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PHOTORESPIRATION DURING C_4 PHOTOSYNTHESIS

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SUMMARY

1. This paper describes aspects of the synthesis and metabolism of glycolate as factors controlling the apparent absence of CO_2 evolution in the light (photorespiration) in the leaves of plants which photosynthesise *via* the C_4 dicarboxylic acid pathway (C_4 plants). The dicotyledon, *Atriplex spongiosa* and the monocotyledon *Sorghum bicolor* were chosen as examples of C_4 plants. *Atriplex hastata*, which fixes CO_2 *via* reactions of the Calvin cycle (C_3 plant) was used as a reference species.

2. Tracer experiments and enzymic data suggest glycolate synthesis may be quantitatively similar in all three species and is associated with Calvin cycle photosynthesis in bundle sheath cells of the C_4 species.

3. Although photosynthetic enzymes showed comparable activity in the bundle sheath of C_4 leaves and in *Atriplex hastata*, the activity of most glycolate pathway enzymes was much reduced in C_4 leaves.

4. Glycolate pathway enzymes associated with the peroxisome and serine transmethylase are largely confined to the bundle sheath cells in *Atriplex spongiosa* leaves. Tetrahydrofolate enzymes of C_1 activation, and glycerate kinase, are principally associated with the mesophyll cells.

5. In C_4 species, the CO_2 release of photorespiration may be controlled in two ways. In *Atriplex spongiosa* CO_2 release, and refixation *via* phosphoenolpyruvate carboxylase may occur in mesophyll cells. In *Sorghum bicolor* the activity of the glycolate pathway is so low that little CO_2 release may occur.

INTRODUCTION

Plants which fix CO_2 *via* the C_4 dicarboxylic acid pathway of photosynthesis (also termed the β-carboxylation pathway) do not normally evolve CO_2 in the light, whereas plants with the C_3 pathway of photosynthesis (the Calvin cycle alone) produce much CO_2 in the light[1,2]. In C_4 plants the evolution of photorespiratory CO_2 may be limited by deficiencies in the synthesis of glycolate[3,4] or by the refixation of the evolved CO_2 by photosynthesis[5]. Earlier reports[3,6] correlated the low glycolate oxidase activity in extracts of the leaves of C_4 plants with the apparent absence of CO_2 evolution in the light (absence of photorespiration) in these species. Glycolate oxidase is the first enzyme of the glycolate pathway, originally formulated by TOLBERT[7] (Fig. 1). 2 moles of photosynthetically derived glycolate are metabolised to 1 mole of

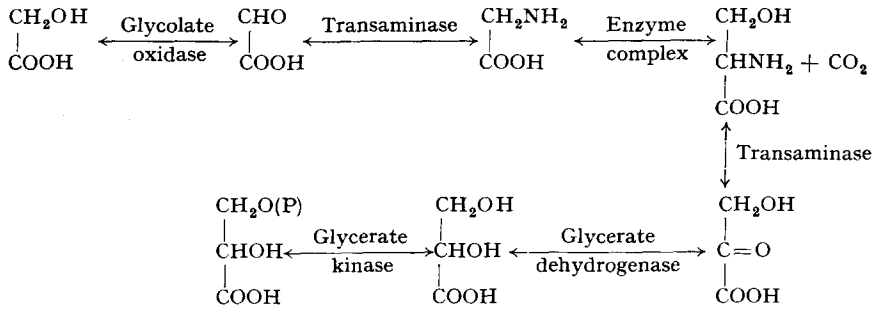

Fig. 1. Intermediates and enzymes of the glycolate pathway.

3-phosphoglycerate with the release of 1 mole of CO_2 during the condensation of glycine to serine. This is thought to be the main source of photorespiratory CO_2. Many of the reactions of this pathway are confined to the peroxisome[8,9], but the locations of others, including the important steps leading to evolution of CO_2, have not been clearly defined.

This paper reports tracer studies which demonstrate the synthesis of glycolate during photosynthesis in C_3 and C_4 species of Atriplex. Further, the metabolism of labeled intermediates of the glycolate pathway in both types of plants in the light is consistent with the operation of this pathway. The activity of all glycolate pathway enzymes and the localisation of these enzymes within the mesophyll and bundle sheath cells of C_4 leaves is reported. These studies suggest that significant glycolate pathway activity and CO_2 release may occur in some C_4 species and that this CO_2 is refixed during photosynthesis. In others, activity of the glycolate pathway enzymes is very low and it is doubtful whether significant quantities of CO_2 are released.

MATERIALS AND METHODS

Seedlings of *Atriplex spongiosa* F.v.M. and *Atriplex hastata* L. were grown in the glasshouse in water culture as described earlier[4]. *Zea mays* L. (var. NES 1002) and *Sorghum bicolor* L. (var. Texas 610) were grown in flats of sand in the glasshouse, with supplementary lighting from 4 Philips I.R. lamps (250 W) during winter months.

