Planta 133, 135–144 (1977)

Planta

C_4-Dicarboxylic Acid Metabolism in Bundle-Sheath Chloroplasts, Mitochondria and Strands of *Eriochloa borumensis* Hack., a Phosphoenolpyruvate-Carboxykinase Type C_4 Species

C.K.M. Rathnam and G.E. Edwards
Horticulture Department, University of Wisconsin, Madison, WI 53706, USA

Abstract. C_4-acid metabolism by isolated bundle-sheath chloroplasts, mitochondria and strands of *Eriochloa borumensis* Hack., a phosphoennolpyruvate-carboxykinase (PEP-CK) species, was investigated. Aspartate, oxaloacetate (OAA) and malate were decarboxylated by strands with several-fold stimulation upon illumination. There was strictly light-dependent decarboxylation of OAA and malate by the chloroplasts, but the chloroplasts did not decarboxylate aspartate in light or dark. PEP was a primary product of OAA or malate decarboxylation by the chloroplasts and its formation was inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea or NH_4Cl. There was very little conversion of PEP to pyruvate by bundle-sheath chloroplasts, mitochondria or strands. Decarboxylation of the three C_4-acids by mitochondria was light-independent. Pyruvate was the only product of mitochondrial metabolism of C_4-acids, and was apparently transaminated in the cytoplasm since PEP and alanine were primarily exported out of the bundle-sheath strands. Light-dependent C_4-acid decarboxylation by the chloroplasts is suggested to be through the PEP-CK, while the mitochondrial C_4-acid decarboxylation may proceed through the NAD-malic enzyme (NAD-ME) system. In vivo both aspartate and malate are considered as transport metobolites from mesophyll to bundle-sheath cells in PEP-CK species. Aspartate would be metabolized by the mitochondria to OAA. Part of the OAA may be converted to malate and decarboxylated through NAD-ME, and part may be transported to the chloroplasts for decarboxylation through PEP-CK localized in the chloroplasts. Malate transported from mesophyll cells may serve as carboxyl donor to chloroplasts through the chloroplastic NAD-malate dehydrogenase and PEP-CK. Bundle-sheath strands and chloroplasts fixed $^{14}CO_2$ at high rates and exhibited C_4-acid-dependent O_2 evolution in the light. Studies with 3-mercaptopicolinic acid, a specific inhibitor of PEP-CK, have indicated that most (about 70%) of the OAA formed from aspartate is decarboxylated through the chloroplastic PEP-CK and the remaining (about 30%) OAA through the mitochondrial NAD-ME. Pyruvate stimulation of aspartate decarboxylation is discussed; a pyruvate-alanine shuttle and an aspartate-alanine shuttle are proposed between the mesophyll and bundle-sheath cells during aspartate decarboxylation through the PEP-CK and NAD-ME system respectively.

Key words: Acids, dicarboxylic – CO_2 fixation – *Eriochloa* – Gramineae – Photosynthesis.

Abbreviations: CK = carboxykinase; α-Kg = α-ketoglutarate; ME = malic enzyme; 3-MPA = 3-mercaptopicolinic acid; OAA = oxaloacetate; PEP = phosphoenolpyruvate; R5P = ribose-5-phosphate

Introduction

In C_4 plants the initial fixation of atmospheric CO_2 is accomplished by PEP carboxylase and the OAA formed is reduced to malate or transaminated to aspartate. Aspartate and malate are assumed to be transported to bundle-sheath cells where they serve as carboxyl donors to the Calvin-Benson cycle operating in these cells (for reviews see Black, 1973; Hatch, 1976). Three C_4-acid decarboxylating enzymes have been found in C_4 plants and these plants have consequently been classified on the basis of their major decarboxylating enzymes as NADP-ME, NAD-ME and PEP-CK type species (Gutierrez et al., 1974; Hatch et al., 1975). Recently we have isolated and separated organelles from bundle-sheath cells and presented evidence for the intracellular distribution of the C_4-acid decarboxylating enzymes in the three types of C_4 species (Rathnam and Edwards, 1975).

Further insight into the mechanism of donation

of CO_2 from C_4-acids to the Calvin-Benson pathway has come from feeding C_4-acids to isolated bundle-sheath mitochondria and cells. The mechanism of C_4-acid decarboxylation in the bundle-sheath cells of NADP-ME and NAD-ME type species was studied in some detail (Huber et al., 1973; Kagawa and Hatch, 1974; Farineau, 1975; Hatch and Kagawa, 1976), but is relatively little understood in the PEP-CK species (Kanai and Black, 1972; Hatch and Kagawa, 1976). PEP-CK, in C_4 plants, catalyzes an ATP-dependent decarboxylation of OAA according to the following reaction (Edwards et al., 1971):

$$OAA + ATP \xrightarrow{Mn^{2+}} PEP + ADP + CO_2.$$

Whether aspartate or malate, or both, serve as precursors for generating OAA is however not known, and the fate of the PEP is also uncertain. On the basis of data on enzyme distribution we proposed that both aspartate and malate may serve as carboxyl donors to bundle-sheath cells of *Panicum maxium*, a PEP-CK type C_4 species (Rathnam and Edwards, 1975). We now present supporting evidence from feeding experiments with C_4-acids, conducted with isolated bundle-sheath chloroplasts, mitochondria and strands of *Eriochloa borumensis* Hack., another PEP-CK species (Gutierrez et al., 1976).

Material and Methods

Chemicals

Biochemicals and the reagent enzymes were obtained from Sigma Chemical Co., St. Louis, Mo., USA; cellulase and macerozyme from Yakult Biochemicals Co., Nishinomiya, Japan; $NAH^{14}CO_3$ from Amersham/Searle, Arlington Heights, Ill. USA; L-[4-^{14}C]aspartate and L-[4-^{14}C]malate from Calatomic, Los Angeles, Ca., USA. The L-[4-^{14}C]malate contained >95% label in the 4-C position as indicated by an in-vitro NADP-ME assay (Huber and Edwards, unpublished data). 3-Mercaptopicolinic acid (3-MPA) was a generous gift from Dr. H.A. Lardy, Institute for Enzyme Research, University of Wisconsin, Madison.

Plant Material

Mature leaves of *Eriochloa borumensis* were excised from plants, 2–3 weeks old, grown in a growth chamber under 16 h of light and 8 h of dark with a day/night temperature of 30°/25° C. A combination of Sylvania (Mountain View, Ca., USA) incandescent and fluorescent (cool white) lamps served as light source, giving a quantum flux density of 50–70 nE cm^{-2} s^{-1} between the wavelengths of 400 and 700 nm. The plants were kept in darkness for 48 h and illuminated for 30 min prior to sampling the leaves for isolating bundle-sheath chloroplasts and mitochondria (for details see Rathnam and Edwards, 1975).

