochondria (Table 2). In addition, all such treatments except osmotic shock resulted in enzyme inactivation. Osmotic shock appeared to be ineffective in rupturing the mitochondria since they were easily recovered by centrifugation at 12 000 g (25 min). The inactivation resulting from acetone powder preparation is in contrast to the results of others,[8,9,19] who found glycine decarboxylation activity in extracts of rat liver mitochondrial acetone powder.

To further investigate whether glycine decarboxylation activity required mitochondrial integrity, a mitochondrial suspension was subjected to increasing levels of sonication and the resulting glycine decarboxylation activity was compared to the light-scattering ability of the treated mitochondrial preparations. Light scattering measured by absorbance at 430 nm was shown to be linearly related to the number of mitochondria present. The rapid decline in enzymatic activity paralleled closely the decrease in absorbance (Fig. 2), indicating that as mitochondrial integrity was lost, so was glycine decarboxylation activity.

[19] G. H. TAIT, *Biochem. J.* **118**, 819 (1970).

Although this result does not rule out enzyme inactivation due to sonication, similar results of inactivation by detergents indicate that mitochondrial integrity is required for glycine decarboxylation activity.

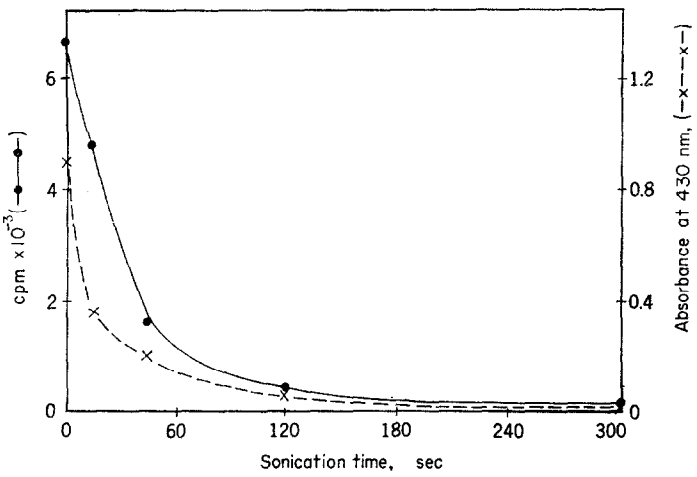

FIG. 2. EFFECT OF SONICATION TIME ON MITOCHONDRIAL GLYCINE DECARBOXYLATION ACTIVITY AND ABSORBANCE AT 430 nm.
The reactions were run in extraction medium containing 10^{-3} M DTT and 10^{-3} M EDTA at pH 8·0 (Tris-Cl) and included 0·05 μC, 0·5 μM glycine-1-^{14}C, 0·2 μM pyridoxal phosphate, and mitochondria from 3 g roots sonicated as indicated. The $^{14}CO_2$ was trapped and counted after 4 hr at 30°. Absorbance at 430 nm of mitochondrial aliquots was corrected for turbidity remaining in an exhaustively sonicated sample.

Requirements for Glycine Decarboxylation

Requirements for glycine decarboxylation were investigated using intact mitochondria and a nearly absolute requirement for pyridoxal phosphate was demonstrable when mitochondria were pre-treated with semicarbazide or cycloserine.[20] Addition of pyridoxal

TABLE 3. PYRIDOXAL PHOSPHATE REQUIREMENT FOR GLYCINE DECARBOXYLATION

Mitochondrial treatment	cpm $^{14}CO_2 \times 10^{-3}$	
	−Pyridoxal phosphate	+0·8 μM Pyridoxal phosphate
None	7·4	12·0
Washed	5·3	12·5
0·02 M Semicarbazide	0·5	9·6
0·01 M Cycloserine	1·6	9·4

The 3·5-ml reaction mixtures at pH 8·0 (in extraction medium containing 0·12 M Tris-Cl) were aerobically incubated 2 hr at 30° and contained 2·0 μM glycine-1-^{14}C (1·0 μCi), 10^{-3} μM EDTA, $1·5 \times 10^{-3}$ μM 2-mercaptoethanol, and mitochondria (3 mg protein). Intact mitochondria were untreated or treated with either semicarbazide or cycloserine for 15 min and washed 3 times with extraction medium and resuspended in fresh extraction medium.