Tracer experiments

Mature leaves of Atriplex or young seedlings of Zea and Sorghum were cut and inserted in a vial of water or labeled metabolite. Exposure to $^{14}CO_2$ in the light was carried out as described previously[4]. The light sources used were a 400-W Philips HPLR mercury vapour lamp fitted with a Schott KG-2 filter or a 500-W Philips Photolita lamp with a 5-cm water filter. Intensities quoted are for light in the 400–700-nm band.

Following exposure to ^{14}C, leaves were killed in boiling 80 % ethanol and subsequently extracted in 50 % ethanol and boiling water. The combined extracts were fractionated on 5 cm Dowex 50-X8 (H^+ form) and Dowex 1-X10 (formate-form) columns prior to chromatography and electrophoresis. The acid column was eluted successively with 15 ml 0.12 M formic acid and with 30 ml 10 M formic acid or 0.5 M HCl. The dilute acid eluate contained glycolate, glycerate and two unidentified com-

pounds of low activity. The 10 M formic acid or 0.5 M HCl eluate contained carboxylic acids and phosphorylated compounds. Acid and amino acid fractions were chromatographed in the two solvent systems used earlier[4] and amino acids were also separated by high-voltage electrophoresis in acetate–formate buffer (15% acetic acid:5% formic acid, v/v). Sugars were separated by high-voltage electrophoresis in 0.1 M borate buffer, pH 8.2. Radioactive compounds were located by autoradiography, cut from the chromatogram, and counted in a liquid scintillation counter.

$^{14}CO_2$ evolved during the metabolism of labeled substrates by leaves was collected on KOH soaked discs of glass filter paper. Discs were changed at intervals and dried at 60° prior to counting in toluene scintillant.

Uptake experiments were done with 0.5-mm wide leaf slices, prepared as described earlier[10]. The slices were exposed to [2-^{14}C]glycine (1 mM) for 1 h in the light and samples were removed at intervals, washed 30 min in unlabeled glycine and dried on planchets. Uptake was linear during this period.

Enzyme assays

Leaves were sliced into 0.5–1.0-cm strips before grinding in a Sorvall Omnimixer using the 400-ml capacity vessel. A differential grinding technique[11,12] was developed which permitted separation of the enzymes associated with mesophyll and bundle sheath cells of the C_4 species with minimum cross contamination. The conditions of the grind are most important. Only a small proportion of the cells in each layer are extracted but calculated total enzyme activities and chlorophyll contents agree well with those obtained by exhaustive extraction.

A. hastata leaves (5–10 g) were ground for 30 sec at full speed in 50 ml of Buffer I which comprised 50 mM "Bicine" buffer, pH 7.5 (*N*,*N*-bis-(2-hydroxyethyl) glycine)[13], 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$, together with 1 g polyclar-AT[14]. The homogenate was filtered through two layers of "Mirracloth" and centrifuged for 20 min at 40000 × g_{max}. *A. spongiosa* leaves (10 g) were first ground for 5 sec in the "Omnimixer" at 10% line-voltage in 50 ml of Buffer I. The filtrate from this grind provides the mesophyll fraction. The residue was returned to the blender and extracted in a further 50 ml of Buffer I for 45 sec at 60% line-voltage to remove all adhering mesophyll cells, leaving clean bundle sheath fragments of 4–10 cells in length. The filtrate was discarded, the residue was washed with Buffer I, and then ground for 60 sec with 0.6 mm glass beads (10 ml) in 15 ml Buffer I in a Janke-Kunkle mill cooled with ice water. The filtrate from this grind, the bundle sheath fraction, was centrifuged as above.

The grinding procedure was modified for the more fibrous leaves of Zea and Sorghum. Buffer II which comprised 50 mM Bicine, pH 7.5, 50 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and 5 mM sodium EDTA, was found to give higher activities of bundle sheath enzymes after the prolonged grinding required to remove mesophyll cells. Sorghum leaves (10 g) were ground for 5 sec at 30% line-voltage in 50 ml Buffer II and polyclar-AT to provide the mesophyll fraction. Clean bundle sheath fragments were obtained after grinding for five consecutive periods (full speed for 60 sec followed by 30 sec temperature equilibration at 10% line-voltage) in 50 ml of Buffer II containing 0.3 M sucrose and saturated with N_2. The washed residue was then ground for 90 sec with glass beads in 25 ml Buffer II to provide the bundle sheath fraction.

These extracts were used as an enzyme source in the following assays and did not appear to contain inhibitors, as evidenced by cross checking with extracts of high and low activity, by linear dependence of activity on volume of extract used and by treatment with Sephadex G-10 or G-25 which failed to stimulate activity. All enzyme assays were performed at 30° and at the pH optima for *A. spongiosa* leaf extracts.