Isolation of Bundle-sheath Strands, Chloroplasts and Mitochondria

Bundle-sheath strands were isolated by digesting the leaf segments (0.5 mm in width) for 2–3 h in the incubation medium consisting of 0.6 M sorbitol, 20 mM 2(N-morpholino)ethane sulfonic acid, 5 mM $MgCl_2$, 2% (w/v) cellulase (Onozuka 4S) and 0.1% (w/v) pectinase (macerozyme) at pH 5.5 (Kanai and Edwards, 1973; Huber and Edwards, 1975). Bovine serum albumin (0.05%, w/v) and sodium ascorbate (50 mM) were routinely included in the incubation medium to improve the yield and activity of the isolated strands.

Bundle-sheath strands free of mesophyll contamination (as observed by light microscopy) were suspended in the isolation medium (0.3 M sorbitol, 50 mM N-2 hydroxyethylpiperazine-N'-ethanesulfonic acid at pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM KH_2PO_4, 5 mM sodium ascorbate and 0.1% (w/v) bovine serum albumin). Bundle-sheath strands were disrupted on ice in a ground glass homogenizer and the unbroken cells were filtered by passing the homogenate through a 20 µm nylon net. Bundle-sheath chloroplasts and mitochondria were isolated by differential centrifugation of the homogenate at $600 \times g$ for 90 s and at $10{,}000 \times g$ for 10 min, respectively. This method, based on activities of marker enzymes for the respective organelles, gives only about 10% cross-contamination of the chloroplast fraction with mitochondria and vice versa (Rathnam and Edwards, 1975). The organelles were suspended in the isolation medium except that bovine serum albumin was omitted, and 0.2 mM KH_2PO_4 was included in place of 2 mM KH_2PO_4 for suspending chloroplasts. The preparations were maintained on ice at 0–4° C.

Assay Medium

All assays were run with a standard reaction mixture consisting of 0.3 M sorbitol, 50 mM N-*tris*-(hydroxymethyl)methyl glycine at pH 8.5, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM KH_2PO_4 (0.2 mM for experiments with chloroplasts) and 3 mM sodium *iso*ascorbate. Other additions were as indicated for the individual experiments. Unless otherwise mentioned, the reaction were run at 37° C in a final volume of 0.15 ml for 10 min. Pyruvate, OAA, α-Kg and sodium isoascorbate were prepared fresh daily.

Illumination

Light, except for the O_2 evolution studies, was provided by sodium discharge lamps (General Electric Co., Richmond Heights, O., USA) giving a total quantum flux density of about 80 nE cm^{-2} s^{-1} between 400 and 700 nm at the surface of the reactions vials. For studies on photosynthetic O_2 evolution illumination was provided by two 150-W General Electric projector flood lamps with a 3-cm layer of copper sulphate (1%, w/v) solution as a filter. The quantum flux density at the surface of the cuvettes was about 140 nE cm^{-2} s^{-1} between 400 and 700 nm.

Decarboxylation of C_4-Acids

Decarboxylation of C_4-acids at the 4-C position by bundle-sheath chloroplasts, mitochondria and strands in light and dark was followed directly using L-[4-^{14}C]aspartate and L-[4-^{14}C]malate in the presence of 25 mM D,L-glyceraldehyde in sealed ampules of 15 ml capacity. Bundle-sheath preparations were preincubated in the assay medium for 2 min at room temperature (22–24° C) and for 5 min at 37° C. The reactions were initiated by adding the labeled C_4-acids at a final concentration of 10 mM. For experiments with light, the reaction vials were preilluminated for 5 min at 37° C prior to the addition of substrates. The reactions were carried in light or dark for 10 min and were stopped by injecting 50 µl of 20% (w/v) trichloroacetic acid. The released $^{14}CO_2$ was trapped into 0.1 ml of 1.0 M hyamine hydroxide placed in a centrally suspended one half of a gelatin capsule (No. 0, Eli Lilly Co.,

Indianapolis, Ind., USA) with a paper wick, and counted by scintillation spectroscopy.

Analysis of C_4-Acid Decarboxylation Products

Bundle-sheath preparations were preincubated in the assay medium with 25 mM D,L-glyceraldehyde as described above and the reactions were initiated by adding the unlabeled substrates (OAA, aspartate or malate) at a final concentration of 10 mM. The reactions were carried in light or dark for 10 min and stopped by adding 0.15 ml of 20% (w/v) trichloracetic acid.

To ensure a complete extraction of the cell contents, the acidified solutions were kept at room temperature for 2 h (except for standing for 15 min when OAA was the substrate). The solutions were neutralized with 5 N KOH and the products formed (pyruvate, PEP, alanine) were measured spectrophotometrically at 340 nm by following the oxidation of NADH in presence of appropriate coupling enzymes. Pyruvate is very stable in acidic solutions at 25° (Silverstein and Boyer, 1964). In all experiments pyruvate was measured first in the presence of 3 units of lactate dehydrogenase with 0.4 mM NADH. After pyruvate measurement, the amounts of alanine and PEP were determined in the presence of excess NADH and lactate dehydrogenase by coupling to alanine aminotransferase (0.85 units) plus α-Kg (1 mM), or pyruvate kinase (3 units) plus 2 mM ADP and 5 mM KCl, respectively.

To test for the export of products of C_4-acid metabolism (pyruvate, PEP, alanine) from bundle-sheath chloroplasts, mitochondria and strands, the reactions with the various C_4-acids were run in light or dark for 10 min. Bundle-sheath strands or the chloroplasts were then immediately centrifuged for 60 s at $600 \times g$ in a clinical centrifuge, and mitochondria for 90 s at $10{,}000 \times g$ in a Beckman Microfuge B at 4° C. The supernatant and pellets were quickly separated, acidified and neutralized, and then assayed for the various products by the enzymatic procedures as described above.

Controls for aspartate and malate decarboxylation experiments consisted of reactions identical to the experimental systems except that the bundle-sheath preparations were incubated in boiling water for 5 min. Controls to correct for the nonenzymatic decarboxylation of OAA consisted of boiling the cellular preparations in the assay medium before the addition of OAA.

Photosynthetic $^{14}CO_2$ Fixation and O_2 Evolution

$^{14}CO_2$ fixation by bundle-sheath chloroplasts and strands was assayed as described by Rathnam and Edwards (1976). Light-dependent O_2 evolution was measured polarographically in a final volume of 1.0 ml at 25° using twin Hansatech O_2 electrodes (Hansatech Ltd., King's Lynn, Norfolk, U.K.) as described by Delieu and Walker (1972). The assay medium containing bundle-sheath chloroplasts or strands was equilibrated with N_2 for 2–3 min and the reactions were initiated by the addition of C_4-acids or sodium bicarbonate as indicated for individual experiments.

Chlorophyll

The strands were incubated overnight with 96% ethanol at 4° C prior to centrifugation to assure complete extraction of chlorophyll. Chlorophyll was determined using the extinction coefficients of Wintermans and DeMots (1965).

