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PHOTOSYNTHETIC CARBON METABOLISM IN *PANICUM MILIOIDES*, A C_3-C_4 INTERMEDIATE SPECIES: EVIDENCE FOR A LIMITED C_4 DICARBOXYLIC ACID PATHWAY OF PHOTOSYNTHESIS *

C.K.M. RATHNAM ** and RAYMOND CHOLLET ***

Laboratory of Agricultural Biochemistry, University of Nebraska, Lincoln, NE 68583 (U.S.A)

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Summary

Panicum milioides, a naturally occurring species with C_4-like Kranz leaf anatomy, is intermediate between C_3 and C_4 plants with respect to photorespiration and the associated oxygen inhibition of photosynthesis. This paper presents direct evidence for a limited degree of C_4 photosynthesis in this C_3-C_4 intermediate species based on:

(a) the appearance of 24% of the total ^{14}C fixed following 4 s photosynthesis in $^{14}CO_2$-air by excised leaves in malate and aspartate and the complete transfer of label from the C_4 acids to Calvin cycle intermediates within a 15 s chase in $^{12}CO_2$-air;

(b) pyruvate- or alanine-enhanced light-dependent CO_2 fixation and pyruvate stimulation of oxaloacetate- or 3-phosphoglycerate-dependent O_2 evolution by illuminated mesophyll protoplasts, but not bundle sheath strands; and

(c) NAD-malic enzyme-dependent decarboxylation of C_4 acids at the C-4 carboxyl position, C_4 acid-dependent O_2 evolution, and $^{14}CO_2$ donation from [4-^{14}C]C_4 acids to Calvin cycle intermediates during photosynthesis by bundle sheath strands, but not mesophyll protoplasts.

However, *P. milioides* differs from C_4 plants in that the activity of the C_4 cycle enzymes is only 15 to 30% of a C_4 *Panicum* species and the Calvin cycle and phospho*enol*pyruvate carboxylase are present in both cell types. From

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** Present address: New England Laboratory of Central Research, The Dow Chemical Co., P.O. Box 400, Wayland, MA 01778 (U.S.A.)

*** To whom correspondence should be addressed.

Abbreviations: Chl, chlorophyll; C_4 acids, malate and aspartate; FCCP, carbonylcyanide 4-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

these and related studies (Rathnam, C.K.M. and Chollet, R. (1979) Arch. Biochem. Biophys. 193, 346—354; (1978) Biochem. Biophys. Res. Commun. 85, 801—808) we conclude that reduced photorespiration in *P. milioides* is due to a limited degree of NAD-malic enzyme-type C_4 photosynthesis permitting an increase in pCO_2 at the site of bundle sheath, but not mesophyll, ribulose-bisphosphate carboxylase-oxygenase.

Introduction

Nonsucculent higher plants can be divided into two major groups based on the pathway of photosynthetic carbon assimilation. Plants with the C_4 dicarboxylic acid pathway of photosynthesis (C_4 plants) exhibit a full syndrome of anatomical, physiological and biochemical characteristics that are clearly distinguished from those possessed by species which fix CO_2 exclusively by the Calvin cycle (C_3 plants) [1—4]. All available evidence indicates that C_4 plants have arisen from C_3 species and that this has occurred independently many times during evolution [5]. However, a puzzling observation has been the failure to identify intermediates linking these two distinct photosynthetic groups.

Kanai and Kashiwagi [6] reported that photosynthetic carbon metabolism in *Panicum milioides*, a naturally occurring species with C_4-like Kranz leaf anatomy, is typical of C_3 plants. Subsequently, reports from several laboratories indicated that *P. milioides* is intermediate between C_3 and C_4 plants with respect to photorespiration [7—15]. However, little evidence has been presented for any C_3-C_4 intermediacy with respect to the pathway of photosynthetic carbon assimilation.

Since *P. milioides* represents the first well-documented example of a higher plant species with reduced photorespiration, it is of considerable importance to determine the biochemial mechanism(s) involved. In this paper we present evidence for a limited degree of C_4 photosynthesis and propose that a limited C_4-like CO_2 pump (mediated by a phospho*enol*pyruvate carboxylation-C_4 acid decarboxylation reaction sequence similar to that in NAD-malic enzyme-type C_4 plants) is responsible for the reduced photorespiration and O_2 sensitivity of net photosynthesis in leaves of *P. milioides*.

Materials and Methods

Plant material

Seeds of *Panicum milioides* Nees ex. Trin. (P.I. No. 285220) and *P. bisulcatum* Thumb. (P.I. No. 194861) were obtained from the Southern Regional Plant Introduction Station, Experiment, GA. The seeds were germinated and grown in a Metro-Mix potting medium in a controlled environment growth room (800 $\mu E\ m^{-2} \cdot s^{-1}$, 16 h photoperiod, 31°C day/27°C night). The plants were watered daily and nutrients were supplied twice weekly in the form of a modified Hoagland solution. Young, fully expanded leaves from 4- to 8-week-old plants were used in the experiments.

Preparation of leaf slices, mesophyll protoplasts and bundle sheath strands

The leaves were mechanically sliced into 0.5-mm wide sections [16], suspended in 0.3 M sorbitol containing 50 mM Hepes, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM KH_2PO_4 and 5 mM isoascorbate adjusted to pH 7.6 (Medium A), and maintained at room temperature.

Leaf slices (1 to 2 g) were placed in a 100 ml flask with 30 ml of digestion medium containing 0.6 M sorbitol, 20 mM Mes, 5 mM $MgCl_2$, 0.05% (w/v) bovine serum albumin, 50 mM isoascorbate, 0.5 mM $CaCO_3$, 0.5 mM $CaSO_4$ and 4% (w/v) 'Meicelase P' cellulase (Meiji Seika Kaisha, Ltd., Toyko, Japan) adjusted to pH 5.5. This medium differs from the one developed by Kanai and Edwards [17] for isolating mesophyll protoplasts and bundle sheath strands from leaves of C_4 plants in that 'Macerozyme' pectinase was omitted, 'Meicelase P' cellulase replaced 'Onozuka' cellulase, and bovine serum albumin, isoascorbate, $CaCO_3$ and $CaSO_4$ were included to enhance the activity and membrane integrity of the isolated cell types [16]. The leaf slices were vacuum infiltrated with the digestion medium for 1 min and subsequently maintained at 37°C. After 1 h the digestion medium was decanted and the leaf slices were collected on a 35-mesh stainless steel sieve and washed twice by gentle swirling in 20 ml of 0.6 M sorbitol. During this process only mesophyll cells were released in the form of protoplasts as judged by light microscopy. The combined 35-mesh filtrates were sequentially passed through 80 μm and 40 μm nylon nets (Tetko Inc., Elmsford, NY) and the resulting filtrate was centrifuged at 400 × *g* for 3 min. The pellet was resuspended in 0.9 ml of 0.6 M sorbitol and intact mesophyll protoplasts were purified from this crude suspension by partitioning at the interface of an aqueous dextran-polyethylene glycol two-phase system essentially as described previously [18].

For isolating bundle sheath strands, the washed, partially digested leaf slices (see above) were digested for an additional 1 h in 30 ml of fresh digestion medium. The slices were collected on the 35-mesh sieve, resuspended and stirred in 40 ml of 0.6 M sorbitol with a magnetic stir bar at 40 to 60 rev./min for 10 to 15 min, and recollected on the sieve. After two additional wash cycles in 0.6 M sorbitol, the combined 35-mesh filtrates were sequentially passed through 210 μm, 110 μm and 80 μm nylon nets. While epidermal tissue, vascular strands with attached mesophyll and bundle sheath cells, and bundle sheath strands attached to epidermal tissue were retained on the 210 μm and 110 μm nets, uncontaminated bundle sheath strands were collected on the 80 μm net. The isolated bundle sheath strands were suspended in Medium A, vortexed for 10 s and recollected on the 80 μm nylon net.