Spectrophotometric assays were done in 3 ml total volume and the following assays, coupled to oxidation of reduced pyridine nucleotides, contained 0.25 μmole of NADH. Blanks contained all reagents except substrate, unless otherwise specified. Phosphoenolpyruvate carboxylase (EC 4.1.1.32) was coupled with 5 units NADH malate dehydrogenase and assayed in Buffer I, pH 7.6, with 5 μmoles $NaHCO_3$ and 3 μmoles phosphoenolpyruvate. Ribulose-1,5-diphosphate carboxylase (carboxydismutase, EC 4.1.1.39) was assayed by the method of RACKER[15] in Buffer I, pH 8.0, with 90 μmoles $NaHCO_3$ and 0.2 μmole ribulose 1,5-diphosphate. Fructose-diphosphate aldolase (EC 4.1.2.13) was coupled with 20 μg mixed crystalline triosephosphate isomerase and glycerol-1-phosphate dehydrogenase[16] and assayed in Buffer I, pH 7.5, with 5 μmoles fructose 1,6-diphosphate. Transketolase (EC 2.2.1.1) was coupled in the same way[17] and 5 units of phosphoriboisomerase were added to 5 μmoles ribose 5-phosphate to generate the isomerase mixture substrate. Glycerate dehydrogenase (EC 1.1.1.29) was assayed in Buffer I, pH 7.5, with 20 μmoles freshly prepared hydroxypyruvate[18]. NADH glyoxylate reductase (EC 1.1.1.26)[19] was assayed in "MES" buffer, pH 6.5 (2-(*N*-morpholino) ethane sulphonic acid)[13], with 300 μmoles sodium glyoxylate. Glycerate kinase (EC 2.7.1.31) was coupled with 5 units each of 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase in Buffer I, pH 8.0, with 5 μmoles ATP and 5 μmoles glycerate.

Glycolate oxidase (EC 1.1.3.1) was assayed by the formation of glycolate phenylhydrazone at 324 nm[20]. The assay, in Buffer I, pH 7.5, contained 0.1 μmole flavin mononucleotide, 10 μmoles phenylhydrazine hydrochloride and 5 μmoles glycolate. Transaminase reactions were initially followed by radiochemical assays. Glutamate, alanine and serine were, in that order, decreasingly effective as donors in the conversion of [2-^{14}C]glyoxylate to [^{14}C]glycine. Glyoxylate was a more effective acceptor than pyruvate in the conversion of [3-^{14}C]serine to [^{14}C]hydroxypyruvate (identified by conversion to 2,4-dinitrophenylhydrazone derivative and to glycerate on addition of NADH). The high glycerate dehydrogenase activity permitted spectrophotometric assay of the serine, glyoxylate coupled formation of glycine and hydroxypyruvate. The assay contained Buffer I, pH 8.0, 5 μmoles of glyoxylate and, after steady rate of NADH oxidation was established (glyoxylate reductase activity) 10 μmoles of serine were added. Pyruvate dependent serine transamination was slow and required high concentrations of pyruvate and crystalline glyoxylate reductase.

Methylene tetrahydrofolate dehydrogenase (EC 1.5.1.5) was assayed in Buffer I, pH 7.5, with 13 μmoles formaldehyde, 1 μmole tetrahydrofolate and 0.25 μmole $NADP^+$ (ref. 21). $NADP^+$ was omitted from the blank. Formyltetrahydrofolate synthetase (EC 6.3.4.3) was assayed by conversion of the N^{10}, formyltetrahydrofolate formed to N^5,N^{10}-methenyltetrahydrofolate by acid treatment[22]. The assay contained, in 1 ml, 125 μmoles Tris–formate buffer, pH 8.0; 200 μmoles KCl; 1 μmole tetrahydrofolate; 5 μmoles ATP; and extract. After 10 min incubation the reaction was stopped with 2 ml 0.18 M H_2SO_4 and after a further 10 min, the mixture was read at 355 nm against a blank without ATP. Serine transmethylase (EC 2.1 2 1) was as-

sayed in the direction of glycine and formaldehyde synthesis. The assay contained, in 0.5 ml, 1 μmole tetrahydrofolate; 0.1 μmole pyridoxal phosphate; 5 μmoles [3-^{14}C]-serine (approx. 0.5 μC/μmole); and buffer I, pH 7.5. The dimedone complex of the [^{14}C]formaldehyde formed was extracted as described[23] and counted. The enzyme was further assayed in this direction by coupling to endogenous methylene tetrahydrofolate dehydrogenase when activity permitted, and was also assayed, with highly variable results, by the formation of [^{14}C]serine from [^{14}C]formaldehyde.

Phosphoglycolate phosphatase (EC 3.1.3.18) was assayed by the release of inorganic phosphate from phosphoglycolate[24]. The crude extracts also contained a less specific phosphatase and 3-phosphoglycerate was used as substrate for this activity. The assay contained 50 μmoles MES buffer, pH 5.5; 8 μmoles $MgCl_2$; and 5 μmoles phosphoglycolate or 3-phosphoglycerate; in 1 ml. The reaction was stopped with 2 ml 1.5 M H_2SO_4 and inorganic phosphate determined by the method of HURST[25]. Chlorophyll was estimated by extraction in 80 % acetone, and measured at 652 nm.