Preliminary assays indicated linear responses with varying chlorophyll concentration and time over a 20-min period, although a 10-min assay was generally used for experiments on C_4-acid metabolism. The data presented, unless otherwise stated, are from single experiments which generally were representative of several such measurements. The activities were expressed as μmole mg^{-1} chlorophyll h^{-1} (μmole mg^{-1} Chl h^{-1}). Mitochondrial activities were based on the chlorophyll present in the bundle-sheath homogenate before subjecting to differential centrifugation.

Results

Decarboxylation of C_4-Acids

Decarboxylation of aspartate and malate by bundle-sheath preparations of *E. borumensis* at the C-4 carboxyl position was followed directly using L-[4-^{14}C]-aspartate and L-[4-^{14}C]malate (Table 1). D,L-Glyceraldehyde, which blocks the Calvin-Benson pathway (Stokes and Walker, 1972; Bamberger and Avron, 1975; Rathnam and Edwards, unpublished data), was included in the assay medium to prevent refixation of the $^{14}CO_2$ liberated from the C_4-acids. The bundle-sheath strands rapidly catalyzed the decarboxylation of both aspartate and malate, the rates being 3–4.5 times higher in light than in dark. In contrast, mitochondrial decarboxylation of the C_4-acids was similar in light and dark, and the rates were relatively lower compared to those obtained with the illuminated strands. Isolated bundle-sheath chloroplasts did not decarboxylate aspartate, but did catalyze malate decarboxylation in a reaction which was strictly light-dependent.

Products of C_4-Acid Decarboxylation

Bundle-sheath strands of *E. borumensis* catalyzed the formation of C_3-products from OAA, aspartate and malate in both light and dark, confirming the decar-

Table 1. Decarboxylation of L-[4-^{14}C] aspartate and L-[4-^{14}C] malate by bundle-sheath strands, chloroplasts and mitochondria of *E. borumensis*

Bundle-sheath strands were enzymatically isolated and the chloroplasts and mitochondria were prepared by disrupting the strands as described by Rathnam and Edwards (1975). Release of label from the C_4-acids was followed in the presence of 25 mM D,L-glyceraldehyde over a 10-min incubation period as described in Material and Methods. The reaction mixtures included either 10 mM L-[4-^{14}C] aspartate + 5 mM α-Kg, or 10 mM L-[4-^{14}C] malate

Conditions	Activity (μmoles/mg^{-1})		
	Strands	Chloroplasts	Mitochondria
L-[4-^{14}C] Aspartate+ α-Kg, light	217	5	122
L-[4-^{14}C] Aspartate+ α-Kg, dark	47	1	127
L-[4-^{14}C] Malate, light	315	184	42
L-[4-^{14}C] Malate, dark	97	4	46

Table 2. Products of C_4-acid decarboxylation by bundle-sheath strands and chloroplasts of *E. borumensis*

Bundle-sheath strands or chloroplasts were incubated with the unlabeled C_4-acids in the presence of 25 mM D,L-glyceraldehyde, and the products of C_4-acid decarboxylation were measured spectrophotometrically in the presence of appropriate coupling enzymes. For complete details, see Material and Methods. Concentrations of various compounds used were: aspartate, 10 mM; malate, 10 mM; OAA, 10 mM; α-Kg, 5 mM; glutamate, 5 mM; PMS (phenazine methosulphate), 20 μM; and DCMU [3-(3,4-dichlorphenyl-1,1-dimethylurea], 25 μM. The activities are expressed as μmoles mg^{-1} Chl h^{-1}

Conditions	Bundle-sheath strands			Bundle-sheath chloroplasts[b]	
	PEP	Pyruvate	Alanine	PEP	Pyruvate
OAA, light	187	70	0	154	13
OAA, dark	16	78	0	38	19
OAA+PMS, light	303	62	–[a]	269	19
OAA+DCMU, light	16	58	–	19	13
Aspartate, light	47	43	0	–	–
Aspartate+ α-Kg, light	199	86	40	13	6
Aspartate+ α-Kg, dark	29	73	82	0	0
Aspartate+ α-Kg+DCMU, light	16	86	–	–	–
Malate, light	151	24	0	77	13
Malate, dark	13	27	9	13	6
Malate+PMS, light	226	31	–	141	6
Malate+DCMU, light	16	74	–	6	6
Malate+glutamate, light	146	0	53	–	–
Malate+gluatamate, dark	20	9	44	–	–

[a] Not determined

[b] No alanine formation was observed with any of the C_4 acids under the conditions studied

boxylation of C_4-acids (Table 2). PEP, pyruvate and alanine were the products. Formation of PEP was strictly light-dependent, while that of pyruvate was independent of light. In light, the rates of PEP formation were 2–6 times higher than those of pyruvate formation. Addition of α-Kg stimulated the rate of aspartate decarboxylation by 3–4 times. Alanine was also a product of aspartate decarboxylation in the presence of α-Kg. There was no alanine formation during OAA or malate decarboxylation by bundle-sheath strands. However, alanine formation could be induced during malate decarboxylation by the strands in the presence of glutamate.

Bundle-sheath chloroplasts catalyzed a light-dependent decarboxylation of OAA and malate, but not of aspartate in light or dark (Table 2). PEP was the major product of both OAA and malate decarboxylation, with little or no formation of pyruvate or alanine. The rates observed with chloroplasts were somewhat less than with bundle-sheath strands; this may be because of partial breakage of chloroplasts during isolation and incubation.

Light-dependent formation of PEP during C_4-acid decarboxylation by the strands and chloroplasts was enhanced 2-fold when phenazine methosulfate, a co-factor of cyclic photophosphorylation (Whatley and Arnon, 1963), was added. PEP formation was inhibited by 3-(3,4-dichlorophenyl-1,1-dimethylurea. Addition of NH_4Cl, an uncoupler of photophosphorylation, had a similar effect (data not shown).

