

Regulation of Oxaloacetate, Aspartate, and Malate Formation in Mesophyll Protoplast Extracts of Three Types of C₄ Plants

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ABSTRACT

The use of mesophyll protoplast extracts from various C₄ species has provided an effective method for studying light- and substrate-dependent formation of oxaloacetate, malate, and aspartate at rates equivalent to whole leaf C₄ photosynthesis. Conditions regulating the formation of the C₄ acids were studied with protoplast extracts from *Digitaria sanguinalis*, an NADP-malic enzyme C₄ species, *Eleusine indica*, an NAD-malic enzyme C₄ species, and *Urochloa panicoides*, a phosphoenolpyruvate (PEP) carboxykinase C₄ species. Light-dependent induction of CO₂ fixation by the mesophyll extracts of all three species was relatively low without addition of exogenous substrates. Pyruvate, alanine and α -ketoglutarate, or 3-phosphoglycerate induced high rates of CO₂ fixation in the mesophyll extracts with oxaloacetate, malate, and aspartate being the primary products. In all three species, it appears that pyruvate, alanine, or 3-phosphoglycerate may serve as effective precursors to the formation of PEP for carboxylation through PEP-carboxylase in C₄ mesophyll cells. Induction by pyruvate or alanine and α -ketoglutarate was light-dependent, whereas 3-phosphoglycerate-induced CO₂ fixation was not.

Several differences between these species representing the three C₄ groups were observed. Substrate induction of CO₂ fixation in mesophyll protoplast extracts of *D. sanguinalis* gave malate as a major product; only by an apparent exchange reaction with cold aspartate did substantial label appear in aspartate (up to 53% of labeled products). In contrast, aspartate was a major product when alanine and α -ketoglutarate served as inducing substrates with *E. indica* (up to 57%) and *U. panicoides* (up to 86%). With induction by pyruvate or 3-phosphoglycerate, mesophyll preparations of *U. panicoides* and *E. indica* were less effective in forming malate (up to 31% of products) than *D. sanguinalis* (up to 87% of products). After 2 seconds of whole leaf ¹⁴CO₂ fixation, malate was the major labeled product (57%) with *D. sanguinalis*, whereas with *E. indica* and *U. panicoides* aspartate was the predominant product (73% and 76%, respectively).

With mesophyll protoplast extracts of *D. sanguinalis*, aspartate inhibited CO₂ fixation (about 50% at 0.6 mM), while malate was relatively uninhibitory at comparable concentrations. CO₂ fixation by mesophyll protoplast extracts of *E. indica* was inhibited by malate (about 50% at 0.6 mM), while aspartate was relatively uninhibitory. With mesophyll preparations of *U. panicoides*, malate or aspartate (2 mM) caused only slight inhibition of CO₂ fixation. The regulation of aspartate and malate synthesis in C₄ mesophyll cells is discussed relative to initial products of photosynthesis in C₄ species *in vivo* and species differences in the mechanisms of C₄ photosynthesis.

Within the first seconds of ¹⁴CO₂ fixation, aspartate and malate are major labeled compounds in C₄ plants, although the percentage of labeling in aspartate relative to malate may vary between C₄ species (1-4, 11, 12, 19). Downton (3, 4) classified C₄ species as "malate formers" or "aspartate formers" based on the tendency for malate or aspartate to be the predominant initial photosynthetic product. The relative percentage of labeling between aspartate and malate may vary with leaf age (18), time of exposure to ¹⁴CO₂ (2, 18, 19), and with various photosynthetic tissue of a given species (27).

By a recent classification based on C₄ acid decarboxylating enzymes and cytological characteristics, C₄ species were divided into three groups: NADP-malic enzyme type, NAD-malic enzyme type, and PEP¹-carboxykinase type (6, 10). According to Downton's initial classification NADP-malic enzyme species would be predominant malate formers, whereas NAD-malic enzyme and PEP-carboxykinase species would be considered predominant aspartate formers when leaves are exposed to ¹⁴CO₂ for a few seconds in the light. From the labeling of aspartate and malate after seconds of exposure to ¹⁴CO₂, both C₄ acids are commonly referred to as "products" of C₄ photosynthesis. OAA is considered the initial product of C₄ photosynthesis *in vivo* although the percentage of labeling in OAA is always low (1-10% [8, 11, 12]). An alternative means of labeling aspartate or malate, without leading to net synthesis, would be through an exchange reaction of ¹⁴C-OAA with endogenous malate or aspartate in the leaf.

Recently, we reported conditions in which high rates of light- and pyruvate-dependent ¹⁴CO₂ fixation into OAA and malate could be induced in mesophyll protoplast extracts of *Digitaria sanguinalis*, an NADP-malic enzyme species (16). The purpose of the present study was to determine the relative level of labeling and mechanism of labeling of OAA, malate, and aspartate during light-dependent CO₂ fixation by mesophyll protoplast extracts of species representing the three C₄ groups.