[20] G. B. BARLEY, O. CHOTAMANGSU and K. VUTTIVEJ, *Biochem.* **9**, 3243 (1970).

phosphate to treated mitochondria restored activity (Table 3). Essentially no activity loss was demonstrable when tetrahydrofolate or NAD^+ were omitted as shown in Table 4. This result is in contrast to those of others,[8,9,19] who have shown a requirement for these other cofactors as well as for pyridoxal phosphate.

Cofactor Addition to Broken Mitochondria

To determine if the activity loss upon mitochondrial rupture resulted from a dilution of endogenous NAD^+ and FH_4, these cofactors were added to intact as well as to broken mitochondria. Results showed that exogenous NAD^+ and FH_4 did not restore glycine decarboxylation activity lost upon mitochondrial rupture (Table 4).

TABLE 4. LACK OF NAD^+ AND FH_4 REQUIREMENT FOR GLYCINE DECARBOXYLATION BY INTACT AND BROKEN MITOCHONDRIA

Mitochondrial preparation	cpm $^{14}CO_2 \times 10^{-3}$ $-NAD^+$ $-FH_4$	$+0{\cdot}5$ μM NAD^+ $+3{\cdot}8$ μM FH_4
Intact	12·7	12·1
0·024% Triton X-100	6·9	7·1
0·045% Triton X-100	4·2	4·2
Sonicated (120 sec)	1·9	2·2
Boiled	2·1	2·0

The 4·0-ml reaction mixtures at pH 7·5 (250 μM phosphate) included 0·08 μM glycine-1-^{14}C (0·2 μCi), 0·5 μM pyridoxal phosphate, and mitochondria (3·5 mg protein). After incubation for 90 min at 30°, the $^{14}CO_2$ was collected and counted. These results obtained from one experiment were typical of three others.

Glyoxylate Decarboxylation to Formaldehyde

Since glycine decarboxylation requires pyridoxal phosphate, then glyoxylate, when condensed with pyridoxamine phosphate, should be an equivalent substrate for the same mitochondrial decarboxylation yielding formaldehyde. When uniformly labeled glyoxylate and glycine of the same specific activities were incubated in identical reaction mixtures, equal amounts of radioactive carbon dioxide and formaldehyde were formed from each substrate (Table 5). Since anaerboic conditions prevented interfering reactions, these results indicate that glyoxylate and pyridoxamine phosphate form an equivalent substrate to glycine and pyridoxal phosphate for mitochondrial decarboxylation. When mitochondrial integrity was destroyed with Triton X-100, neither glycine nor glyoxylate decarboxylation was noted, suggesting that glyoxylate (with pyridoxamine phosphate) is decarboxylated by the same mitochondrial system active for glycine.

Oxidative Decarboxylation of Glyoxylate to Formate

In addition to the decarboxylation of glyoxylate to carbon dioxide and formaldehyde by intact mitochondria just described, glyoxylate was also oxidatively decarboxylated to carbon dioxide and formate by soluble mitochondrial enzymes. Using *Nicotiana rustica* root mitochondrial protein extracts, a broad pH optimum was observed from pH 6·3 to 7·0.

TABLE 5. A COMPARISON OF GLYOXYLATE AND GLYCINE DECARBOXYLATION BY MITOCHONDRIA UNDER ANAEROBIC CONDITIONS

Substrate	cpm products	
	$^{14}CO_2$	$H^{14}CHO$
Glyoxylate-U-^{14}C (0·08 μCi)	1250	1330
Glycine-U-^{14}C (0·08 μCi)	1210	1300
Glyoxylate-1-^{14}C (0·04 μCi)	1280	—
Glycine-1-^{14}C (0·04 μCi)	1185	—
Glyoxylate-1-^{14}C (0·04 μCi) 0·2% Triton X-100	128	—
Glycine-1-^{14}C (0·04 μCi) 0·2% Triton X-100	50	—

The reaction mixtures at pH 7·5 250 μM (Tris-Cl) included mitochondria (8 mg protein), 0·4 μM each pyridoxal and pyridoxamine phosphate, 1·0 mg catalase, 0·5 μM glycine or glyoxylate, and 10 μM unlabeled formate. The $^{14}CO_2$ was collected and counted after incubation under purified nitrogen for 2 hr at 30°. Results have been corrected for boiled enzyme.

Products of Glyoxylate Oxidative Decarboxylation

The products of the oxidative decarboxylation of glyoxylate catalyzed by *Nicotiana rustica* root mitochondrial extracts were shown to be carbon dioxide arising from the carboxyl carbon (glyoxylate-1-^{14}C as substrate) and formate arising from the carbonyl carbon (glyoxylate-2-^{14}C as substrate). Labeled formate was shown to arise from glyoxylate-2-^{14}C by paper chromatography of reaction products and by competition experiments with unlabeled formate and formaldehyde.