Bundle-sheath mitochondria decarboxylated the added OAA, aspartate or malate, and the rates were similar in light or dark (Table 3). Pyruvate was the only product detected. However, alanine formation

Table 3. Products of C_4-acid decarboxylation by mitochondria isolated from bundle-sheath strands of *E. borumensis*

The reactions were run in the dark (values in parantheses were rates in the light) in the presence of 25 mM D,L-glyceraldehyde for 10 min using the assay medium described in Material and Methods. Substrate concentrations used were as described in Table 2; and AAT (alanine aminotransferase) was 0.85 units/ml

Conditions	Product formation (μmoles mg^{-1} Chl h^{-1})		
	PEP	Pyruvate	Alanine
OAA	0 (0)	81 (69)	0 (0)
Aspartate+ α-Kg	0 (0)	86 (67)	0 (0)
Aspartate+ α-Kg+AAT	0 (0)	14 (14)	79 (67)
Malate	0 (0)	31 (35)	0 (0)
Malate+glutamate	0	29	0
Malate+glutamate+AAT	0 (0)	0 (0)	38 (34)
Malate+aspartate (2 mM) + α-Kg (2 mM)	0 (0)	105 (110)	0 (0)
Malate+aspartate (2 mM) + α-Kg (2 mM)+AAT	0	24	81

Table 4. Export of C_4-acid decarboxylation products out of bundle-sheath chloroplasts, and mitochondria of *E. borumensis*

Immediately after the completion of incubation, the reaction mixtures were centrifuged quickly. The supernatant and pellets were separated, acidified, neutralized and assayed for the products. The values reported here are for the products present in the supernatant. There was no PEP, pyruvate or alanine detected in the pellets except 60–80% of total pyruvate (values given in parantheses) was retained in the pellets of bundle-sheath strands. Substrate concentrations were those described in Table 2. The rates are expressed as μmoles mg^{-1} Chl h^{-1}

Conditions	Strands			Chloroplasts[a]		Mitochondria[a]	
	PEP	Pyruvate	Alanine	PEP	Pyruvate	PEP	Pyruvate
OAA, light	223	19 (56)	0	127	17	0	69
OAA, dark	23	14 (51)	0	34	34	0	58
Aspartate+ α-Kg, light	181	28 (46)	70	0	0	0	81
Aspartate+ α-Kg, dark	9	23 (70)	93	0	0	0	104
Malate, light	158	9 (28)	0	138	11	0	75
Malate, dark	28	18 (28)	0	11	23	0	72

[a] No alanine was detected in the supernatants of chloroplasts and mitochondria

could be induced during the decarboxylation of aspartate by including alanine aminotransferase, an extramitochondrial enzyme in PEP-CK species (Rathnam and Edwards, 1975), in the reaction mixture. Some alanine formation also occurred during malate decarboxylation by mitochondria with the addition of alanine aminotransferase and glutamate (Table 3).

The total rates of formation of C_3-products determined during C_4-acid decarboxylation (Tables 2, 3) resembled the rates of $^{14}CO_2$ release from 4-^{14}C labeled C_4-acids with the bundle-sheath chloroplasts, mitochondria and strands (Table 1).

Export of Decarboxylation Products from Bundle-sheath Chloroplasts, Mitochondria and Strands

The C_3-products present in the pellets and the supernatants, obtained by a quick centrifugation of the reaction mixtures after a 10-min incubation period, were measured in order to determine which of them are retained in and which are exported from the bundle-sheath chloroplasts, mitochondria and strand (Table 4). During α-Kg-dependent aspartate decarboxylation, the major products appearing in the supernatant fractions of the strands were PEP and alanine in light, with mostly alanine in dark, while no product was released from the chloroplasts under these conditions in light or dark. PEP was the major product exported out of the strands and the chloroplasts during OAA and malate decarboxylation in light with very little PEP in dark. Pyruvate was the only product recovered in the supernatants during the decarboxylation of OAA, aspartate and malate by the mitochondria in light and dark. Though pyruvate was a major product of C_4-acid decarboxylation by bundle-sheath strands (Table 2), little pyruvate was present in their supernatants. No decarboxylation products were retained in the pellets of the organelles, but there was retention of pyruvate (60–80% of total) in pellets of bundle-sheath strands.

Lack of PEP Metabolism in Bundle-sheath Chloroplasts, Mitochondria and Strands

There was no formation of alanine (data not reported) and very little of pyruvate when PEP was fed to bundle-sheath chloroplasts, mitochondria or strands in light or dark (Table 5). Addition of ADP has no effect. The results complement the data in Tables 2

Table 5. Lack of PEP conversion to pyruvate by bundle-sheath strands, chloroplasts and mitochondria of *E. borumensis*

Bundle-sheath preparations were incubated for 10 min with 10 mM PEP in the assay medium (see Methods) with or without ADP (2 mM), acidified and neutralized. Formation of pyruvate was measured spectrophotometrically at 340 nm by following the oxidation of NADH in presence of lactic dehydrogenase

Conditions	Pyruvate formation (μmole mg^{-1} Chl h^{-1})		
	Strands	Chloroplasts	Mitochondria
PEP, light	13.4	8.2	0
PEP, dark	10.4	9.9	—[a]
PEP+ADP, light	8.9	8.2	—

[a] Not determined

and 4 showing PEP formation from C_4-acids by the chloroplasts and strands.

Several attempts to identify pyruvate-kinase-type activity using various assays (Duggleby and Dennis, 1973; Yamada and Carlsson, 1975; Noce and Utter, 1975) in bundle-sheath extracts of *E. borumensis* and of other PEP-CK species including *Panicum maximum*, *Urochloa panicoides* and *Bouteloua curtipendula* were without success. Although there is 3-phosphoglycerate phosphatase activity (assay pH 5.9) in bundle-sheath extracts of C_4 plants (Randall et al., 1971; Kestler et al., 1975; Rathnam and Edwards, unpublished data), there was no indication of any significant hydrolysis of PEP by this enzyme (which shows some specificity for PEP and for 3-phosphoglycerate in leaves of sugarcane) in the bundle-sheath chloroplasts or strands of *E. borumensis*.

Table 6. $^{14}CO_2$ fixation by isolated bundle-sheath strands and chloroplasts of *E. borumensis*

Bundle-sheath strands were isolated either by enzymatic digestion (see Material and Methods) or by gentle grinding of leaves (Huber et al., 1973). Chloroplasts were then prepared by disrupting the strands as described in Material and Methods. Light-dependent $^{14}CO_2$ fixation assays were performed as described earlier (Rathnam and Edwards, 1976) using the assay medium described in Material and Methods with 6 mM $NaH^{14}CO_3$. The activities are expressed as μmole $^{14}CO_2$ fixed mg^{-1} Chl h^{-1}

Preparation	Enzymatic		Mechanical	
	Control	+3 mM R5P	Control	+3 mM R5P
Bundle-sheath strands	227	229	255	256
Bundle-sheath chloroplasts	174	197	146	153

Photosynthetic $^{14}CO_2$ Fixation

Bundle-sheath strands isolated from leaves of *E. borumensis* by enzymatic or mechanical treatment at pH 5.5 subsequently fixed $^{14}CO_2$ at rates of 227–256 μmole mg^{-1} Chl h^{-1}. Chloroplasts isolated from enzymatically or mechanically prepared strands also exhibited high rates of endogenous $^{14}CO_2$ fixation (Table 6). Addition of Calvin-cycle intermediate such as R5P did not significantly increase the carbon fixation capacities of the isolated strands and chloroplasts. The inclusion of 50 mM sodium ascorbate in the digestion medium provided the most active preparations.