MATERIALS AND METHODS

Plant Culture. Leaves of *Digitaria sanguinalis*, *Eleusine indica*, and *Urochloa panicoides* were collected from plants grown in a growth chamber with a 16 hr daylength at 30 C day and 20 C night temperatures. Leaves were taken from plants 2 to 4 weeks old. Light was provided by a combination of incandescent and fluorescent lamps giving a total quantum flux density of 50 to 70 einsteins cm⁻² sec⁻¹ between 400 and 700 nm.

Mesophyll Protoplast Extracts and ¹⁴CO₂ Fixation Assays. Mesophyll protoplast isolation was as previously described (16).

¹ Abbreviations: PEP: phosphoenolpyruvate; OAA: oxaloacetate; 2-PGA; 2-phosphoglycerate; 3-PGA: 3-phosphoglycerate.

The protoplast extraction medium contained 0.3 M sorbitol, 1 mM MgCl₂, 2 mM KH₂PO₄, and 50 mM Tricine-KOH (pH 7.5). After breaking the protoplasts, BSA was added to give 0.5 mg/ml. Mesophyll protoplast extracts were used for CO₂ fixation since previous studies showed protoplast extracts gave the highest rates of CO₂ fixation relative to mesophyll protoplasts or cells (16). The reaction mixture for CO₂ fixation was the same as the protoplast extraction medium including 6 mM NaH¹⁴CO₃ and substrates as indicated. EDTA and MnCl₂, previously used in the extraction and reaction mixture (16) were eliminated since they were found not to be required. All reactions were run at 40 C with a quantum flux density of approximately 80 einsteins cm⁻² sec⁻¹ between 400 and 700 nm provided by a sodium vapor lamp (General Electric Lucalox, 400 w). Rates were calculated from the linear phase of CO₂ fixation which generally lasted at least 10 min.

Chromatography of CO₂ Fixation Products. Products of mesophyll protoplast extract ¹⁴CO₂ fixation were separated by chromatography and identified as previously described (16). An equal volume of chloroform-ethanol (4:1) was added to the extracts. Phenylhydrazone derivatives in the organic phase were concentrated by evaporation at 40 C and separated by silica gel TLC in 1-butanol-ethanol-0.5 N NH₄OH (7:1:2). Identification of compounds in the aqueous phase was as previously described (16).

Dowex Column Chromatography. In one experiment with mesophyll protoplast extracts, Dowex columns were used to fractionate ¹⁴CO₂ fixation products into acidic, basic, and neutral fractions. Reaction mixtures for column chromatography were terminated in 80% methanol. The heat-labile fraction was determined by heating the extracts at 80 C for 20 min and taking the radioactivity lost as an indication of the percentage of ¹⁴C-4 OAA in the extract. Controls with authentic labeled ¹⁴C-4 OAA, malate, and aspartate showed that only OAA lost activity under this treatment. The heat-stable compounds were resuspended in 80% methanol and successively applied to 1-cm columns of Dowex 1 × 9 (formate form) and Dowex 50 × 8 (H⁺ form). The samples were washed through the columns with 2 ml of H₂O, and the effluent was taken as the neutral fraction. Organic acids were eluted from the Dowex 1 × 8 (formate) column, and amino acids were eluted from the Dowex 50 × 8 (H⁺) column with 0.5 ml of 10 N HCl followed with 1.5 ml of 1 N HCl. Ion exchange chromatography of a mixture of ¹⁴C-malate and ¹⁴C-aspartate followed by one-dimensional paper chromatography of the basic and acidic fractions showed malate was retained in the organic acid fraction and aspartate in the amino acid fraction.

For whole leaf CO₂ fixation, leaves were exposed to ¹⁴CO₂ by the "dip method" similar to that of Kortschak *et al.* (19). Detached leaves with the basal portion in water were preilluminated at room temperature (approximately 25 C) for 20 min prior to exposure to ¹⁴CO₂. Again the light source was the sodium vapor lamp giving a quantum flux density of 80 einsteins cm⁻² sec⁻¹ between 400 and 700 nm. NaH¹⁴CO₃ was added to a 15-ml test tube and sealed with parafilm. The total concentration of NaHCO₃ was adjusted to give a final CO₂ concentration of 0.045% or 0.5% after lactic acid addition. Lactic acid was injected with a syringe and the sealed tube was pre-equilibrated for several minutes at 30 C prior to exposing the leaves.

A single leaf was inserted in the tube through a small slit in the parafilm. After approximately 2 sec, the leaf was killed similar to the method of Hatch and Slack (11) in 10 ml of a mixture of 80% ethanol, 0.04% 2,4-dinitrophenylhydrazine, and 0.4 N HCl kept at -80 C with liquid nitrogen to trap oxaloacetate as the phenylhydrazone derivative and further extracted with an equal volume of 50% ethanol.