The labeled compounds used were obtained from the following sources: $NaH^{14}CO_3$, sodium [1-^{14}C]glyoxylate, sodium [2-^{14}C]glyoxylate, L-[3-^{14}C]serine from Radiochemical Centre, Amersham, U.K.; D-[$^{14}C_6$]fructose 6-phosphate, D-[$^{14}C_6$]-fructose 1,6-diphosphate, L-[1-^{14}C]glycine, L-[2-^{14}C]glycine and [^{14}C]formaldehyde from New England Nuclear, Boston, U.S.A.; and D-[$^{14}C_5$]ribose 5-phosphate from Schwartz Biochemicals. Glyoxal bisulfite was obtained from Fluka and recrystallised from aqueous ethanol before use. Crystalline enzymes, chemicals and radiochemicals were obtained from Sigma, Calbiochem, Boehringer and General Biochemicals.

RESULTS AND DISCUSSION

Glycolate synthesis

Glycolate formation during photosynthesis is conventionally measured in the presence of an inhibitor of glycolate oxidase[26]. The bisulfite compound inhibitors used are, however, strong inhibitors of photosynthetic CO_2 fixation *via* the C_4 dicarboxylic acid pathway[27]. To avoid interference with the rate of photosynthesis, labeled sugar phosphate products of photosynthesis, thought to give rise to glycolate[28,29], were fed to leaves in the light, together with 10 mM glyoxal bisulfite. Fig. 2 shows that labeled ribose 5-phosphate and fructose 6-phosphate were equally rapidly metabolised to glycolate in the leaves of both *A. hastata* and *A. spongiosa*. Similar results were ob-

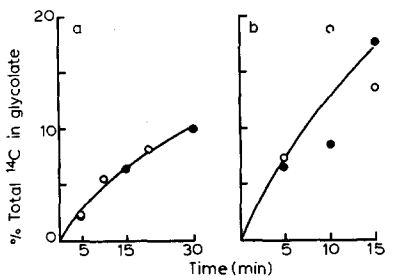

Fig. 2. Formation of glycolate during photosynthesis of labeled sugar phosphates. (a) [$^{14}C_5$]Ribose-5-*P* (0.016 μmole, 0.5 μC). (b) [$^{14}C_6$]Fructose-6-*P* (0.006 μmole, 1.0 μC). Glycolate formation in *A. hastata* (○) and *A. spongiosa* (●) leaves. Leaves pretreated in 10 mM glyoxal bisulfite for 10 min prior to transfer to labeled solutions. Light intensity, 17 mW·cm^{-2}; temp., 28°.

tained with labeled fructose 1,6-diphosphate. The rate of labeled glycolate formation in the light was at least 10-fold that in the dark. Treatment with glyoxal bisulfite also inhibited the labeling of glycine and serine from the labeled sugar phosphates suggesting that they were produced from these substrates *via* glycolate. A small proportion of the total label (1–5 %) was found in malate and aspartate and this too was inhibited by glyoxal bisulfite.

The glycolate pathway, tracer experiments

The labeling of glycine and serine in the above experiments and in $^{14}CO_2$ fixation studies[4,27] suggested that the glycolate pathway was active in both C_3 and C_4 species of Atriplex.

Experiments with specifically labeled glyoxylate (Table I) establish that serine may be formed by the C_2, C_1 condensation of the glycolate pathway in *A. spongiosa* leaves. Thus the ratio of label in serine to that in glycine synthesised from [2-^{14}C]-glyoxylate should be double that formed from [1-^{14}C]glyoxylate. The actual ratios found in Table I are very close to this ideal relationship. The release of $^{14}CO_2$ from [1-^{14}C]glycine or glyoxylate fed to *A. spongiosa* in the dark, or *A. hastata* leaves in the light or dark was 20–60-fold greater than that from [2-^{14}C]glycine consistent with the predictions of Table I. Other experiments showed that L-[3-^{14}C]serine was rapidly converted to sugars and phosphorylated compounds in leaves of C_3 and C_4 Atriplex species.

TABLE I

LABELING OF GLYCINE AND SERINE FROM SPECIFICALLY LABELED GLYOXYLATE IN *A. spongiosa* LEAVES IN THE LIGHT

Leaves were pretreated 10 min in water or 10 mM glyoxal bisulfite prior to transfer to 50 μl solution containing 0.13 μmole labeled glyoxylate (1 μC) in water or 10 mM glyoxal bisulfite. Temp., 28°; light intensity, 17 mW·cm^{-2}; duration, 10 min. Data expressed as percentage of total soluble radioactivity extracted.