We did not observe a difference in the endogenous $^{14}CO_2$ fixation capacities of bundle-sheath strands isolated from *E. borumensis* leaves either by enzymatic digestion or by mechanical grinding (Table 6). This is in contrast to findings of Hatch and Kagawa (1976) who reported a reduction in activity during prolonged treatment of leaf tissues from some C_4 species with cellulase at lower pH.

O_2 Evolution by Bundle-sheath Strands and Chloroplasts in Light

Bundle-sheath strands rapidly evolved O_2 when either HCO_3^--or C_4-acids were added to the incubation mixture (Fig. 1). O_2 evolution was light-dependent and

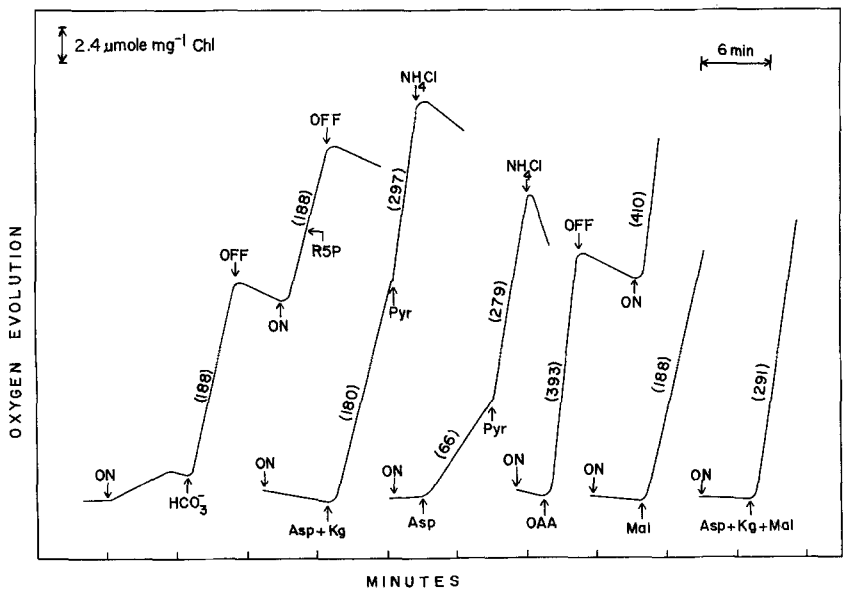

Fig. 1. Polarographic traces of light-dependent O_2 evolution by bundle-sheath strands of *E. borumensis*. Substrate concentrations used were: aspartate, 10 mM; malate, 10 mM; OAA, 10 mM; α-Kg, 5 mM; pyruvate, 2 mM; R5P, 3 mM; HCO_3^- 6 mM and NH_4Cl, 20 mM. Values in parantheses on the curves indicate the rates of O_2 evolution in μmoles mg^{-1} Chl h^{-1}. *Asp* aspartate, *Mal* malate

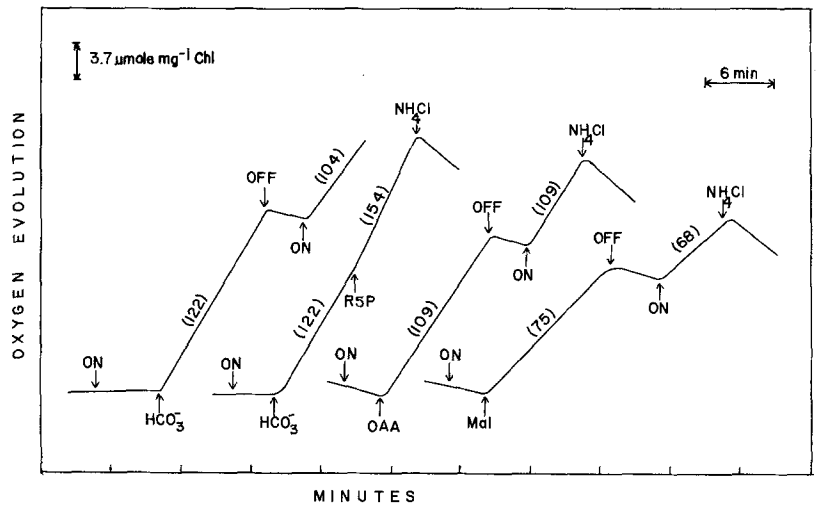

Fig. 2. Polarographic traces of O_2 evolution by bundle-sheath chloroplasts of *E. borumensis* in light. Substrate concentrations were as those described in Figure 1. Values in parantheses on the curves are the rates of O_2 evolution in µmoles mg^{-1} Chl h^{-1}. *Mal* malate

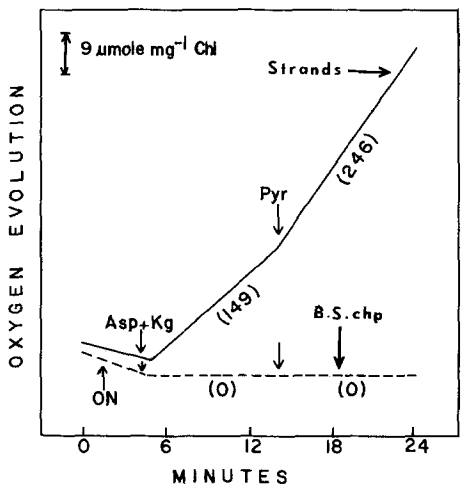

Fig. 3. Polarographic traces showing the effect of pyruvate on substrate-induced O_2 evolution ih light by bundle-sheath chloroplasts and strands of *E. borumensis*. The substrate concentrations were as those described in Figure 1. Values in parantheses on the curves are the rates of O_2 evolution in µmoles mg^{-1} Chl h^{-1}. *Asp* aspartate

was inhibited by NH_4Cl, an uncoupler of photophosphorylation. There was little or no O_2 evolution in light before the addition of the substrates. R5P did not stimulate the HCO_3^--dependent O_2 evolution. The rate of O_2 evolution with aspartate alone was low. However, when α-Kg was included along with aspartate, the bundle-sheath strands actively evolved O_2. Surprisingly, low concentrations of pyruvate (2 mM) greatly enhanced aspartate- or aspartate+α-Kg-dependent O_2 evolution. Pyruvate alone did not induce O_2 evolution (data not shown). OAA and malate also readily induced O_2 evolution. Pyruvate had no effect on OAA- or malate-induced O_2 evolution in bundle-sheath strands (data not shown). Aspartate+α-Kg stimulated the malate-dependent O_2 evolution. The rates of O_2 evolution with C_4-acids approached or exceeded those observed with HCO_3^-.

Bundle-sheath chloroplasts of *E. borumensis* exhibited an active, HCO_3^--dependent O_2 evolution which was stimulated slightly when R5P was included. Both malate and OAA induced O_2 evolution with the chloroplasts (Fig. 2). Pyruvate has no effect on OAA- or malate-induced O_2 evolution by chloroplasts.