Radioactive carbon dioxide was evolved when glyoxylate-2-^{14}C was incubated with root mitochondrial extracts. The immediate precursor for this $^{14}CO_2$ generation was shown to be formate by the dilution effect of unlabeled formate on $^{14}CO_2$ production (Table 6). These results indicate that formate is a product of the oxidative decarboxylation of glyoxylate and that formaldehyde is not an intermediate or product.

Localization of Activity

The specific activity of the oxidative glyoxylate decarboxylation activity was determined on the clear supernatant (30 000 *g*, 30 min) of sonicated (30 sec) mitochondria, as well as on the soluble protein fraction of cell-free homogenates. The data indicate that the mitochondrial extracts exhibited nine times greater specific activity than did the cytoplasmic protein fraction.

Requirements for the Oxidative Decarboxylation of Glyoxylate

The oxidative decarboxylation of glyoxylate to carbon dioxide and formate catalyzed by *Nicotiana rustica* mitochondrial protein preparations exhibited a requirement for oxygen, thiamine pyrophosphate, and a divalent metal ion (Table 7). Although manganese was the most effective metal ion tested, Mg^{2+}, Ca^{2+} and Co^{2+} were about 30% as effective as manganese while Fe^{3+}, Cu^{2+}, Zn^{2+}, Ni^{2+} and Cd^{2+} were ineffective (Table 8).

ABLE 6. EFFECT OF UNLABELED FORMATE AND FORALHEHYDE ON THE FURTHER OXIDATION OF THE EACTION PRODUCTS OF GLYOXYLATE-2-^{14}C OXIDATIVE DECARBOXYLATION

Reaction mixture	$^{14}CO_2$ formed (% of control)
Complete + 25 μM HCHO	93
Complete + 25 μM HCOOH	10

The 2·5-ml reaction mixture at pH 7·0 (200 μM phosphate) included 1·0 μM glyoxylate-2-^{14}C (0·018 μCi), 1 mg catalase, and mitochondria (4 mg protein). The $^{14}CO_2$ was collected and counted after aerobic incubation for 2 hr at 30°.

TABLE 7. REQUIREMENTS FOR THE OXIDATIVE DECARBOXYLATION OF GLYOXYLATE TO FORMATE BY MITOCHONDRIAL PROTEIN

Changes in complete reaction mixture	Glyoxylate decarboxylation (cpm $^{14}CO_2$)
None	3900
+1·0 μM NAD^+	3850
+1·0 μM $NADP^+$	4100
$-O^2$ (N_2 gas phase)	120
ThPP, (pH 7·0 + Mg^{2+} − Mn^{2+})	500
−Mn^{2+}, (+30 μM EDTA pH 7·0)	600

The 2·3-ml reaction mixture at pH 6·5 (300 μM phosphate) included 0·5 μM glyoxylate-1-^{14}C (0·015 μCi), 1·0 μM ThPP, 0·5 μM $MnCl_2$, 1 mg catalase, and 3·4 mg dialyzed soluble mitochondrial protein. Complete reaction mixtures run at pH 7·0 with $MgCl_2$ gave the same activity as complete mixtures run at pH 6·5 with Mn^{2+}. Results have been corrected for boiled enzyme.

Glyoxylate Decarboxylation via Hydroxyketoadipic Acid

Nicotiana rustica root mitochondria (but not cytoplasmic protein) contained enzymatic activity (2-oxoglutarate:glyoxylate carboligase) which resulted in the 2-oxoglutarate-dependent decarboxylation of glyoxylate. This activity was readily released from the mitochondria by either detergent treatment or sonication and remained in the subsequent clear supernatant (30 000 *g*, 30 min).

TABLE 8. THE EFFECT OF METAL IONS ON GLYOXYLATE OXIDATIVE DECARBOXYLATION BY MITOCHONDRIAL PROTEIN

Addition to reaction mixture*	Glyoxylate decarboxylation activity (% of maximum)
$MnCl_2$	100
$MgCl_2$	37
$CoCl_2$	24
$CaCl_2$	32
$Fe(NO_3)_3$	4
$CuSO_4$	1
$ZnCl_2$	5
$NiSO_4$	0
$CdCl_2$	0
EDTA	3

The 2-ml reaction mixture at pH 6·5 (250 μM phosphate) included 0·5 μM glyoxylate-1-^{14}C (0·015 μCi), 1·0 μM ThPP, 1 mg catalase, and 1·15 mg dialized soluble mitochondrial protein. The $^{14}CO_2$ was collected after aerobic incubation for 90 min at 30°.