	Serine	*Glycine*	*Ratio serine/glycine*
[1-^{14}C]Glyoxylate			
Control	18.0	8.3	2.2
+ 10 mM glyoxal bisulfite	31.1	12.5	2.5
[2-^{14}C]Glyoxylate			
Control	28.0	5.8	4.8
+ 10 mM glyoxal bisulfite	38.1	11.3	3.8

CO_2 evolution during glycolate pathway metabolism

The above experiments clearly indicate glycolate pathway activity in leaves of C_3 and C_4 species of Atriplex. Yet these species differ in that *A. spongiosa* shows an apparent absence of CO_2 evolution in the light, in common with all other C_4 species[4,30]. In the light, [1-^{14}C]glycolate or [1-^{14}C]glycine yield very much less $^{14}CO_2$ in leaves of C_4 plants than in C_3 plants, suggesting that the CO_2 of photorespiration is evolved from these intermediates of the glycolate pathway[31,32]. If the $^{14}CO_2$ released from these substrates was refixed[5], particularly if refixed by reactions of the C_4 dicarboxylic

acid pathway, heavy labeling of malate and aspartate might be observed. Further, if photosynthetic refixation *via* the C_4 dicarboxylic acid pathway was significant, treatment of leaves of C_4 plants with bisulfite compound inhibitors of this pathway should stimulate $^{14}CO_2$ release from [1-^{14}C]glyoxylate or [1-^{14}C]glycine. Fig. 3 shows the distribution of label amongst important products extracted from leaves of

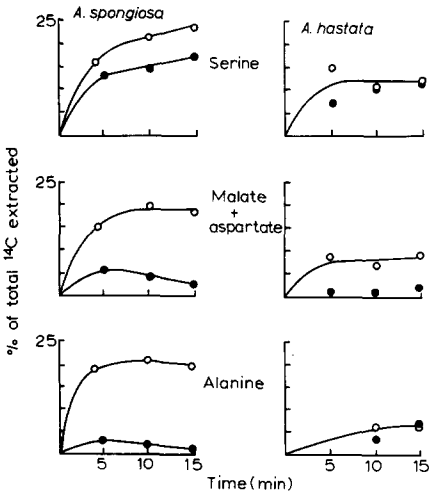

Fig. 3. Metabolism of [1-^{14}C]glyoxylate in *A. spongiosa* and *A. hastata* leaves in the light in control (○) and 10 mM glyoxal bisulfite (●). Leaves pretreated 10 min in water or 10 mM glyoxal bisulfite prior to transfer to 100 μl solution containing 0.34 μmole [1-^{14}C]glyoxylate (2.5 μC) in water or 10 mM glyoxal bisulfite. Light intensity, 17 mW·cm^{-2}; temp., 28°.

A. spongiosa and *A. hastata* fed [1-^{14}C]glyoxylate in the light. Serine was rapidly labeled in both species and labeling of serine was not inhibited by glyoxal bisulfite treatment, consistent with metabolism of glyoxylate *via* the glycolate pathway. The C_4 acids were also heavily labeled, particularly in *A. spongiosa* and the labeling of these acids was strongly depressed by glyoxal bisulfite treatment, consistent with formation *via* phosphoenolpyruvate carboxylase. In *A. spongiosa*, but not in *A. hastata*, labeling of alanine was also sensitive to glyoxal bisulfite, presumably because alanine is synthesised from pyruvate derived from the C_4 acids in the C_4 species[33]. The C_4 acids may have been labeled by the fixation of photorespiratory $^{14}CO_2$ or of $^{14}CO_2$ released from [1-^{14}C]glyoxylate by other reactions[34]. In either case, however, although treatment with glyoxal bisulfite stimulated the release of $^{14}CO_2$ in the light by *A. spongiosa* leaves, the rate did not exceed 10% that of *A. hastata*. Both species yield the same amount of $^{14}CO_2$ from these substrates in the dark.

To remove the uncertainty as to the origin of the $^{14}CO_2$ produced, [1-^{14}C]glycine was used as a substrate in similar experiments (Table II).

Treatment with glyoxal bisulfite stimulated the release of $^{14}CO_2$ in *A. spongiosa* about 10-fold but even then the amount of $^{14}CO_2$ released in the light in *A. spongiosa* was only about 10% of that in the dark or in *A. hastata* in the light. In this long term experiment (30 min) the proportion of label in C_4 compounds was small and did not show the clear cut effect of glyoxal bisulfite found in Fig. 3. In an experiment of this duration any $^{14}CO_2$ refixed by the phosphoenolpyruvate carboxylase system

TABLE II

PRODUCTS OF [1-^{14}C]GLYCINE METABOLISM IN ATRIPLEX LEAVES

Leaves pretreated 15 min in water or 10 mM glyoxal bisulfite prior to transfer to 50 μl solution containing 0.5 μmole [1-^{14}C]glycine (2.5 μC) in water or 10 mM glyoxal bisulfite. Temp., 25°; light intensity, 40 mW·cm^{-2}; time, 30 min. Data expressed as percentage of label in products of glycine metabolism.