Mesophyll protoplasts from leaves of *P. bisulcatum* were isolated and purified as described by Kanai and Edwards [17,18].

Purified mesophyll protoplasts and bundle sheath strands were resuspended in Medium A and maintained at room temperature.

Photosynthetic activities of leaf slices, mesophyll protoplasts and bundle sheath strands

(a) Assay conditions. $^{14}CO_2$ fixation and L-[4-^{14}C]malate and L-[4-^{14}C]-aspartate decarboxylation assays were run in serum-stoppered glass vials containing a standard reaction mixture of 0.3 M sorbitol, 50 mM Tricine, 1 mM

$MgCl_2$, 1 mM $MnCl_2$, 2 mM KH_2PO_4 and 3 mM isoascorbate adjusted to pH 8.0 [15,16]. Other additions were as indicated for the individual experiments. The CO_2 fixation and decarboxylation assays were run in an illuminated constant temperature water bath maintained at 30°C; illumination was provided by sodium discharge lamps (400-W Ceramalux, Westinghouse Electric Co.), giving a quantum flux density of about 5 to $10.5 \cdot 10^3$ $\mu E\ m^{-2} \cdot s^{-1}$ (400—700 nm) at the surface of the reaction vials. For photosynthetic O_2 evolution studies, illumination was provided by two 150-W Westinghouse low-temp spot lamps filtered through a 1% (w/v) $CuSO_4$ solution. The quantum flux density at the surface of the cuvette was 1.1 to $1.4 \cdot 10^3$ $\mu E\ m^{-2} \cdot s^{-1}$ (400—700 nm).

(b) Photosynthetic $^{14}CO_2$ fixation. The reaction vials contained the standard assay medium, leaf slices or the isolated cell types (2—6 μg of Chl), and 0.48 or 6 mM $NaH^{14}CO_3$ (2.5 Ci/mol) in a final volume of 0.5 ml. The sealed reaction vials containing the leaf slices or cell types and assay medium were flushed for 8—10 min with humidified O_2/N_2 mixtures (Matheson Gas Products), pre-illuminated for 5 min, and the assays initiated by injecting $NaH^{14}CO_3$. The reactions were terminated after 10 min by injecting 0.25 ml of 50% (w/v) trichloroacetic acid or 44% (v/v) lactic acid. The vials were flushed with air to ensure complete removal of unfixed $^{14}CO_2$ and dpm determined by liquid scintillation spectroscopy. Preliminary experiments indicated that $^{14}CO_2$ uptake was linear with respect to time for at least 15 min.

(c) C_4 acid decarboxylation. Decarboxylation of C_4 acids at the C-4 position by leaf slices, mesophyll protoplasts and bundle sheath strands (3—6 μg of Chl) in light and darkness was followed directly using 10 mM L-[4-^{14}C]aspartate (0.96 Ci/mol) and 10 mM L-[4-^{14}C]malate (1.04 Ci/mol) as previously described [19,20], except that the evolved $^{14}CO_2$ was trapped in 0.1 ml of 3 M ethanolamine placed in a centrally-suspended polyethylene sample vial (No. 17570, Bel—Art Products) fitted with a filter paper wick. The reaction mixtures contained 50 mM D,L-glyceraldehyde and 4 mM maleate to prevent refixation of released $^{14}CO_2$ by ribulosebisphosphate carboxylase and phospho*enol*pyruvate carboxylase, respectively [19,20]. All values were corrected for zero-time controls.

(d) Substrate-dependent O_2 evolution. Light-dependent O_2 evolution was measured polarographically in a final volume of 1.2 ml at 30°C using a Hansatech O_2 electrode (Hansatech Ltd., King's Lynn, Norfolk, U.K.) [20]. The CO_2-free assay medium (minus isoascorbate) containing isolated mesophyll chloroplasts, mesophyll protoplasts or bundle sheath strands (14 μg of Chl) was equilibrated with N_2 for 3—5 min and the reaction initiated by the addition of the appropriate substrate(s) as indicated for the individual experiments.

(e) Analysis of ^{14}C-labeled products. For the analysis of products labeled during $NaH^{14}CO_3$- and [4-^{14}C]C_4 acid-experiments with leaf slices, mesophyll protoplasts and bundle sheath strands (8—15 μg of Chl), 5 ml of boiling abs. ethanol was added to the lactic acid-terminated reaction vials and boiling continued for 3 min. The tissue was homogenized with a mortar and pestle and extracted successively with 5-ml aliquots of boiling 80% (v/v) ethanol, 50% (v/v) ethanol and H_2O. The extracts were pooled, cooled and centrifuged at 20 000 × *g* for 30 min. The supernatant was decanted and the pellet was washed three times with 5 ml of H_2O. The washed insoluble fraction was

transferred to a scintillation vial containing 1 ml of H_2O, vortexed, scintillation fluid was added, and dpm determined. The ethanol/water-soluble supernatant fractions were pooled and fractionated by sequential ion exchange chromatography on 1 × 6.5 cm columns of AG 50W-X8 (H^+; 200-400 mesh) and AG 1-X8 (acetate; 200—400 mesh) (Bio-Rad Labs) as cation and anion exchange resins, respectively [21]. Three fractions were obtained from the ion exchange columns: (a) a neutral fraction (mainly sugars) eluted with 30 ml of H_2O through the coupled columns; (b) a basic fraction (mainly amino acids) eluted from the cation column with 30 ml of 2 N HCl; and (c) an acidic fraction (mainly organic acids, sugar phosphates and other phosphorylated compounds) eluted from the anion column with 30 ml of 2 N HCl. The eluants were evaporated to dryness in vacuo at 40° C, redissolved in 100 µl of H_2O and aliquots counted by liquid scintillation spectroscopy to determine the distribution of ^{14}C between the various fractions. Recovery from the columns was greater than 95%.

In experiments with [4-^{14}C]aspartate, the basic fraction (containing unmetabolized [^{14}C]aspartate) was discarded. In [4-^{14}C]malate-fed samples, unmetabolized [^{14}C]malate was separated from other labeled compounds in the acidic fraction by one-dimensional descending paper chromatography (Whatman No. 3MM paper) using the organic phase of *n*-pentanol: 5 N formic acid (1:1, v/v) as the solvent [22]. Sugar phosphates and other phosphorylated compounds, free of malate, remained at the origin and were eluted from the air-dried chromatogram with H_2O, taken to dryness in vacuo and redissolved in 100 µl of H_2O. The phosphorylated compounds present in the acidic fraction of $NaH^{14}CO_3$- and [4-^{14}C]C_4 acid-fed samples were separated by one-dimensional TLC on precoated plates of Cellulose MN 300 using 2-butanol/formic acid/H_2O (6:1:2, v/v) as the solvent [23]. Authentic phosphorylated standards were co-chromatographed and located with ammonium molybdate reagent [24]. The distribution of ^{14}C in the acidic fraction was determined from similarly developed, unsprayed chromatograms by scraping the corresponding areas into scintillation vials and extraction with 1 ml of H_2O for 1 h. Scintillation fluid was added and radioactivity (dpm) determined.

Leaf photosynthesis

(a) $^{14}CO_2$ pulse-$^{12}CO_2$ chase experiments. Fully expanded leaves were cut under water and transferred to a 250 ml beaker with their cut bases immersed in water. The excised leaves were illuminated at a quantum flux density of about 3.4 to $10.5 \cdot 10^3$ µE $m^{-2} \cdot s^{-1}$ (400—700 nm) for at least 1 h before exposure to $^{14}CO_2$. Illumination was provided by sodium discharge lamps.