* All at 1 μM, except EDTA (10 μM).

TABLE 9. REQUIREMENTS FOR 2-OXOGLUTARATE:GLYOXYLATE CARBOLIGASE

Reaction mixture	CPM 4-aminoantipyrine-dependent $^{14}CO_2$ released
+ 5 μM $MnCl_2$	1500
+ 5 μM $MgCl_2$	830
+ 30 μM EDTA	40
+ 5 μM $MgCl_2$−2-oxoglutarate	80
+ 5 μM $MgCl_2$−ThPP	100

The complete reaction mixture at pH 7·5 (50 μM phosphate) was incubated for 2 hr at 30° and included 1·0 μM glyoxylate-1-^{14}C (0·032 μCi), 40 μM 2-oxoglutarate, 2·0 μM ThPP, 1 mg catalase, and 1·75 mg dialyzed mitochondrial protein (30 000 *g* supernatant of mitochondria broken with 0·1% Triton X-100). The $^{14}CO_2$ released upon treatment with 4-AAP after liberation of $^{14}CO_2$ present as bicarbonate was trapped and counted. Results have been corrected for boiled enzyme.

On the basis of chromatographic evidence, and on similarities in enzyme properties,[21,22] the reaction product appeared to be hydroxylevulinic acid. Co-chromatography of the 2,4-DNP of the reaction product with the 2,4-DNP of hydroxylevulinic acid prepared according to the procedure of Schlossberg *et al.*[23] revealed only a single spot in two solvent systems. One of the R_f values was in agreement with one reported[21] for the 2,4-DNP of hydroxylevulinic acid. In addition to the chromatographic evidence, the requirements (Table 9) of glyoxylate, 2-oxoglutarate, thiamine pyrophosphate and Mg^{2+} for enzyme activity were the same as those previously reported.[21] The pH optimum for 2-oxoglutarate–glyoxylate carboligase from *Nicotiana rustica was* near 7·0.

DISCUSSION

Glycine Decarboxylation

Glycine decarboxylation by *Nicotiana rustica* root mitochondria resulted in the formation of carbon dioxide, formaldehyde, glyoxylate, formate, and probably pyridoxamine phosphate. Pyridoxal phosphate appeared to be the only required cofactor. The Schiff's base formed by the condensation of glycine with pyridoxal phosphate would appear to be the true substrate, since glyoxylate and pyridoxamine phosphate were equally as effective as glycine and pyridoxal phosphate. When uniformly labeled glycine and glyoxylate of the same specific activities were used, formaldehyde and carbon dioxide were found in a 1:1 ratio from each substrate. This decarboxylation of glyoxylate yielding formaldehyde catalyzed by intact mitochondria is different from the oxidative decarboxylation of glyoxylate yielding formate catalyzed by soluble mitochondrial enzymes to be discussed later.

Reports of glycine decarboxylation in animal liver,[7–9,19] plants,[10,12] and bacteria[13,14] suggest that the reaction involves two molecules of glycine being transformed to one each of serine, carbon dioxide, and ammonia. The results of this study suggest that the conversion of glycine to serine occurs in at least three steps in *Nicotiana rustica* root mitochondria. Formaldehyde, carbon dioxide, and ammonia (probably as pyridoxamine phosphate) are produced by glycine cleavage in the first step. N^5–N^{10} methylene-FH_4 is formed from the formaldehyde and FH_4 in the second, and serine is formed from N^5–N^{10} methylene-FH_4 and another molecule of glycine in the third. The sum of these reactions appears to represent the glycine decarboxylation reaction reported by others and is a combination of the glycine decarboxylation described here followed by serine formation from the products. This exclusively mitochondrial system required mitochondrial (or mitochondrial membrane) integrity and pyridoxal phosphate. Contrary to the results of others, no requirement or stimulation of activity could be demonstrated for NAD^+ ($NADP^+$) or FH_4 for the strict decarboxylation of glycine; however, subsequent serine formation was dependent on FH_4. The loss of glycine decarboxylation activity upon mitochondrial rupture was not due to a dilution of these cofactors.