Enzyme Assays. The following enzymes were assayed as

previously described: Cyt *c* oxidase (23), PEP-carboxylase, NADP-malate dehydrogenase, alanine aminotransferase, and NAD-malate dehydrogenase (5). The assay for aspartate aminotransferase was as previously described (5), except 0.5 unit of malic dehydrogenase was included. Pyruvate Pi dikinase was assayed by following the fixation of ¹⁴CO₂ in a system coupled to PEP-carboxylase and malate dehydrogenase. The reaction mixture contained enzyme extract, 30 mM HEPES-KOH (pH 8), 7 mM MgCl₂, 4 mM dithiothreitol, 2.5 mM K₂HPO₄, 4 mM pyruvate, 1 mM NADH, 1.5 mM ATP, 1.5 units of PEP-carboxylase, 2 units of malate dehydrogenase, and 6 mM NaH¹⁴CO₃ in a final volume of 0.15 ml. Enolase was assayed in a mixture containing 50 mM Tricine-KOH (pH 7.8), 10 mM MgCl₂, 5 mM dithiothreitol, 4 mM NaH¹⁴CO₃, 5 mM NADH, 2 units of NAD-malate dehydrogenase, 2 units of PEP-carboxylase (partially purified from maize leaves), and 0.5 mM 2-P-glycerate in a total volume of 0.15 ml. Phosphoglyceromutase was assayed similarly, except the reaction mixture included 2 units of enolase and 0.5 mM 3-PGA was substituted for 2-P-glycerate. All assays were performed at 35 C.

Chlorophyll Determinations. Chlorophyll was determined using the extinction coefficients of Wintermans and De Mots (30).

RESULTS

Substrate Induction of CO₂ Fixation. Table I shows the light-dependent induction of CO₂ fixation by mesophyll protoplast extracts of *Digitaria sanguinalis*, an NADP-malic enzyme species; *Eleusine indica*, an NAD-malic enzyme species; and *Urochloa*

Table I. Effect of Various Substrates on CO₂ Fixation by Mesophyll Protoplast Extracts of *D. sanguinalis*, *E. indica*, and *U. panicoides*

Substrate concentrations were: 2.5 mM pyruvate, 2.5 mM alanine, 1 mM α-ketoglutarate, 1 mM 3-PGA, and 0.5 mM OAA. Rates were calculated from the linear phase of CO₂ fixation which lasted at least 5 min.

Conditions	<i>D. sanguinalis</i>	<i>E. indica</i>	<i>U. panicoides</i>
	μmole/mg Chl · hr ⁻¹		
Endogenous			
Light	4	6	4
Dark	<1	<1	<1
Pyruvate			
Light	78	172	132
Dark	<1	<1	<1
Pyruvate, OAA			
Light	248	205	119
Dark	<1	<1	<1
3-PGA			
Light	95	220	265
Dark	97	180	252
Pyruvate, 3-PGA			
Light	328	345	295
Dark	82	180	243
Alanine, α-ketoglutarate			
Light	74	79	95
Dark	<1	<1	<1
Alanine			
Light	6	25	6
α-Ketoglutarate			
Light	16	32	6
OAA			
Light	23	14	32

Table II. Distribution of Label after 10 Min of $^{14}\text{CO}_2$ Fixation by Mesophyll Protoplast Extracts of *D. sanguinalis*, *E. indica*, and *U. panicoides*

Substrate concentrations were: 2.5 mM pyruvate, 2.5 mM alanine, 2 mM α -ketoglutarate, 1 mM 3-PGA, 0.5 mM OAA, 2 mM glutamate, and 1 mM aspartate. Reactions were terminated after 10 min of $^{14}\text{CO}_2$ fixation. OAA was extracted as the phenylhydrazone derivative and identified by TLC. All other products were separated by two-dimensional paper chromatography.