A $^{14}CO_2$-feeding technique similar to that described by Chollet and Ogren [25] was employed. A 60-ml serum-stoppered glass test tube with a narrow slit at the top served as the $^{14}CO_2$-feeding chamber. Light intensity at the surface of the leaves was about 3.4 to $5.2 \cdot 10^3$ µE $m^{-2} \cdot s^{-1}$. $^{14}CO_2$ was generated just prior to feeding each pair of leaves by injecting 5 µCi (0.1 µmol) or 15 µCi (1.13 µmol) of $NaH^{14}CO_3$ into 2 ml of 44% (v/v) lactic acid present in the bottom of the sealed chamber. Two leaves were fed each time by inserting them through the slit at the top of the chamber containing 5 µCi or 15 µCi of $^{14}CO_2$ in air (initial pCO_2, approx. 367 µl/l or 746 µl/l, respectively). After

a 4 s pulse in $^{14}CO_2$ at 25°C, the leaves were withdrawn, the slit was quickly resealed with tape and the leaves were either immediately extracted in boiling 80% (v/v) ethanol or transferred to an adjacent, open test tube for a 5 s or 15 s chase in $^{12}CO_2$-air (approx. 330 μl/l) at 25°C before extraction.

(b) Analysis of ^{14}C-labeled products. Three pairs of identically-fed leaves were pooled and successively extracted by grinding with a mortar and pestle in 10 ml each of boiling 80% (v/v) ethanol, 50% (v/v) ethanol and H_2O. The extracts were pooled, cooled, and centrifuged at 20 000 × *g* for 30 min. The supernatant was decanted and the pellet was washed three times with 5 ml of water.

The combined ethanol/water-soluble fraction was evaporated to dryness in vacuo at 35°C, redissolved in 100 μl of water, and aliquots of known radioactivity were spotted on 22.5 × 50 cm sheets of Whatman No. 1 chromatography paper. The ^{14}C-labeled compounds were separated by one-dimensional descending paper chromatography in liquefied phenol (approx. 90%)/water/acetic acid/1 M EDTA (840:160:10:1, by vol.) [25]. To enhance separation in this one-dimensional system, the chromatograms were developed for 48 h by dripping the solvent off the serrated bottom of the filter paper. The developed chromatograms were thoroughly air-dried and the radioactive areas located with a Vanguard-880 radiochromatogram scanner. Authentic standards were co-chromatographed and located by color development. Amino acids were detected with ninhydrin [26], organic acids with bromophenol blue [22], and phosphorylated compounds with ammonium molybdate [24]. Corresponding areas from unsprayed chromatograms were cut out, extracted in 1 ml of H_2O and radioactivity determined by liquid scintillation spectroscopy.

Inhibitors

The inhibitors used in the present study were: maleate for phospho*enol*pyruvate carboxylase (for details, see Refs. 15 and 16); D,L-glyceraldehyde for the Calvin cycle [27]; 3-mercaptopicolinic acid for phospho*enol*pyruvate carboxykinase [28,29]; oxalate for NADP-malic enzyme [29,30]; dibromothymoquinone as a plastoquinone antagonist [31]; FCCP and NH_4Cl as photophosphorylation uncouplers [31]; and diuron for electron transport through Photosystem II [31].

Results and Discussion

Isolation of leaf cell types from P. milioides

An important feature of C_4 photosynthesis is the compartmentation of carbon metabolism between mesophyll and bundle sheath cells [2—4]. Although it has been reported [32] that pure mesophyll protoplasts and bundle sheath strands can be obtained from leaves of *P. milioides* using the conventional 'Onozuka' (*Trichoderma viride*) cellulase-pectinase enzyme digestion system and sequential filtration protocol originally developed for isolating pure leaf cell types from C_4 plants [17], attempts in this laboratory and elsewhere (Servaites, J.C. and Reger, B.J., personal communications) using the conventional C_4 protocol yielded cross-contaminated mesophyll and bundle sheath preparations from this intermediate species. Several factors including

leaf age, leaf slice thickness, digestion time and temperature, concentration and source of *T. viride* cellulase (Yakult Biochemicals Co., Nishinomiya, Japan; Boehringer Mannheim) and/or pectinase were varied systematically without success. These trials always resulted in either a complete digestion of mesophyll and bundle sheath cell walls yielding a mixed population of mesophyll and bundle sheath protoplasts and naked vascular strands, or vascular strands with varying degrees of mesophyll and bundle sheath cells attached to epidermal strips. In attempting to develop a technique for separating mesophyll and bundle sheath cells including mechanical grinding, enzymic digestion and sequential filtrations, we have succeeded in isolating uncontaminated mesophyll protoplasts and bundle sheath strands from *P. milioides* (as evidenced by light microscopy) by digesting thin leaf slices with 'Meicelase P' cellulase.

The activity and intercellular compartmentation of key photosynthetic and photorespiratory enzymes between mesophyll and bundle sheath cells of *P. milioides* obtained by the 'Meicelase P' cellulase digestion system have recently been presented [13]. The specific activity of phospho*enol*pyruvate carboxylase, the first enzyme of the carboxylative phase of the C_4 pathway, is 4-times greater in the mesophyll than in the bundle sheath (on a Chl basis). Pyruvate, orthophosphate dikinase, catalyzing phospho*enol*pyruvate regeneration from pyruvate, is exclusively localized in mesophyll cells. Of the three known C_4 acid decarboxylating enzymes (NADP-malic enzyme, NAD-malic enzyme and phospho*enol*pyruvate carboxykinase) present in leaves of C_4 plants [2—4], only NAD-malic enzyme activity was detected in leaf extracts of *P. milioides* [15] and this activity was exclusively localized in the bundle sheath [13]. Reciprocal mixing experiments with the crude cell homogenates indicated that the absence of detectable pyruvate, orthophosphate dikinase and NAD-malic enzyme activity in the bundle sheath and mesophyll extracts, respectively, was not due to the presence of endogenous inhibitors (unpublished data). An approximately equal distribution of aspartate- and alanine-aminotransferases and NAD-malate dehydrogenase between the two cell types (on a specific activity basis) further suggested a compartmentation of C_4 pathway enzymes similar to that in NAD-malic enzyme-type C_4 plants [2—4]. Consistent with recent in situ immunofluorescent localization studies of ribulosebisphosphate carboxylase in *P. milioides* leaf sections [33], the in vitro enzyme data [13] indicated that several marker enzymes of the Calvin cycle and photorespiration are present in both cell types, with the bundle sheath having about 3-times the specific activity of the mesophyll. Evidence from independent experiments supporting this intercellular distribution of C_4- and C_3-photosynthetic enzymes based on exogenous substrate effects on light-dependent $^{14}CO_2$ fixation and O_2 evolution by the isolated cell types and the exclusive localization of [4-^{14}C]C_4 acid metabolism in intact bundle sheath strands is presented below.

Exogenous substrate effects on $^{14}CO_2$ fixation

Table I shows the effects of various 3-carbon precursors of PEP on $^{14}CO_2$ fixation by isolated mesophyll protoplasts and bundle sheath strands of *P. milioides* in light and darkness. In the absence of exogenous substrates, both mesophyll protoplasts and bundle sheath strands exhibited a light-dependent, D,L-glyceraldehyde-sensitive, maleate-insensitive fixation of $^{14}CO_2$, indicating

TABLE I

EFFECT OF D,L-GLYCERALDEHYDE AND MALEATE ON SUBSTRATE-DEPENDENT $^{14}CO_2$ FIXATION IN LIGHT AND DARKNESS BY ISOLATED MESOPHYLL PROTOPLASTS AND BUNDLE SHEATH STRANDS OF *P. MILIOIDES*

The isolated cell types were incubated in the standard assay medium containing the specified substrates and inhibitors for 5—10 min at 25°C. The reaction vials were transferred to a constant temperature water bath and the assays were started following 5 min preillumination by addition of $NaH^{14}CO_3$ (6 mM). The reactions were run for 10 min at 21% O_2 and 30°C. Concentrations of added compounds, in mM, were: D,L-glyceraldehyde, 50; maleate, 4; phospho*enol*pyruvate (PEP), 5; pyruvate, 5; alanine, 5; α-ketoglutarate (α-Kg), 10; dihydroxyacetone-*P*, 2.5. The results are the average of 2—3 experiments.