Treatment	*Product*					
	$^{14}CO_2$	*Serine*	*Sugars*	*Phospho-rylated compounds*	*Malate + aspartate*	*Glycolate*
A. spongiosa						
Light	0.2	58.5	12.0	9.2	5.9	0.9
Light + 10 mM glyoxal bisulfite	3.7	58.0	3.7	5.9	4.6	11.7
Dark	22.1	57.0	0.3	1.9	7.0	0.6
A. hastata						
Light	20.9	22.6	3.1	22.4	19.2	0
Light + 10 mM glyoxal bisulfite	25.5	46.2	2.1	6.2	9.9	3.4
Dark	24.2	56.4	0.4	2.0	6.3	1.4

would appear in sugars and phosphorylated compounds. These compounds would also be labeled from glycine *via* the glycolate pathway or by the refixation of $^{14}CO_2$ *via* carboxydismutase. In parallel experiments, *Z. mays* and *S. bicolor* behaved as *A. spongiosa* and *T. vulgare* as *A. hastata*. The data do not permit conclusions as to the likely pathways of $^{14}CO_2$ refixation in leaves of C_4 plants. Interpretation of these experiments is made more difficult in that only 25 % of the glycine metabolised was recovered as $^{14}CO_2$, compared with an expected yield of 50 % during glycolate pathway activity in the absence of refixation.

The glycolate pathway, enzyme activities

Activities and distribution of the two principal photosynthetic carboxylases show features expected on the basis of earlier work[1,11,35] (Table III). Thus *A. hastata*, a C_3 species, has high carboxydismutase and low phosphoenolpyruvate carboxylase activity, comparable to that found in the bundle sheath fraction of both C_4 species. The mesophyll fraction from *A. spongiosa* also contains quite high carboxydismutase activity[11] and more gentle grinding conditions have not been found to reduce this activity. One is forced to accept these high values in the mesophyll fraction of C_4 dicotyledons although the activity may not arise from mesophyll cells themselves. Some contamination from bundle sheath cells is unavoidable but the C_4 dicotyledons often contain an additional layer of chloroplast containing cells outside the mesophyll layer. These cells may contain high levels of carboxydismutase. In Sorghum, on the other hand, carboxydismutase activity is very low in the mesophyll fraction. In both C_4 species, the contamination of the bundle sheath fraction by mesophyll cells is low as indicated by the low level of phosphoenolpyruvate carboxylase activity found in this fraction. The photosynthetic carboxylases thus serve as markers for the distribution of enzymes associated with glycolate synthesis and metabolism in C_4 leaves.

TABLE III

SPECIFIC ACTIVITY OF CARBOXYLASES AND ENZYMES OF GLYCOLATE SYNTHESIS IN *A. hastata* LEAVES AND IN MESOPHYLL AND BUNDLE SHEATH FRACTIONS FROM THE LEAVES OF C_4 PLANTS

Rates as μequiv/mg chlorophyll per min, showing means and S.E. with number of experiments shown in parentheses.

Enzyme	*A. hastata*	*A. spongiosa*		*S. bicolor*	
		Mesophyll	*Bundle sheath*	*Mesophyll*	*Bundle sheath*
Phosphoenolpyruvate carboxylase	0.42 ± 0.16 (5)	14.6 ± 2.4 (8)	0.12 ± 0.05 (8)	7.50 ± 1.0 (6)	0.81 ± 0.22 (5)
Carboxydismutase	7.02 ± 0.22 (4)	2.39 ± 0.32 (7)	5.94 ± 1.04 (7)	0.57 ± 0.16 (5)	4.84 ± 0.63 (3)
Fructose-diphosphate aldolase	25.7 ± 7.2 (4)	13.5 ± 2.5 (4)	31.7 ± 2.7 (4)	3.0 ± 0.6 (5)	27.0 ± 4.7 (5)
Transketolase	11.4 ± 3.8 (4)	5.0 ± 0.6 (4)	9.1 ± 2.0 (4)	1.3 ± 0.7 (4)	9.9 ± 3.1 (3)
Phosphoglycolate phosphatase	5.2 ± 2.1 (4)	3.2 ± 0.3 (4)	12.3 ± 1.8 (5)	0.3 ± 0.2 (5)	5.7 ± 0.8 (5)
3-Phosphoglycerate phosphatase	1.5 ± 0.5 (5)	1.3 ± 0.6 (4)	1.5 ± 0.4 (5)	0.6 ± 0.3 (5)	2.1 ± 0.3 (3)

TABLE IV

SPECIFIC ACTIVITY OF GLYCOLATE PATHWAY ENZYMES IN *A. hastata* LEAVES AND IN MESOPHYLL AND BUNDLE SHEATH FRACTIONS FROM THE LEAVES OF C_4 PLANTS

Rates as μequiv/mg chlorophyll per min, showing means and S.E. with number of experiments shown in parentheses.