Figure 3 clearly shows that aspartate+α-Kg induced O_2 evolution in bundle-sheath strands but not in their chloroplasts. Pyruvate stimulated aspartate-dependent O_2 evolution in bundle-sheath strands while it had no effect on the chloroplasts.

The highest rates of C_4-acid metabolism, photosynthetic $^{14}CO_2$ fixation and O_2 evolution obtained in the present study are comparable to the rates of whole leaf photosynthesis of C_4 species.

Effect of 3-MPA on O_2 Evolution by Bundle-sheath Strands in Light

Figure 4 shows the effect of 3-MPA, an inhibitor of PEP-CK (Kostos et al., 1975; Jomain-Baum et al., 1976) on substrate-dependent O_2 evolution by isolated bundle-sheath strands in light. 3-MPA, at a concentration of 100 µM, completely inhibited malate-dependent O_2 evolution while it has no effect on the HCO_3^--dependent O_2 evolution. It caused only a 70–75% inhibition of OAA- and aspartate+α-Kg-induced O_2 evolution by strands, and no further inhibition was apparent even when the inhibitor concentration was increased by 4-fold. However, malate- and OAA-dependent O_2 evolution by bundle-sheath chloroplasts was completely inhibited by 100 µM 3-MPA (data not shown). 3-MPA had no effect on

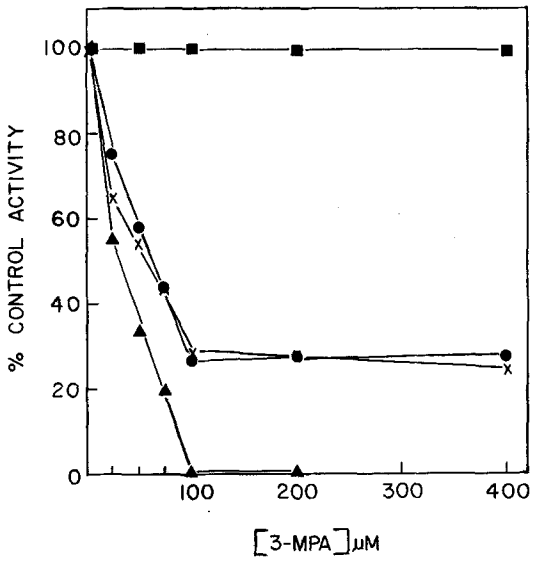

Fig. 4. Effect of 3-mercaptopicolinic acid (3-MPA) on the rates of light-dependent O_2 evolution by bundle-sheath strands of *E. borumensis* induced by HCO_3^- (■), aspartate + α-Kg (●), OAA (x) and malate (▲). Substrate concentrations used were: HCO_3^-, 6 mM; aspartate, 10 mM; α-Kg, 10 mM; OAA, 6 mM and malate, 10 mM

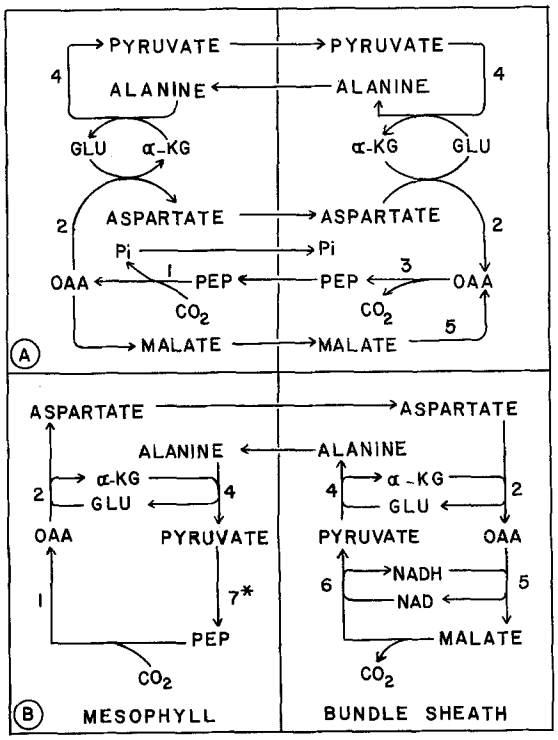

Fig. 5A and **B**. Proposed scheme for **A** malate decarboxylation or pyruvate stimulation of aspartate decarboxylation through PEP-CK, and **B** aspartate decarboxylation through NAD-ME system in leaves of *E. borumensis*, a PEP-CK C_4 species. The numbered reactions are catalyzed by (*1*) PEP carboxylase, (*2*) aspartate aminotransferase, (*3*) PEP-CK, (*4*) alanine aminotransferase, (*5*) NAD-malate dehydrogenase, (*6*) NAD-ME, (*7*) pyruvate, Pi dikinase[1]. *Glu* glutamate

C_4-acid-induced O_2 evolution activities of bundle-sheath strands isolated from leaves of NADP-ME and NAD-ME species (Rathnam and Edwards, unpublished data).

Discussion

The discovery of PEP-CK as the primary C_4-acid decarboxylating enzyme in the bundle-sheath cells of certain C_4 species lead to the classification of these plants as PEP-CK type C_4 species (Edwards et al., 1971; Gutierrez et al., 1974; Hatch et al., 1975). However, PEP-CK species were also shown recently to have another decarboxylase, NAD-ME (Gutierrez et al., 1974; Rathnam and Edwards, 1975; Gutierrez et al., 1976), and this finding lead to the proposal that these two decarboxylases may be functioning in these species simultaneously (Rathnam and Edwards, 1975). This idea is supported by the ability of bundle-sheath strands of *E. borumensis*, a PEP-CK species, to decarboxylate added aspartate, OAA and malate in the dark, and by the several-fold stimulation of these decarboxylations upon illumination since NAD-ME is light-independent while PEP-CK (because of ATP requirement) is light-dependent. The pathways of decarboxylations mediated by the two decarboxylases in leaves of PEP-CK species are thus shown in Figure 5.