Assay conditions	μmol $^{14}CO_2$ fixed/mg chlorophyll per h			
	Mesophyll protoplasts		Bundle sheath strands	
	Light	Dark	Light	Dark
Control	23.5	0	120	0
+Glyceraldehyde (Gld)	0	0	0	0
+Maleate	25.6	0	123	0
+Gld + maleate	0	0	0	0
PEP	126	96.0	147	18.3
+Gld	98.6	86.5	18.8	18.4
+Maleate	25.3	0	119	0
+Gld + maleate	0	0	0	0
Pyruvate	104	0	121	0
+Gld	81.7	0	0	0
+Maleate	25.5	0	121	0
+Gld + maleate	0	0	0	0
Alanine + α-Kg	83.4	0	123	0
+Gld	60.9	0	0	0
+Maleate	25.0	0	120	0
+Gld + maleate	0	0	0	0
Dihydroxyacetone-*P*	26.9	0	160	27.3
+Gld	0	0	27.6	27.6
+Maleate	27.3	0	124	0
+Gld + maleate	0	0	0	0

the presence of a functional Calvin cycle in both cell types. The rate of $^{14}CO_2$ fixation by mesophyll protoplasts was about 20% of that observed with bundle sheath strands, in agreement with the reduced activity of Calvin cycle enzymes in this cell type [13]. The relatively low CO_2 fixation capacity of mesophyll protoplasts is not likely due to any deficiency in photochemical activity since thylakoid membranes isolated from mesophyll chloroplasts and bundle sheath strands of *P. milioides* catalyzed various photosynthetic electron transport partial reactions at comparable rates: 349 and 215 (photosystem II + I, from H_2O to methyl viologen); 368 and 302 (photosystem II, from H_2O to ferricyanide in the presence of dibromothymoquinone); and 263 and 271 (photosystem I, from reduced 2,6-dichlorophenolindophenol to methyl viologen in the presence of diuron) μmol O_2/mg Chl per h by mesophyll and bundle sheath thylakoid membranes, respectively (for assay methods, see Refs. 34 and 35).

Exogenous phospho*enol*pyruvate greatly enhanced (5-fold) CO_2 fixation by mesophyll protoplasts whereas the rate of bundle sheath CO_2 uptake was increas-

ed by only 23%. A limited effect of phospho*enol*pyruvate on bundle sheath CO_2 fixation is expected due to the relatively low phospho*enol*pyruvate carboxylase activity in this cell type [13]. The incremental phospho*enol*pyruvate-dependent fixation of $^{14}CO_2$ was largely light-independent, D,L-glyceraldehyde-insensitive and inhibited by maleate, indicating phospho*enol*pyruvate carboxylase participation. The light-independent CO_2 uptake observed in the presence of phospho*enol*pyruvate plus D,L-glyceraldehyde is likely due to phospho*enol*pyruvate carboxylase since carboxylation of phospho*enol*pyruvate per se does not require any energy [4], whereas the light-dependent fixation of CO_2 observed in the presence of phospho*enol*pyruvate plus maleate is due to Calvin cycle activity.

Addition of 3-carbon precursors of phospho*enol*pyruvate such as pyruvate or alanine (plus α-ketoglutarate) resulted in a 3.5- to 4-fold increase in light-dependent $^{14}CO_2$ fixation by mesophyll protoplasts, but was without effect on bundle sheath CO_2 uptake. Since both cell types exhibit phospho*enol*pyruvate-enhanced CO_2 fixation and contain phospho*enol*pyruvate carboxylase and alanine aminotransferase [13], the absence of pyruvate or alanine stimulation of CO_2 uptake by bundle sheath strands is consistent with the exclusive localization of pyruvate, orthophosphate dikinase in the mesophyll cells of *P. milioides* [13]. The light-dependency of pyruvate- and alanine-enhancement of CO_2 fixation by mesophyll protoplasts is due to the conversion of pyruvate to phospho*enol*pyruvate via pyruvate, orthophosphate dikinase which requires ATP and thus photosynthetic electron transport. Maximum induction by alanine required α-ketoglutarate, suggesting that alanine is converted to pyruvate by mesophyll alanine aminotransferase and then to phospho*enol*-pyruvate via pyruvate, orthophosphate dikinase. Although maleate did not inhibit CO_2 fixation by mesophyll protoplasts with $H^{14}CO_3^-$ alone, it completely inhibited the incremental pyruvate- or alanine-dependent uptake of CO_2, indicating the participation of phospho*enol*pyruvate carboxylase. The residual rate of CO_2 fixation observed in the presence of maleate was comparable to the endogenous control rate of CO_2 uptake. Although D,L-glyceraldehyde completely inhibited endogenous CO_2 fixation, there was only a partial inhibition of pyruvate- or alanine-enhanced CO_2 uptake. The sum of the residual rates of CO_2 fixation observed in the presence of D,L-glyceraldehyde or maleate approached the uninhibited rate, suggesting that the rate of CO_2 uptake observed in the presence of pyruvate or alanine is a composite of both ribulosebisphosphate carboxylase- and phospho*enol*pyruvate carboxylase-mediated CO_2 fixation.

Exogenous dihydroxyacetone-*P*, a Calvin cycle intermediate, resulted in a 33% increase in the rate of CO_2 fixation by bundle sheath strands, while having only a slight effect with mesophyll protoplasts. The light-independent, maleate-sensitive, D,L-glyceraldehyde-insensitive nature of dihydroxyacetone-*P* stimulation suggests that triose-*P* may be converted to phospho*enol*pyruvate in a nonenergy-requiring reaction sequence via phosphoglyceromutase and enolase, and subsequently carboxylated by bundle sheath phospho*enol*pyruvate carboxylase. Given that the potential for phospho*enol*pyruvate generation from 3-*P*-glycerate (evidence for a phosphoglyceromutase-enolase reaction sequence) is present in both mesophyll and bundle sheath extracts (81 and 121 μmol/mg

Chl per h, respectively), the observation that dihydroxyacetone-*P* stimulates CO_2 fixation only in the bundle sheath is puzzling. The fact that D,L-glyceraldehyde-insensitive, light-independent, maleate-sensitive CO_2 fixation in the bundle sheath was observed in the presence of dihydroxyacetone-*P*, but not when HCO_3^- alone was added, suggests that triose-*P* formed during bundle sheath photosynthesis can not be used as a precursor for phospho*enol*pyruvate; perhaps triose-*P* formed in the mesophyll Calvin cycle is exported as a precursor for phospho*enol*pyruvate generation in the bundle sheath.