Enzyme	*A. hastata*	*A. spongiosa*		*S. bicolor*	
		Mesophyll	*Bundle sheath*	*Mesophyll*	*Bundle sheath*
Glycolate oxidase	1.66 ± 0.33 (7)	0.36 ± 0.12 (9)	0.30 ± 0.14 (9)	0.03 ± 0.01 (9)	0.15 ± 0.09 (9)
Coupled transaminases	1.91 ± 0.62 (3)	0.24 ± 0.13 (5)	0.44 ± 0.12 (4)	0.01 ± 0.01 (4)	0.03 ± 0.03 (4)
Glycerate dehydrogenase	10.2 ± 0.4 (2)	6.48 ± 0.36 (4)	8.68 ± 0.81 (5)	0.80 ± 0.08 (4)	2.84 ± 0.46 (2)
NADH glyoxylate reductase	3.5 ± 1.1 (4)	0.85 ± 0.25 (4)	1.21 ± 0.24 (4)	0.45 ± 0.06 (4)	0.86 ± 0.08 (4)
Serine transmethylase	0.44 ± 0.10 (4)	0.25 ± 0.05 (3)	0.35 (3)	0.01 (4)	0.03 ± 0.01 (4)
Formyl tetrahydrofolate synthetase	0.51 ± 0.19 (5)	0.41 ± 0.05 (3)	0.15 ± 0.03 (3)	0.10 ± 0.04 (3)	0.09 ± 0.02 (3)
Methylene tetrahydrofolate dehydrogenase	0.31 ± 0.08 (6)	0.26 ± 0.07 (5)	0.02 ± 0.02 (5)	0.08 ± 0.04 (2)	0.04 ± 0.04 (2)
Glycerate kinase	1.05 ± 0.16 (5)	0.82 ± 0.10 (7)	0.05 ± 0.02 (6)	0.21 ± 0.07 (7)	0.05 ± 0.06 (7)

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Table III shows that transketolase and phosphoglycolate phosphatase, enzymes probably associated with glycolate synthesis[24,28,29], are most active in the bundle sheath fraction of C_4 leaf extracts[36]. In this fraction the activity may exceed that found in *A. hastata*. The ratios of activity, mesophyll to bundle sheath fraction are identical to those found for carboxydismutase and transaldolase, establishing a clear association of glycolate synthesis with the Calvin cycle. The activity associated with the mesophyll fraction probably represents contamination from the bundle sheath and suggests that the mesophyll chloroplasts may not produce glycolate. The actvities of the enzymes listed in Table III are sufficient to catalyse rates of photosynthesis (3–5 μmoles CO_2 fixed·min^{-1}·mg chlorophyll^{-1}) observed *in vivo* and the high rates of glycolate synthesis reported during photosynthesis.

Table IV shows that many enzymes associated with the glycolate pathway are sufficiently active to permit rates of glycolate metabolism approaching 50 % the rate of photosynthesis *in vivo*. The tetrahydrofolate enzymes showed lowest activities and may be limiting, but the role of these enzymes in the glycolate pathway is not yet clear. In the C_4 species, two important trends are evident. Firstly, the activity of all glycolate pathway enzymes in the bundle sheath extracts of *A. spongiosa* and Sorghum is lower than that in *A. hastata*. This is in marked contrast to the photosynthetic enzymes which showed comparable activity in all three species. Glycerate dehydrogenase is the only exception but when assayed as the glyoxylate reductase, this too is deficient in extracts of the C_4 species. In most instances, the specific activity in the mesophyll fraction is lower than in the bundle sheath fraction.

The second feature of the glycolate pathway in C_4 leaves, shown in Table IV, is that neither cell layer contains high activities of all enzymes of the pathway. Thus glycerate kinase and enzymes of C_1 activation have high activities in the mesophyll fraction whereas glycolate oxidase, glycerate dehydrogenase and coupled transaminase are more active in the bundle sheath. In all cases glycerate dehydrogenase was about 5 times more active with hydroxypyruvate than with glyoxylate (NADH glyoxylate reductase)[20]. In the C_4 leaf, it is unlikely that glycolate conversion to 3-phosphoglycerate occurs in a single cell layer.

Studies have shown that the distribution of chlorophyll in the leaves of C_4 plants differs markedly between species and genera. From the ratio chlorophyll *a*/chlorophyll *b* in plastids of each layer and the ratio chlorophyll *a*/chlorophyll *b* in the whole leaf, the relative distribution of total chlorophyll may be calculated[37]. In Table V the distribution of chlorophyll has been taken into account when expressing the percentage distribution between mesophyll and bundle sheath of the enzymes listed in Tables III and IV. Mean specific activity was used in each case.

Two forms of distribution are apparent. Carboxydismutase and the Calvin cycle enzymes thought to be involved in glycolate synthesis are very largely restricted to the bundle sheath cells[1,35] and the glycolate pathway enzymes associated with the peroxisome, glycolate oxidase, transaminase and glycerate dehydrogenase–NADH glyoxylate reductase are also largely found in the bundle sheath[36]. Serine transmethylase, in *A. spongiosa* at least, is largely associated with the bundle sheath. These enzymes are less clearly associated with either cell layer in Sorghum. Thus these data indicate that one might expect to find more peroxisomes in the bundle sheath than the mesophyll of *A. spongiosa*, and an equal distribution in Sorghum. The frequency, as judged by enzyme activity, would be much smaller than in a C_3 species. These

TABLE V

PERCENTAGE DISTRIBUTION OF TOTAL ENZYME ACTIVITY BETWEEN MESOPHYLL AND BUNDLE SHEATH CELLS IN *A. spongiosa* AND *S. bicolor* LEAVES