Mitochondria have been considered as the site of aspartate decarboxylation in bundle-sheath cells of NAD-ME species, leading to pyruvate formation through the NAD-ME system (Kagawa and Hatch, 1974, 1975; Rathnam and Edwards, 1975). The decarboxylation of aspartate, OAA and malate by bundle-sheath mitochondria, with the export of pyruvate during C_4-acid decarboxylation, indicates the operation of NAD-ME in *E. borumensis*, a PEP-CK species (Fig. 5B). Transamination of the pyruvate exported out of the mitochondria may be facilitated by the cytoplasmic alanine aminotransferase (Rathnam and Edwards, 1975), with alanine being transported from bundle-sheath to mesophyll cells. An aspartate-alanine shuttle between mesophyll and bundle-sheath cells in the NAD-ME system would thus maintain a proper amino balance between the two cell types (Hatch and Kagawa, 1974) (Fig. 5B). Although pyruvate is an immediate precursor of alanine formation, in our experiments we could observe only 30–50% of the total pyruvate formed being converted

[1] Hatch et al. (1976) and Ku and Edwards (1975) have shown evidence for the presence of significant levels of pyruvate, Pi dikinase in leaves of several PEP-CK C_4 species with preferential localization in their mesophyll cells

to alanine. There may be a limiting step during alaning formation from pyruvate. A similar observation was made by Hatch and Kagawa (1976) who attributed this limitation to the mass-action effect of an excess of added α-Kg. This interpretation may be correct since we observed the formation of alanine and only little pyruvate when the strands were fed with malate+glutamate (Table 2). Conversion of pyruvate to PEP is unlikely since little or no pyruvate, Pi dikinase was detected in the bundle-sheath cells of several PEP-CK species (Ku and Edwards, 1975; Rathnam and Edwards, unpublished data).

The lack of aspartate decarboxylation by bundle-sheath chloroplasts is expected in view of the extra-chloroplastic localization of aspartate amino-transferase in PEP-CK species (Rathnam and Edwards, 1975), However, the bundle-sheath chloroplasts showed light dependent decarboxylation of OAA and malate with the production of PEP (Tables 1, 2, 4) indicating the participation of PEP-CK. An NAD-malate dehydrogenase present in the bundle-sheath chloroplasts of PEP-CK species (Rathnam and Edwards, 1975) may serve to generate OAA from malate.

The source of malate to bundle-sheath chloroplasts may be that formed as an initial product of CO_2 fixation in the mesophyll cells. Appearance of half as much radioactivity in malate compared to aspartate during brief exposure of *Panicum maximum* leaves to $^{14}CO_2$, and steep negative slopes for both aspartate and malate during a $^{12}CO_2$ chase (Kanai and Black, 1972; Foster and Black, 1976) indicate that both C_4-acids are decarboxylated. The decarboxylation of both malate and aspartate by bundle-sheath strands of *E. borumensis* supports this interpretation. Since aspartate is also a carboxyl donor to bundle-sheath strands, with PEP formation in light, but not to bundle-sheath chloroplasts (Tables 1, 2, 4) we suggest that in vivo aspartate is deaminated to OAA in the bundle-sheath mitochondria, with most of the OAA formed transported to the bundle-sheath chloroplasts for decarboxylation PEP-CK. Thus, both malate and aspartate may serve as carboxyl donors through PEP-CK (Fig. 5A). Malate formed in mitochondria is probably not transported to the choroplasts since it would upset the stoichiometric balance of nucleotides for OAA reduction.

Initial evidence for inhibition of PEP-CK by 3-MPA in C_4 photosynthesis was recently reported (Ray and Black, 1976). Our complete inhibition of OAA- and malate-dependent O_2 evolution by this compound with the bundle-sheath chloroplasts indicates their decarboxylation through PEP-CK. Only a 70–75% inhibition by 3-MPA of OAA- and aspartate+ αKg-dependent O_2 evolution by strands confirms that bout 70% of OAA formed from aspartate undergoes decarboxylation through PEP-CK, with the remaining 30% OAA decarboxylated through the NAD-ME system. A total inhibition of malate-dependent O_2 evolution by strands indicates that malate is a carboxyl donor through PEP-CK only. 3-MPA even at 400 μM levels had no effect on HCO_3^--dependent O_2 evolution, indicating that the inhibitor has specificity for PEP-CK without affecting CO_2 fixation through the Calvin-Benson pathway. The inhibitor had no effect on the C_4-acid-dependent O_2 evolution by bundle-sheath strands of NADP-ME and NAD-ME species confirming than 3-MPA is specific for PEP-CK.

Kanai and Black (1972) have suggested that PEP is the transport metabolite from bundle-sheath to mesophyll in PEP-CK species, while Hatch and Kagawa (1976) speculated a conversion of PEP by an unknown pathway to pyruvate and transamination to alanine for subsequent transport to mesophyll cells. Hatch and Kagawa (1976) found no PEP formation from C_4-acids and little stimulation of C_4-acid decarboxylation upon illumination in bundle-sheath cells of PEP-CK species, indicating that decarboxylation mediated by PEP-CK was not working under their conditions. The formation of pyruvate and alanine which they observed may have been based upon metabolism through the NAD-ME system.

In our study, there was no evidence for conversion of PEP to pyruvate at significant rates in the bundle-sheath of *E. borumensis*. The presence of PEP and alanine in the supernatants of strands indicates that PEP and alanine can be the transport metabolites from bundle-sheath to mesophyll. Although both decarboxylases may function in the leaves of *E. borumensis*, we consider PEP-CK as the primary C_4-acid decarboxylase and NAD-ME as a secondary one since the amount of PEP formed is considerably greater than that of alanine (+pyruvate).

Studies on C_4-acid-induced O_2 evolution by bundle-sheath strands and chloroplasts complement the data on C_4-acid decarboxylation data and establish that the C_4-acids are an active source of CO_2 for photosynthesis in the bundle-sheath. The finding that pyruvate stimulates aspartate decarboxylation (data not reported) and aspartate-dependent O_2 evolution may be based on a pyruvate-dependent conversion of glutamate to α-Kg, which would regenerate the keto acceptor for aspartate aminotransferase (Fig. 5A).

Deamination of aspartate in bundle-sheath cells would result in a net production of glutamate and OAA. Decarboxylation of OAA through NAD-ME and transport of alanine from bundle-sheath to mesophyll would maintain an amino balance between the two cell types (Fig. 5B; Hatch and Kagawa, 1976). However, if part of the OAA from aspartate deamina-

tion is decarboxylated through PEP-CK, with transport of PEP to the mesophyll (Fig. 5A), only a part of the net glutamate produced by aspartate aminotransferase would be utilized in the transamination of pyruvate. A pyruvate-alanine shuttle between the mesophyll and bundle-sheath cells (Fig. 5A) would consume the remaining glutamate and maintain an amino balance between the cell types. Thus, the pyruvate stimulation of aspartate-dependent O_2 evolution and decarboxylation in vitro is expected.

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison and by the University of Wisconsin Research Committee with funds from the Wisconsin Alumni Research Foundation; and by National Science Foundation grant BMS-74-09611 to G.E.E.