Effect of pyruvate on oxaloacetate- or 3-P-glycerate-dependent O_2 evolution

The exclusive localization of phospho*enol*pyruvate regeneration from pyruvate via pyruvate, orthophosphate dikinase in the mesophyll cells of *P. milioides* (Table I; Ref. 13) was further documented by studying the effect of pyruvate on the photoreduction of oxaloacetate and 3-*P*-glycerate in the Hill reaction (Table II). The ability of these latter compounds to support O_2 evolution is presumably due to their reduction within chloroplasts by NAD(P)-malate dehydrogenase and NADP-triosephosphate dehydrogenase, respectively, with the associated turnover of NAD(P)H providing an acceptor for electrons from water. Both mesophyll protoplasts and bundle sheath strands actively catalyzed oxaloacetate- or 3-*P*-glycerate-dependent O_2 evolution. The oxaloacetate-dependent rate of O_2 evolution was similar in both cell types, consistent with the comparable activities of NAD-malate dehydrogenase [13] (some of which is presumably chloroplastic; Refs. 36,37) and photosynthetic electron transport in mesophyll and bundle sheath preparations. In contrast, the rate of

TABLE II

EFFECT OF PYRUVATE ON OXALOACETATE- OR 3-*P*-GLYCERATE-DEPENDENT O_2 EVOLUTION BY ILLUMINATED MESOPHYLL PROTOPLASTS, MESOPHYLL CHLOROPLASTS AND BUNDLE SHEATH STRANDS OF *P. MILIOIDES*

Light-dependent O_2 evolution was followed polarographically at 30°C using a Hansatech O_2 electrode. The CO_2-free assay medium containing the cell preparations was equilibrated with N_2 for 3—5 min and the reactions were initiated by the addition of oxaloacetate or 3-*P*-glycerate. Intact mesophyll chloroplasts were obtained by gentle protoplast rupture as described elsewhere [37]. Concentrations of added compounds, in mM, were: pyruvate, 10; oxaloacetate, 0.5; FCCP, 0.002; 3-*P*-glycerate, 1.

Assay conditions	μmol O_2 evolved/mg chlorophyll per h		
	Mesophyll protoplasts	Mesophyll chloroplasts	Bundle sheath strands
Pyruvate	0	0	0
Oxaloacetate	15.6	21.4	20.7
+ FCCP *	62.4	68.5	72.6
+ Pyruvate	98.6	109.6	20.8
+ Pyruvate + FCCP	64.5	68.0	70.9
3-*P*-Glycerate	30.9	38.5	109.3
+ FCCP *	0.4	0.3	0.1
+ Pyruvate	94.5	124.6	108.7
+ Pyruvate + FCCP *	0.3	0.2	0.1

* Similar responses were observed when 20 mM NH_4Cl was used in place of FCCP to uncouple photophosphorylation.

3-*P*-glycerate-dependent O_2 evolution was about 3.5-fold higher in bundle sheath strands than in mesophyll protoplasts (Table II), in agreement with the findings that the activity of NADP-triosephosphate dehydrogenase and other Calvin cycle enzymes is about 3-times higher in bundle sheath extracts [13] and that the rate of photosynthesis in the absence of exogenous substrates is about 5-fold higher in the bundle sheath (Table I).

When pyruvate was added to cell preparations exhibiting light-dependent O_2 evolution in the presence of oxaloacetate or 3-*P*-glycerate the rate of O_2 evolution was markedly enhanced in mesophyll protoplasts, but not bundle sheath strands. A similar effect of pyruvate was also observed on oxaloacetate- or 3-*P*-glycerate-dependent O_2 evolution by intact chloroplasts isolated from mesophyll protoplasts. Neither mesophyll chloroplasts, mesophyll protoplasts nor bundle sheath strands evolved O_2 when pyruvate alone was added. It is proposed that the pyruvate stimulation of oxaloacetate- or 3-*P*-glycerate-dependent O_2 evolution in the mesophyll is due to the ATP-dependent conversion of pyruvate to phospho*enol*pyruvate via pyruvate, orthophosphate dikinase, i.e., a stimulation of coupled photosynthetic electron transport by an increased demand for ATP [38]. This proposal was supported by the complete reversal of the pyruvate effect by the photophosphorylation uncouplers, FCCP and NH_4Cl (Table II). The stimulatory effect of the uncouplers on oxaloacetate-dependent O_2 evolution by both cell types can be explained on the basis that the photoreduction of oxaloacetate requires only NAD(P)H and that the rate of photosynthetic electron transport in uncoupled chloroplasts is several-fold higher than in control chloroplasts. The inhibitory effect of FCCP and NH_4Cl on 3-*P*-glycerate-dependent O_2 evolution is presumably due to a lack of ATP for the prerequisite phosphorylation of 3-*P*-glycerate prior to the reductive step. These results, together with those presented in Table I, further document the exclusive localization of pyruvate, orthophosphate dikinase in the mesophyll cells of *P. milioides* (see Ref. 13) and indicate that the enzyme is chloroplastic as in leaves of C_4 plants [2,4,38].

[4-^{14}C]C_4 acid metabolism

A basic feature of C_4 photosynthesis is that the C_4 acids formed from phospho*enol*pyruvate carboxylation in the mesophyll are enzymatically decarboxylated in the bundle sheath at the C-4 carboxyl position and the released CO_2 is reassimilated by the Calvin cycle [2—4]. C_4 acid decarboxylation is mediated, depending on the C_4 species, by specific decarboxylases: NADP-malic enzyme, NAD-malic enzyme and phospho*enol*pyruvate carboxykinase [2—4]. Oxalate, an inhibitor of NADP-malic enzyme [30], and 3-mercaptopicolinic acid, an inhibitor of phospho*enol*pyruvate carboxykinase [28], have been used to distinguish between the different decarboxylation systems [3,4,29]. An α-ketoglutarate-stimulated, light-independent decarboxylation of aspartate, insensitive to both inhibitors, is likely due to NAD-malic enzyme which functions in conjunction with aspartate aminotransferase, NAD-malate dehydrogenase and alanine aminotransferase, whereas a light-dependent, 3-*P*-glycerate-stimulated decarboxylation of malate is either due to NADP-malic enzyme (oxalate-sensitive), phospho*enol*pyruvate carboxykinase (3-mercaptopicolinic acid-sensitive) or NAD-malic enzyme (insensitive to both inhibitors) (for complete

details, see Refs. 3,4,29). We have previously reported that of the three known C_4 acid decarboxylases present in leaves of C_4 plants, only NAD-malic enzyme activity was detected in leaf extracts of *P. milioides* [15]. We also provided evidence for an NAD-malic enzyme-catalyzed decarboxylation of malate and aspartate at the C-4 carboxyl position by *P. milioides* leaf slices [15].

Bundle sheath strands isolated from leaves of *P. milioides* exhibited an α-ketoglutarate-stimulated, light-independent aspartate decarboxylation and a light-dependent, 3-*P*-glycerate-stimulated decarboxylation of malate, both of which were insensitive to oxalate and 3-mercaptopicolinic acid (Table III), suggesting that C_4 acid decarboxylation is catalyzed by NAD-malic enzyme. Under identical assay conditions, mesophyll protoplasts isolated from *P. milioides* and *P. bisulcatum*, a representative C_3 species, did not decarboxylate added aspartate or malate in light or darkness, although both preparations contain substantial NAD-malate dehydrogenase and aspartate- and alanine-aminotransferase activity [13,15].

We have recently reported that when malate plus aspartate are added to the cell types isolated from leaves of *P. milioides*, only bundle sheath strands exhibit a light-dependent evolution of O_2 [13]. The suggestion that the C_4 acid-dependent O_2 evolution was due to CO_2 donation and fixation via the Calvin cycle rather than photoreduction of oxaloacetate, an intermediate formed during C_4 acid decarboxylation via the NAD-malic enzyme system [3,4], was confirmed by the complete inhibition of O_2 evolution by the uncouplers, NH_4Cl and FCCP (Ref. 13; cf. Table II), and the Calvin cycle inhibitor, D,L-glyceraldehyde (unpublished data). Direct evidence for the transfer of CO_2

TABLE III

DECARBOXYLATION OF 10 mM L-[4-^{14}C]ASPARTATE AND 10 mM L-[4-^{14}C]MALATE BY BUNDLE SHEATH STRANDS ISOLATED FROM LEAVES OF *P. MILIOIDES*

The assays were carried out at 21% O_2 and 30°C in light or darkness in serum-stoppered reaction vials containing CO_2-free assay medium, mesophyll protoplasts or bundle sheath strands, 50 mM D,L-glyceraldehyde and 4 mM maleate (to prevent refixation of released $^{14}CO_2$ by ribulosebisphosphate carboxylase and phospho*enol*pyruvate carboxylase, respectively) and the indicated substrates and inhibitors. The reactions were started by the addition of [4-^{14}C]aspartate ± α-ketoglutarate (α-Kg) or [4-^{14}C]malate ± 3-*P*-glycerate and terminated after 10 min by injecting 0.25 ml of 50% (w/v) trichloroacetic acid. Concentrations of added compounds, in mM, were: α-Kg, 10; 3-mercaptopicolinic acid, 0.4; oxalate, 0.4; 3-*P*-glycerate, 10. The results are the average of 2—3 experiments.