Enzyme	*A. spongiosa*		*S. bicolor*	
	Mesophyll	*Bundle sheath*	*Mesophyll*	*Bundle sheath*
Phosphoenolpyruvate carboxylase	96	4	98	2
Carboxydismutase	15	85	38	62
Fructose-diphosphate aldolase	15	85	37	63
Transketolase	19	81	40	60
Phosphoglycolate phosphatase	10	90	22	78
Glycolate oxidase	34	66	51	49
Coupled transaminase	18	82	63	37
Formyl tetrahydrofolate synthetase	54	46	85	15
Methylene tetrahydrofolate dehydrogenase	85	15	92	8
Serine transmethylase	23	77	63	37
Glycerate dehydrogenase	24	76	60	40
Glycerate kinase	88	12	95	5
Chlorophyll	30	70	84	16

speculations are in accord with the published ultrastructural studies showing microbodies (peroxisomes?) in both cell layers of C_4 species[38,39]. On the other hand, phosphoenolpyruvate carboxylase is almost entirely restricted to the mesophyll cell layer as pointed out in other studies[1]. The C_1 activation enzymes are also largely associated with these cells, as is glycerate kinase[1].

DISCUSSION

The experiments reported here suggest that significant synthesis of glycolate occurs in the leaves of C_3 and C_4 species during photosynthesis. In C_4 species, glycolate synthesis is closely associated with Calvin cycle metabolism of the bundle sheath cells, and may be absent from the C_4 acid metabolism in mesophyll cells. However, Photosystem II activity is an additional requirement for glycolate synthesis[29] and it is interesting to speculate that Sorghum bundle sheath chloroplasts, lacking this component of the photosynthetic system[12], may be deficient in glycolate production. This may induce much reduced glycolate pathway activity and explain the absence of photorespiration in Sorghum.

However, in tracer experiments with glycolate pathway intermediates, glycine is converted to serine equally rapidly in *A. hastata*, *A. spongiosa*, Sorghum and several other species (Table II), whereas glycolate pathway enzymes in Sorghum are only 1–10 % as active as in *A. hastata*. This serious anomaly is readily explained by the very slow rate of uptake of the intermediary compounds by leaves. Glycine uptake rates in leaf slice experiments with Atriplex spp. and Sorghum were 0.4–0.8 μmole·h^{-1}·mg^{-1} chlorophyll. In petiole feedings this rate would be much lower. Thus uptake occurs at a rate about 1% of the likely rate of photorespiration (1–2 μmoles·min^{-1}·mg^{-1} chlorophyll) and [^{14}C]glycine entering the cell would be immediately consumed, even in Sorghum. The tracer experiments simply demonstrate that the pathway is present

in the species studied and data from such experiments should be viewed with caution[31,32].

We believe the very low activity of all glycolate pathway enzymes in extracts of *S. bicolor* indicates that in this species, the pathway is not sufficiently active to generate significant amounts of CO_2 in the light. On the other hand, in *A. spongiosa* the glycolate pathway is quite active, and CO_2 may be released and refixed in significant quantities.

It is not yet clear whether the release and refixation of CO_2 in C_4 plants such as *A. spongiosa* involves release and refixation in the mesophyll, release in the bundle sheath with refixation in the mesophyll, or release and refixation in the bundle sheath. The clear association of C_1 activation enzymes with the mesophyll, together with adequate serine transmethylase activity in this tissue, favours a mesophyll location of serine synthesis from glycine, accompanied by CO_2 release. Presumably phosphoenolpyruvate carboxylase could then be implicated in refixation of photorespiratory CO_2. Treatment with bisulfite compounds always stimulated $^{14}CO_2$ release from [1-^{14}C]glyoxylate or [1-^{14}C]glycine in *A. spongiosa* leaves but never brought $^{14}CO_2$ release up to the dark level. This suggests phosphoenolpyruvate carboxylase may be involved in refixation, but that the high activity of this enzyme may be sufficient, even when 90% inhibited by the bisulfite compound, to refix most of the photorespiratory CO_2.

In view of the limited access of external CO_2 to bundle sheath chloroplasts it is unlikely that there would be rapid back diffusion of CO_2 to the mesophyll cells. However, the possibility of CO_2 release and refixation in the Calvin cycle of the bundle sheath cells cannot be excluded at present. Release and refixation of photorespiratory CO_2 in the mesophyll cells is an attractive hypothesis in that it accommodates the distribution of glycerate kinase. If correct, it requires the transport of 2 moles of glycine from the bundle sheath and its metabolism to CO_2 and 3-phosphoglycerate in the mesophyll. The 3-phosphoglycerate may equilibrate with the pool of C_3 acceptors for CO_2 fixation so that the C_4 product represents complete recovery of carbon lost from the bundle sheath. Movement of substrates between cell layers is an interesting feature of current formulations of the C_4 dicarboxylic acid pathway of photosynthesis[1,40]. It would not be surprising if substrate movements were involved in the control of photorespiration in some C_4 species.

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