The radioactivity of the carbon dioxide released from uniformly labeled glycine was equal to the radioactivity in formaldehyde, formate, and one third the serine formed (Table 2). When run in the absence of FH_4 and in the presence of aminopterin, almost no serine

[21] J. KOCH and E. L. R. STOKSTAD, *Biochem. Biophys. Res. Commun.* **23**, 585 (1966).
[22] T. MORIYAMA and G. YUI, *Biken J.* **9**, 263 (1966).
[23] M. A. SCHLOSSBERG, D. A. RICHERT, R. J. BLOOM and W. W. WESTERFELD, *Biochem.* **7**, 333 (1968).

was produced and the elevated levels of formaldehyde and formate accounted for all the C-2 atom remaining after decarboxylation of the C-1 atom. Under these conditions, the formaldehyde level was 4-fold higher while the formate level was less than doubled. In a similar experiment run anaerobically, the amount of formaldehyde produced was equivalent to the amount of carbon dioxide produced from uniformly labeled glycine. These results indicate that the formate previously detected resulted from the aerobic oxidation of the formaldehyde product.

Under conditions of inhibited serine formation ($-FH_4$ + aminopterin), the amount of carbon dioxide released increased by 25%. This increase probably was a result of the carbon dioxide produced from the accumulating formate derived from formaldehyde, since formate oxidation to carbon dioxide also occurred in *Nicotiana rustica* root mitochondria (results not shown).

Sucrose density gradient centrifugations of root and leaf preparations showed the glycine decarboxylation activity to be closely associated with the mitochondrial fraction. As recently reported,[15] no activity was observed in either the peroxisomal or the chloroplast fractions obtained from leaves.

The requirement for mitochondrial integrity (or mitochondrial membrane integrity) is not well understood, but might represent a required association of two or more proteins involved in glycine decarboxylation. Tait,[19] for example, has suggested that the glycine decarboxylase system of rat liver mitochondria is a complex of several proteins loosely attached to one another. The mitochondrial decarboxylation of glycine may account for much of the CO_2 produced in photorespiration since glycine has been indicated as the precursor for photorespiratory CO_2.[17]

Oxidative Decarboxylation of Glyoxylate

Extracts of *Nicotiana rustica* roots were able to catalyze the oxidative decarboxylation of glyoxylate to formate and CO_2. These results are in agreement with those of Tolbert *et al.*,[24] who studied glycollic acid oxidation in tobacco and concluded that glyoxylate was an intermediate which was further oxidized to carbon dioxide and formate with the consumption of oxygen. In rat liver,[25] the major pathway of glycine catabolism was *via* conversion to glyoxylate and subsequent oxidation to formate and carbon dioxide. In these experiments catalase was routinely added to reaction mixtures to insure that no hydrogen peroxide would be available to non-enzymatically oxidize glyoxylate to formate and CO_2.[26]

In the present study, requirements for a divalent metal ion, thiamine pyrophosphate, and oxygen were demonstrated. In keeping with the thiamine pyrophosphate requirement reported here, Liang[27] found that animals deficient in thiamine pyrophosphate did not oxidize glyoxylate to carbon dioxide and formate as usual, and that large amounts of glyoxylate accumulated.

Glyoxylate Decarboxylation Via Hydroxyketoadipic Acid

In *Nicotiana rustica* root preparations, the enzymatic condensation of glyoxylate with 2-oxoglutarate appears to be similar to that reported by others.[21,23] This condensation

[24] N. E. Tolbert, C. O. Clagett and R. H. Burris, *J. Biol. Chem.* **181**, 905 (1949).
[25] H. I. Nakada, B. Freidman and S. Weinhouse, *J. Biol. Chem.* **216**, 583 (1955).
[26] W. H. Hatcher and G. W. Holden, *Trans. Roy. Soc. Canada* 3 Ser. 19, 11 (1925).
[27] C. C. Liang, *Nature, Lond.* **188**, 660 (1960).

is expected to give rise to carbon dioxide and hydroxyketoadipic acid which upon decarboxylation yields hydroxylevulinic acid. The pH optimum for hydroxyketoadipic acid formation in *Nicotiana rustica* root mitochondria was at pH 7·0, while the enzyme from pig liver showed an optimum of 7·0,[28] and that from rat liver an optimum of 6·2.[21] In *Nicotiana rustica* roots, all activity was confined to the mitochondria. Others have used beef heart particulate preparations[23] and rat liver mitochondria.[21]

The importance of hydroxylevulinic acid formation and its metabolic fate in plants is unclear at present. Levulinic acid was shown to competitively inhibit Δ-aminolevulinic acid dehydratase of *Rhodopseudomonas spheroides*[29] and porphyrin synthesis in cell-free extracts of *Mycobacterium phlei*.[30] In *Myobacterium phlei* hydroxylevulinic acid was a more potent inhibitor of porphyrin synthesis than levulinic acid. Thus, hydroxylevulinic acid formation may play a role in the regulation of plant porphyrin synthesis.