Assay conditions *	μmol C_4 acid decarboxylated/mg chlorophyll per h	
	Light	Dark
Aspartate	6.6	5.9
+ α-Kg	27.3	27.5
+ α-Kg + 3-mercaptopicolinic acid	27.2	28.1
+ α-Kg + oxalate	27.1	29.9
Malate	4.0	0
+ 3-*P*-Glycerate	23.5	0
+ 3-*P*-Glycerate + 3-mercaptopicolinic acid	23.2	0
+ 3-*P*-Glycerate + oxalate	23.9	0

* Under identical assay conditions mesophyll protoplasts isolated from *P. milioides* and *P. bisulcatum*, a C_3 species, did not decarboxylate added aspartate or malate in light or darkness.

from the C-4 carboxyl position of C_4 acids into the bundle sheath Calvin cycle of this C_3-C_4 intermediate species came from comparative studies of $H^{14}CO_3^-$- and [4-^{14}C]C_4 acid-metabolism in leaf slices and isolated mesophyll protoplasts and bundle sheath strands (Table IV). As expected, both mesophyll protoplasts and bundle sheath strands fixed $H^{14}CO_3^-$ into Calvin cycle intermediates and products, with the rate being 4-fold higher in the bundle sheath (cf. Table I). Leaf slices and isolated bundle sheath strands actively decarboxylated exogenous [4-^{14}C]malate and [4-^{14}C]aspartate (cf. Table III and Ref. 15) and, more noteworthy, the radiocarbon from the C-4 carboxyl position of the C_4 acids was incorporated into Calvin cycle intermediates in the light (Table IV). Under identical conditions, mesophyll protoplasts of *P. milioides* neither decarboxylated exogenous C_4 acids (cf. Table III) nor incorporated C-4 carboxyl radiocarbon from [4-^{14}C]C_4 acids into 3-*P*-glycerate and sugar phosphates in the light. Essentially identical results were obtained with mesophyll protoplasts isolated from a C_3 *Panicum* species. These findings with the isolated intact cell types (Tables III and IV) further document the exclusive localization of C_4 acid decarboxylation (i.e., NAD-malic enzyme) in the bundle sheath of *P. milioides* (cf. Ref. 13) and directly indicate the potential for C_4 acid-dependent CO_2 donation to the bundle sheath Calvin cycle of this C_3-C_4 intermediate species.

$^{14}CO_2$ incorporation studies with intact leaves

The data presented in Tables I—IV and elsewhere [13,15] indicate that the C_3-C_4 intermediate nature of *P. milioides* with respect to photorespiration is likely due to the potential for a limited phospho*enol*pyruvate carboxylation-C_4 acid decarboxylation-phospho*enol*pyruvate regeneration cycle similar to that in NAD-malic enzyme-type C_4 plants permitting an increase in pCO_2 at the site of bundle sheath ribulosebisphosphate carboxylase-oxygenase. If, indeed, some C_4 photosynthesis occurs in this intermediate species in vivo, radiotracer kinetic studies should reveal a limited labeling of malate and aspartate during short-term leaf photosynthesis in $^{14}CO_2$ and the transfer of ^{14}C from the C_4 acids to Calvin cycle intermediates during $^{14}CO_2$ pulse-$^{12}CO_2$ chase experiments. Although the initial experiments along these lines by Kanai and Kashiwagi [6] and Black and co-workers [8,9] gave no such indication, the total pCO_2 may have been too high or the $^{14}CO_2$ pulses too long to critically evaluate these parameters. We have therefore reinvestigated the labeling patterns of $^{14}CO_2$ assimilation during short-term leaf photosynthesis by *P. milioides* at physiological levels of CO_2 and O_2 and high light intensity.

Table V represents data from a typical experiment in which excised leaves of *P. milioides* were exposed to $^{14}CO_2$-air in the light at an initial total pCO_2 of 367 μl/l. Following 4 s photosynthesis, 24% of the total ^{14}C fixed was associated with C_4 acids and the remainder in Calvin cycle intermediates. During a 15 s interval following transfer of the leaves from $^{14}CO_2$-air to $^{12}CO_2$-air in the light, there was a progressive loss of label from both aspartate and malate associated with a concomitant increase in the percent ^{14}C fixed in 3-*P*-glycerate and sugar phosphates. In contrast, when leaves of a C_3 *Panicum* species were exposed to $^{14}CO_2$ for 4 s, only 8% of the total ^{14}C fixed was associated with C_4 acids, and upon transfer of the leaves from $^{14}CO_2$-air to $^{12}CO_2$-air the C_4 acids accumulated label at the expense of Calvin cycle intermediates (Table V).

TABLE IV

METABOLITES LABELED DURING L-[4-^{14}C]MALATE AND L-[4-^{14}C]ASPARTATE METABOLISM AND PHOTOSYNTHESIS IN $H^{14}CO_3^-$ BY LEAF SLICES AND MESOPHYLL PROTOPLASTS AND BUNDLE SHEATH STRANDS ISOLATED FROM *PANICUM* SPECIES

The assays were carried out under CO_2-free conditions in 21% O_2 at 30°C in the light. After 10 min, the reactions were terminated and the ethanol/water-soluble fractions processed by paper and cation/anion exchange column chromatography for final analysis by TLC as described in Methods. Concentrations of added compounds, in mM, were: $H^{14}CO_3^-$, 0.48 (10.5 μM CO_2 at pH 8.0) (2.5 Ci/mol); [4-^{14}C]malate, 10 (2.5 Ci/mol); [4-^{14}C]aspartate, 10 (2.5 Ci/mol). α-ketoglutarate (10 mM) was always included with aspartate. PEP, phospho*enol*pyruvate.

Assay conditions	μmol/mg chlorophyll per h		Distribution of ^{14}C in metabolites labeled from $H^{14}CO_3^-$ or [4-^{14}C]C_4 acids (%)					
	$^{14}CO_2$ fixation	[4-^{14}C]C_4 acid decarboxylation *	3-*P*-glycerate	Sugar phosphates	PEP	Amino acids	Neutrals	Insoluble
P. milioides								
Leaf slices **								
Malate	—	14.6	19	70	0	0	5.6	2.7
Aspartate	—	14.5	23	69	0	—	5.0	2.5
Mesophyll protoplasts								
HCO_3^-	26.1	—	16	65	4.0	2.6	4.7	2.4
Malate	—	0	None detected					
Aspartate	—	0	None detected					
Bundle sheath strands								
HCO_3^-	116	—	13	73	2.0	1.0	2.0	1.1
Malate	—	24.1	27	64	0	0	5.2	2.6
Aspartate	—	23.9	14	71	0	—	4.8	2.5
P. bisulcatum (C_3)								
Mesophyll protoplasts								
HCO_3^-	105	—	14	75	2.0	0.9	2.5	1.4
Malate	—	0	None detected					
Aspartate	—	0	None detected					

* See Table III and Ref. 15 for details of the malate (+3-*P*-glycerate) and aspartate (+ α-ketoglutarate) decarboxylation experiments.