References

Bamberger, E.S., Avron, M.: Site of action of inhibitors of carbon dioxide assimilation by whole lettuce chloroplasts. Plant Physiol. **56**, 481–485 (1975)

Black, C.C.: Photosynthetic carbon fixation in relation to net CO_2 uptake. Ann. Rev. Plant Physiol. **24**, 253–286 (1973)

Delieu, T., Walker, D.A.: An improved cathode for the measurement of photosynthetic oxygen evolution by isolated chloroplasts. New Phytol. **71**, 201–225 (1972)

Duggleby, R.C., Dennis, D.T.: Pyruvate kinase, a possible regulatory enzyme in higher plants. Plant Physiol. **52**, 312–317 (1973)

Edwards, G.E., Kanai, R., Black, C.C.: Phosphoenolpyruvate carboxykinase in leaves of certain plants which fix CO_2 by the C_4-dicarboxylic acid pathway of photosynthesis. Biochem. Biophys. Res. Commun. **45**, 278–285 (1971)

Farineau, J.: Photoassimilation of CO_2 by isolated bundle-sheath strands of *Zea mays*. II. Role of malate as a source of CO_2 and reducing power for the photosynthetic activity of isolated bundle-sheath cells of *Zea mays*. Physiol. Plantarum **33**, 310–315 (1975)

Foster, A., Black, C.C.: Changes in pathway of carbon flow in response to CO_2 concentration during leaf photosynthesis in *Panicum maximum* (Abstr.) Amer. Soc. Plant Physiol., Suppl. **57**, **306** (1976)

Gutierrez, M., Edwards, G.E., Brown, W.V.: PEP carboxykinase containing species in the *Brachiaria* group of the subfamily Panicoideae. Biochem. System. Ecol. **4**, 47–49 (1976)

Gutierrez, M., Gracen, V.E., Edwards, G.E., Biochemical and cytological relationships in C_4 plants. Planta (Berl.) **119**, 279–300 (1974)

Hatch, M.D.: The C_4-pathway of photosynthesis: mechanism and function. In: CO_2 metabolism and plant productivity, pp. 59–81, Burris, R.H., Black, C.C., Eds. Baltimore: University Park Press 1976

Hatch, M.D., Kagawa, T.: Photosynthetic activities of isolated bundle-sheath cells in relation to different mechanisms of C_4 pathway photosynthesis. Arch. Biochem. Biophys. **157**, 39–53 (1976)

Hatch, M.D., Kagawa, T., Craig, S.: A survey of C_4 decarboxylating systems, mitochondria frequency and ultrastructure features in C_4 pathway leaves and the subclassification of these species. Aust. J. Plant Physiol. **1**, 357–369 (1975)

Huber, S.C., Edwards, G.E.: An evaluation of some parameters required for the enzymatic isolation of cells and protoplasts with CO_2 fixation capacity from C_3 and C_4 grasses. Physiol. Plantarum **35**, 203–209 (1975)

Huber, S.C., Kanai, R., Edwards, G.E.: Decarboxylation of malate by isolated bundle-sheath cells of certain plants having the C_4-dicarboxylic acid pathway of photosynthesis. Planta (Berl.) **113**, 53–66 (1973)

Jomain-Baum, M., Schramm, V.L., Hanson, R.W.: Mechanism of 3-mercaptopicolinic acid inhibition of hepatic phosphoenolpyruvate carboxykinase (GTP). J. biol. Chem. **251**, 37–44 (1976)

Kagawa, T., Hatch, M.D.: C_4 acids as the source of CO_2 for Calvin cycle photosynthesis by bundle sheath cells of the C_4 pathway species *Atriplex spongiosa*. Biochem. Biophys. Res. Commun. **59**, 1326–1332 (1974)

Kagawa, T., Hatch, M.D.: Mitochondria as a site of C_4 acid decarboxylation in C_4-pathway photosynthesis. Arch. Biochem. Biophys. **167**, 687–696 (1975)

Kanai, R., Black, C.C.: Biochemical basis for net CO_2 assimilation in C_4 plants. In: Net carbon dioxide assimilation in higher plants, pp. 75–93, C.C. Black, Ed. Raleigh: Proc. South Sect. Amer. Soc. Plant Physiol. 1972

Kanai, R., Edwards, G.E.: Separation of mesophyll protoplasts and bundle-sheath cells from maize leaves for photosynthetic studies. Plant Physiol. **51**, 1133–1137 (1973)

Kestler, D.P., Mayne, B.C., Ray, T.B., Goldstein, L.D., Brown, R.H., Black, C.C.: Biochemical components of the photosynthetic CO_2 compensation point of higher plants. Biochem. Biophys. Res. Commun. **66**, 1439–1446 (1975)

Kostos, V., Di Tullio, N.W., Rush, J., Cieslinski, L., Saunders, H.L.: The effect of 3-mercaptopicolinic acid on phosphoenolpyruvate carboxykinase (GTP) in the rat and guinea pig. Arch. Biochem. Biophys. **171**, 459–465 (1975)

Ku, S.B., Edwards, G.E.: Photosynthesis in mesophyll protoplasts and bundle-sheath cells of various types of C_4 plants. IV. Enzymes of respiratory metabolism and energy utilizing enzymes of photosynthetic pathways. Z. Pflanzenphysiol. **77**, 16–32 (1975)

Noce, P.S., Utter, M.F.: Decarboxylation of oxalacetate to pyruvate by purified avian phosphoenolypyruvate carboxykinase. J. biol. Chem. **250**, 9099–9105 (1975)

Randall, D.D., Tolbert, N.E., Gremel, D.: 3-phosphoglycerate phosphatase in plants. II. Distribution, physiological considerations, and comparison with *p*-glycolate phosphatase. Plant Physiol. **48**, 480–487 (1971)

Rathnam, C.K.M., Edwards, G.E.: Intracellular localization of certain photosynthetic enzymes in bundle sheath cells of plants possessing the C_4 pathway of photosynthesis. Arch. Biochem. Biophys. **171**, 214–225 (1975)

Rathnam, C.K.M., Edwards, G.E.: Protoplasts as a tool for isolating functional chloroplasts from leaves. Plant Cell Physiol. **17**, 177–186 (1976)

Ray, T.B., Black, C.C.: Inhibition of oxalacetate decarboxylation during C_4 photosynthesis by 3-mercaptopicolinic acid. J. Biol. Chem. **251**, 5824–5826 (1976)

Silverstein, E., Boyer, P.D.: Instability of pyruvate-C^{14} in aqueous solution as detected by enzymic assay. Analyt. Biochem. **8**, 470–476 (1964)

Stokes, D.M., Walker, D.A.: Photosynthesis by isolated chloroplasts. Inhibition by D,L-glyceraldehyde of carbon dioxide assimilation. Biochem. J. **128**, 1147–1157 (1972)

Whatley, F.R., Arnon, D.I.: Photosynthetic photophosphorylation in plants. Meth. Enzymol. **6**, 308–313 (1963)

Wintermans, J.F.G.M., DeMots, A.: Spectrophotometric characteristics of chlorophyll and their pheophytins in ethanol. Biochim. Biophys. Acta **109**, 448–453 (1965)

Yamada, T., Carlsson, J.: Glucose-6-phosphate-dependent pyruvate kinase in *Streptococcus mutans*. J. Bacteriol. **124**, 502–503 (1975)

Received 2 June; accepted 24 August 1976