EXPERIMENTAL

Materials. Nicotiana rustica plants were grown from seed and transplanted to aerated nutrient culture as previously described.[31] Roots and leaves were obtained from plants less than 25 cm in height.

Mitochondrial preparations. Mitochondria were obtained by grinding roots in a mortar with sand and 0·36 M mannitol medium containing other ingredients as described with the experiment under discussion. The brei was squeezed through nylon cloth and the pellet sedimenting between 1000 *g* (5 min) and 12 000 *g* (25 min) was resuspended in fresh grinding medium. Mitochondrial acetone powder was prepared by mixing root mitochondria with 75 vol. of cold (−15°) acetone. The centrifuged pellet was resuspended in 100 ml cold acetone, centrifuged, air-dried, and stored at −15° until ready for use. To osmotically shock mitochondria, 3 ml of a suspension in 0·4 M sucrose was mixed with 150 ml of 1 mM Tris-Cl buffer, pH 8·0. After stirring for 5 min at 4°, the suspension was centrifuged at 12 000 *g* for 30 min. The pellet was resuspended in 3 ml 0·4 M sucrose, and the clear supernatant was discarded. All sonications were performed with a Branson Model S-125 at a power level of 80 W. Typical volumes sonicated ranged from 10 to 15 ml. The mitochondrial suspension and the instrument probe were cooled in an ice bath prior to and at 20-sec intervals during the sonication.

Sucrose density gradient centrifugations. Isopycnic discontinuous density gradient centrifugations were performed generally as previously reported.[17] The sucrose concentrations presented in Fig. 1 were used and centrifugations were carried out for 3 hr (37 000–90 000 *g*) at 4°. Fractions were collected from the bottom of the tube through a needle.

Paper chromatography of reaction products. The 2,4-DNP derivatives of reaction products were prepared and separated into a-keto acid derivatives and other carbonyl derivatives as previously described.[32] For formaldehyde, this procedure was modified to eliminate excess unreacted 2,4-DNP from the preparation by adding excess unlabeled glyoxylic acid and again separating the other carbonyl derviatives from this unwanted acid derivative. Separation was accomplished with *n*-BuOH–EtOH–0·5 N NH_4OH (7 : 1 : 2). Radioactive formate was detected as a product of the oxidative decarboxylation of glyoxylate-2-^{14}C by making the reaction mixture alkaline (NH_4OH) and developing aliquots on paper in EtOH–NH_4OH (100:1) and EtOH–MeOH–NH_4OH (75:25:1). Hydroxylevulinic acid was identified by co-chromatography of the 2,4-DNP derivative from the reaction mixture with an authentic 2,4-DNP derivative[27] on paper with *n*-BuOH–EtOH–0·5 N NH_4OH (7:1:2) R_f 0·1 and MeOH–benzene–EtOH–H_2O–(4:2:2:2) R_f 0·65. To measure the activity, the paper segments were counted in a liquid scintillation counter.

Enzyme assays. The reactions using either glycine-1-^{14}C or glyoxylate-1-^{14}C as substrate were carried out in sealed Warburg vessels. After completion, 0·3 ml 75% TCA was tipped in from a sidearm to stop the reaction and to liberate the $^{14}CO_2$ which was swept from the flask with air. The effluent $^{14}CO_2$ was trapped in 3 ml 15% phenethylamine in methanol contained in the scintillation vial. Toluene-based scintillation fluid was added (10 ml) and the samples were counted in a liquid scintillation spectrometer. 2-Oxoglutarate: glyoxylate carboligase activity was assayed by measuring the $^{14}CO_2$ released from the reaction mixture after elimination of all $^{14}CO_2$ present as the bicarbonate. This was accomplished by first lowering the pH to 5·5–6·0 with 0·5 ml 1·0 M citrate buffer. The decarboxylation of 5-hydroxyl-4-ketoadipic acid was then accom-

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plished using either strong acid or 4-amino-antipyrine.[21] Cytochrome oxidase[33] and glyoxylate reductase[34] were assayed as previously described.

Protein and chlorophyll were determined by the methods of Lowry *et al.* [35] and Arnon,[36] respectively.

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Key Word Index—Nicotiana rustica; Solanaceae; enzymes; glycine decarboxylase; glyoxylate decar boxylase.