** To follow the transfer of ^{14}C from [4-^{14}C]C_4 acids to Calvin cycle intermediates and products, leaf slices of *P. milioides* were treated with 5 mM maleate to prevent fixation of released $^{14}CO_2$ by phospho*enol*pyruvate carboxylase.

TABLE V

DISTRIBUTION OF ^{14}C IN EXCISED LEAVES OF *PANICUM* SPECIES FOLLOWING 4 s PHOTOSYNTHESIS IN $^{14}CO_2$ AND VARIOUS CHASE PERIODS IN $^{12}CO_2$-AIR IN THE LIGHT

The initial pCO_2 during the 4 s $^{14}CO_2$-pulse was as indicated, at 25°C and 21% O_2. The $^{12}CO_2$-chase was in air (330 μl/l CO_2, 21% O_2). Light intensity during the $^{14}CO_2$-pulse and pulse-chase experiments was 3.4—5.2 × 10^3 μE · m^{-2} · s^{-1}. The combined ethanol/water-soluble fraction was subjected to descending paper chromatography using liquefied phenol : water : acetic acid : 1 M EDTA as the solvent. The chromatogram was scanned for radioactivity, the radioactive areas identified, eluted and counted.

Species	Initial pCO_2 during 4 s $^{14}CO_2$-pulse (μl/l)	Chase in $^{12}CO_2$-air (s)	Distribution of ^{14}C fixed (%) *,**		
			Aspartate	Malate	3-*P*-glycerate + sugar phosphates
P. milioides	367	0	8.2 ***	16 ***	69
	367	5	3.1	6.2	86
	367	15	0	0	93
	746	0	2.4	5.5	86
P. bisulcatum (C_3)	367	0	3.3	4.8	87
	367	5	4.1	6.0	81
	367	15	5.9	7.8	77
	746	0	2.4	2.8	91

* Total ^{14}C fixed (cpm) at 367 μl/l and 746 μl/l CO_2, respectively, was 83 180 and 162 948 (*P. milioides*) and 64 912 and 130 500 (*P. bisulcatum*).

** An identical distribution pattern was observed when the ethanol/water-soluble extracts were fractionated into neutral, basic, acid-1 and acid-2 fractions by cation/anion exchange column chromatography (see Ref. 21) and the individual fractions analyzed by one-dimensional TLC using several solvent systems (data not shown).

*** Following 4 s photosynthesis by leaf slices at 21% O_2 and 0.48 mM $H^{14}CO_3^-$ (10.5 μM CO_2 at pH 8.0, 30°C), 21% of the total ^{14}C fixed was incorporated into malate and aspartate and this incorporation was completely inhibited by 5 mM maleate (data not shown).

These comparative $^{14}CO_2$ incorporation studies provide convincing in vivo evidence for a limited C_4-like photosynthesis of malate and aspartate and transfer of carbon from C_4 acids to the Calvin cycle in leaves of *P. milioides*.

Kanai and Kashiwagi [6] observed that when leaves of *P. milioides* were exposed to a 8 s pulse of $^{14}CO_2$ at 710 μl/l total pCO_2 about 5% of the ^{14}C fixed was associated with malate and aspartate. We have confirmed this observation in that when the total pCO_2 in the photosynthesis chamber was increased from physiological levels to 746 μl/l, the percent of the total ^{14}C associated with C_4 acids following 4 s photosynthesis by leaves of *P. milioides* decreased from 24% to 8% (Table V). This decrease in labeling of C_4 acids can be explained on the basis that the potential activity of ribulosebisphosphate carboxylase and the Calvin cycle would be considerably enhanced at elevated pCO_2 [1—4]. In the studies of Black and co-workers [8,9], no radiocarbon was detected in malate or aspartate following 10 s photosynthesis by leaves of barley, a C_3 plant, or *P. milioides* in $^{14}CO_2$-'air' (calcd. total pCO_2, 425 μl/l). These findings are in marked contrast with the results of Kanai and Kashiwagi [6] and ourselves (Table V) for *P. milioides*, and with other data in the literature [39—41] and Table V which indicate a limited labeling of C_4 acids during short-term photosynthesis by leaves of C_3 plants in $^{14}CO_2$.

General discussion

Based on the photosynthetic studies presented in this paper and elsewhere [13,15] with intact leaves, leaf slices and isolated mesophyll and bundle sheath cell types, we propose that CO_2 fixation in *P. milioides* is mediated via two photosynthetic pathways: a limited, but functional, C_4 pathway and the conventional Calvin cycle (Fig. 1). The mechanism of CO_2 fixation via the limited C_4 cycle is basically similar to that in NAD-malic enzyme-type C_4 plants [2—4] with respect to the sequence and intercellular compartmentation of reactions leading to the synthesis and subsequent decarboxylation of C_4 acids, the refixation of the released CO_2, and the regeneration of phospho*enol*-pyruvate, the initial CO_2 acceptor. The NAD-malic enzyme-type centripetal distribution of chloroplasts and numerous mitochondria in the bundle sheath cells [42] of this intermediate species (Refs. 6,43, and unpublished data) is consistent with this designation. Although a functional Calvin cycle is present in both cell types, the likely role of the limited C_4 pathway is to concentrate CO_2 at the site of bundle sheath, but not mesophyll, ribulosebisphosphate carboxylase-oxygenase. This interpretation is in agreement with several lines of evidence which indicate that NAD-malic enzyme, the only C_4 acid decarboxylase detected in this intermediate species [15], is exclusively localized in the bundle sheath (see Tables III, IV and Ref. 13). If a major function of the C_4 pathway of photosynthesis in C_4 plants is to reduce the competitive interaction of O_2

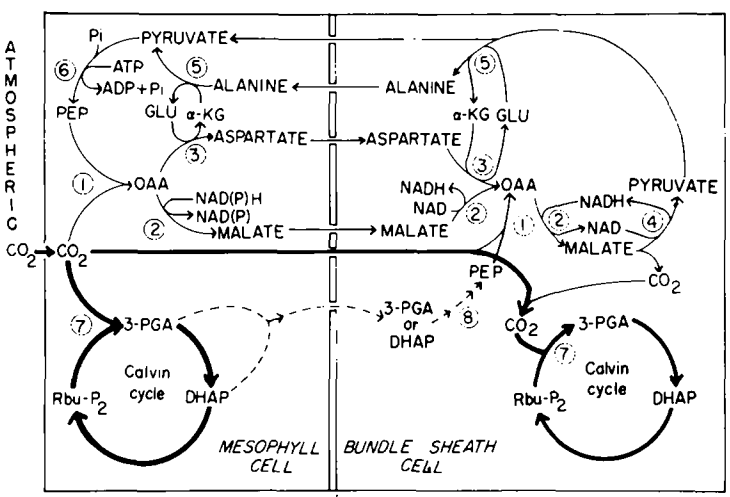

Fig. 1. The proposed path of carbon during photosynthesis in *Panicum milioides*, a C_3-C_4 intermediate species. The bold arrows indicate direct fixation of atmospheric CO_2 by the Calvin cycle and represent the major route for CO_2 uptake. The thin, solid arrows indicate a limited NAD-malic enzyme-type C_4 cycle concentrating CO_2 at the site of bundle sheath ribulosebisphosphate carboxylase-oxygenase. It can not be ascertained from our results whether the limited C_4-like phospho*enol*pyruvate carboxylation-regeneration system depicted in the mesophyll is present in all mesophyll cells or only in the radially-arranged cells immediately adjacent to the bundle sheath. The numbered reactions are catalyzed by: (1) phospho*enol*pyruvate carboxylase; (2) NAD(P)-malate dehydrogenase; (3) aspartate aminotransferase; (4) NAD-malic enzyme; (5) alanine aminotransferase; (6) pyruvate, orthophosphate dikinase, adenylate kinase and inorganic pyrophosphatase; (7) ribulosebisphosphate carboxylase; and (8) phosphoglyceromutase and enolase. Abbreviation: PEP, phospho*enol*pyruvate; α-KG, α-ketoglutarate; Rbu-P_2 ribulosebisphosphate; DHAP, dihydroxyacetone-*P*; OAA, oxaloacetate; 3-PGA, 3-*P*-glycerate.

with ribulosebisphosphate carboxylase-oxygenase by increasing the pCO_2 at the site of this bifunctional enzyme [1—4], it is of additional significance that this and other enzymes associated with the Calvin cycle and photorespiration are largely co-compartmented with the C_4 acid decarboxylase in the bundle sheath of *P. milioides* (see Ref. 13 and Tables I and IV). Although the present data indicate that the mesophyll Calvin cycle operates independently of the mesophyll-bundle sheath C_4 cycle, indirect evidence from in vitro experiments (Table I) suggests that 3-*P*-glycerate or triose-*P* photosynthesized in the mesophyll could give rise to phospho*enol*pyruvate in the bundle sheath via phosphoglyceromutase and enolase, thus providing substrate for the limited phospho*enol*pyruvate carboxylase in this cell type (Fig. 1) (see Ref. 13 and Table I).

The capacity for C_4 photosynthesis (i.e., phospho*enol*pyruvate carboxylation, C_4 acid decarboxylation, and phospho*enol*pyruvate regeneration) in *P. milioides*, although substantial compared to its total absence in C_3 *Panicum* species (Tables III—V; Refs. 13,15), is not sufficient to account for the observed rates of net photosynthesis. This observation, together with estimates that about 75—80% of the total CO_2 fixed by *P. milioides* is directly assimilated via ribulosebisphosphate carboxylase and the Calvin cycle (see Ref. 15 and Table V) and that considerable Calvin cycle activity is localized in the bundle sheath (Tables I, IV; Ref. 13), suggest that bundle sheath ribulosebisphosphate carboxylase fixes both atmospheric CO_2 directly entering the leaf and that CO_2 donated during C_4 acid decarboxylation by the limited mesophyll-bundle sheath C_4 cycle (Fig. 1). This situation is in marked contrast to that in leaves of C_4 plants in which the bundle sheath is viewed as a CO_2-tight compartment, inaccessible to atmospheric CO_2 [1—4].

Several previously reported anatomical and physiological features of *P. milioides* are seemingly inconsistent with the operation of even a limited C_4 cycle in this species. It is well recognized that the operation of the C_4 pathway requires rapid fluxes of photosynthetic intermediates between the two cell types and that the classical radial arrangement of Kranz leaf anatomy is particularly suited for such intercellular transport [2—4]. In general, the mesophyll and bundle sheath cells of C_4 plants are situated such that each mesophyll cell is separated, at most, by one cell from the nearest bundle sheath cell [44]. In addition, numerous plasmodesmata traverse the mesophyll-bundle sheath interfacial cell wall, providing a direct means for symplastic transport between the two cell types [2,4]. However, in leaves of *P. milioides* this classical radial arrangement of mesophyll and bundle sheath cells is largely lacking. The number of mesophyll cells between the bundle sheath cells of laterally adjacent vascular bundles is 5—7 compared to 2—4 in most C_4 species [43,44]. This represents a striking deviation from the classical situation in C_4 plants in that most of the mesophyll cells in *P. milioides* are separated by two or more cells from the nearest bundle sheath cell, although the mesophyll cells directly surrounding the bundle sheath are radially-arranged with numerous plasmodesmata traversing the interfacial cell wall (Refs. 6,7,43, and unpublished data). However, it can not be ascertained from our results whether the limited C_4-like phospho*enol*pyruvate carboxylation-regeneration system is present in all mesophyll cells or only in those cells immediately adjacent to the bundle sheath. In addition, it should be noted that deviation from the classical radial geometry

of Kranz anatomy does not necessarily preclude the operation of the C_4 pathway since considerable divergence has been observed amongst C_4 plants per se, including two radial layers of functional mesophyll cells (of which the outer layer is considerably larger than the inner) separated from an inner Kranz sheath by a non-chlorophyllous layer of cells in the tribe Fimbristylideae of the Cyperaceae and a single layer of sheath cells separated from the mesophyll by a non-chlorophyllous layer of mestome sheath cells in the tribe Cypereae of the Cyperaceae [33,44—47]. In addition, a tightly-packed double layer of mesophyll cells around the bundle sheath cells has been noted in some C_4 dicots, as well as Kranz anatomy dicots with palisade and spongy mesophyll layers [48,49].

Another feature that distinguishes C_4 plants from C_3 species is the $^{13}C/^{12}C$ carbon isotope fractionation ratio ($\delta^{13}C$). Fractionation values in the range of —9 to —19‰ specify C_4 plants, whereas ratios of —22 to —34‰ are characteristic of C_3 species [4,7]. Since the $\delta^{13}C$ values obtained reflect the first functional photosynthetic carboxylating enzyme (ribulosebisphosphate carboxylase in C_3 plants and phospho*enol*pyruvate carboxylase in C_4 species), it is generally considered that this is a reliable qualitative method for determining the overall pathway of photosynthetic carbon metabolism in a given plant. The $\delta^{13}C$ value of about —29‰ obtained for *P. milioides* (compared to —15‰ and —28‰ for a C_4 and C_3 *Panicum* species, respectively, grown under identical conditions) (unpublished data) indicates that the Calvin cycle is the major pathway of photosynthetic carbon assimilation in this intermediate species, consistent with data reported in this paper (Table V) and elsewhere [15]. However, this C_3-like value does not necessarily rule out the functional participation of a limited phospho*enol*pyruvate carboxylation given the qualitative nature of the fractionation ratio and the possibility discussed above that C_4 acid decarboxylation may not be taking place in a CO_2-tight bundle sheath compartment in *P. milioides*, thus allowing $^{13}CO_2$-$^{12}CO_2$ discrimination by bundle sheath ribulosebisphosphate carboxylase prior to refixation [2].

$^{14}CO_2$-radiotracer kinetic experiments with C_4 plants indicate the presence of a rapidly turning-over pool of C_4 acids during C_4 photosynthesis [2], reflecting the high rates of phospho*enol*pyruvate carboxylation and C_4 acid decarboxylation in these species. A rapid turnover of C_4 acids is observed regardless of the specifics of the C_4 leaf anatomy, even in those species in which the bundle sheath (i.e., the site of C_4 acid utilization) is physically separated from the mesophyll (i.e., the site of C_4 acid synthesis) by a layer of non-chlorophyllous cells [46,47]. Analogous radiotracer experiments with *P. milioides* (Table V) indicate a qualitatively-similar situation in this C_3-C_4 intermediate species. However, when one considers the lower rates of net photosynthesis in *P. milioides* relative to C_4 *Panicum* species [7,15,50] and the observations that only about 25% of the total ^{14}C fixed after 4 s photosynthesis is in C_4 acids and that about 15% of the total ^{14}C fixed is transferred from the C_4 acids in 5 s (compared to 80% and 37%, respectively, in C_4 plants [51,52]), it is evident that the flux of carbon through the C_4 pathway is much less than in C_4 plants. This is consistent with the reduced activity of C_4 cycle enzymes in this intermediate species [15].

In summary, we propose that photorespiration and the associated O_2 inhibi-

tion of photosynthesis is reduced in *P. milioides* by a limited C_4-like phospho*enol*pyruvate carboxylation-C_4 acid decarboxylation-phospho*enol*pyruvate regeneration system permitting an increase in pCO_2 at the site of bundle sheath ribulosebisphosphate carboxylase-oxygenase. The reduced rate of glycolate formation relative to photosynthesis recently reported for *P. milioides* [14] is consistent with this proposal.

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