Treatment	<i>Digitaria sanguinalis</i>					<i>Eleusine indica</i>					<i>Urochloa panicoides</i>				
	Asp	Mal	OAA	Others	Rate	Asp	Mal	OAA	Others	Rate	Asp	Mal	OAA	Others	Rate
	%				$\frac{\mu\text{moles CO}_2 \text{ fixed/mg Chl} \cdot \text{hr}^{-1}}$	%				$\frac{\mu\text{moles CO}_2 \text{ fixed/mg Chl} \cdot \text{hr}^{-1}}$	%				$\frac{\mu\text{moles CO}_2 \text{ fixed/mg Chl} \cdot \text{hr}^{-1}}$
Pyruvate	1	87	10	2	267	6	31	60	3	146	4	3	49	44	210
Pyruvate, OAA	2	47	51	0	382	0	22	78	0	208	2	9	83	6	216
3-PGA	5	85	10	0	110	18	12	70	0	243	4	26	62	8	142
Pyruvate, 3-PGA	2	87	10	1	529	8	24	68	0	315	4	30	57	9	375
Pyruvate, OAA, glutamate	9	41	50	0	182	34	13	52	1	199	53	4	43	0	230
Alanine, α -Kg	10	87	2	1	205	46	10	44	0	112	48	17	34	1	130
Alanine, α -Kg, OAA	15	71	14	0	346	57	8	35	0	134	86	6	5	3	154
Alanine, α -Kg, 3-PGA	13	74	11	2	493	46	24	30	0	320	34	30	21	15	370
Endogenous	26	32	41	1	11	66	22	10	2	5	77	18	5	0	4
Pyruvate, aspartate	53	45	2	0	176	62	5	33	0	118	60	2	38	0	190

panicoides, a PEP-carboxykinase species. Without the addition of organic substrates, the rate of CO_2 fixation was low. Pyruvate, alanine + α -ketoglutarate, and pyruvate + OAA induced high rates of light-dependent CO_2 fixation by mesophyll protoplast extracts of all three species. Alanine or α -ketoglutarate when used alone induced much lower rates of CO_2 fixation than when used in combination. High rates of CO_2 fixation in both light and dark were induced with 3-PGA, although rates were somewhat higher in the light (Table I). Maximum rates of CO_2 fixation were obtained with a combination of pyruvate + PGA or alanine + α -ketoglutarate + PGA (Tables I and II). The induction by 5 mM pyruvate + 0.5 mM OAA was much higher than that of pyruvate alone with *D. sanguinalis*, while 0.5 mM OAA had little effect on pyruvate induction in mesophyll preparations of *E. indica* and *U. panicoides* (Tables I and II).

Concentration Curves for Pyruvate and Alanine Induction of CO_2 Fixation. With all three species, the induction of CO_2 fixation by varying concentrations of pyruvate gave hyperbolic curves, which saturated at 0.2 to 0.4 mM (Fig. 1). With *D. sanguinalis* and *U. panicoides*, varying concentrations of alanine (α -ketoglutarate kept constant at 2 mM) gave sigmoidal type curves, and pyruvate was much more effective than alanine at low concentrations (0.02–0.14 mM). Variable concentrations of alanine (+2 mM α -ketoglutarate) in the presence of 0.02 mM pyruvate tended to reduce the sigmoidicity of the concentration curve with mesophyll preparations of *D. sanguinalis* and *U. panicoides* which resulted in more effective induction at low alanine concentration (Fig. 1). With *E. indica*, pyruvate and alanine were equally effective in inducing CO_2 fixation at low concentrations.

Products of CO_2 Fixation. Table II shows the effect of various substrates on the rate of CO_2 fixation and distribution of products in the light with mesophyll protoplast extracts of the three species. With *D. sanguinalis*, the products of endogenous CO_2 fixation were OAA, malate, and aspartate with about equal label appearing in aspartate and malate. In contrast, aspartate was the major product and malate and OAA minor products of endogenous fixation with *E. indica* and *U. panicoides*.

Malate, aspartate, and OAA were always the major products of CO_2 fixation by mesophyll preparations of the three species. The per cent distribution of the products between the C_4 acids is dependent both on the species and the substrates used. With pyruvate, PGA, pyruvate + OAA, and pyruvate + PGA the

majority of the label with *D. sanguinalis* was in malate (85–87% of the total). With *E. indica* and *U. panicoides*, these substrates only gave from 3 to 30% of the label in malate, with little label in aspartate and the major part of the label in OAA.

Alanine + α -ketoglutarate, alanine + α -ketoglutarate + PGA, and alanine + α -ketoglutarate + OAA as substrate combinations with *D. sanguinalis* gave 10 to 15% of the label in aspartate with 70 to 80% of the label in malate. However, using these substrate combinations with *E. indica* and *U. panicoides* aspartate was the primary labeled product (up to 86%). The combination of pyruvate + OAA + glutamate resulted in greater labeling in malate compared to aspartate in *D. sanguinalis*, whereas *E. indica* and *U. panicoides* had a higher labeling in aspartate than malate with these substrates.

The only conditions which gave substantial labeling in aspartate with *D. sanguinalis* was without added substrates (26% label in aspartate) and with the combination of 1 mM cold aspartate + pyruvate (53% label in aspartate, Table II). This apparent labeling of aspartate by an exchange reaction was not dependent on exogenous glutamate or α -ketoglutarate (data not shown). Figure 2 shows the effect of aspartate concentration on the distribution of label with the pyruvate + aspartate induction with protoplast extracts of *D. sanguinalis*. When the concentration of cold aspartate was varied from 0 to 0.8 mM, label in the amino acid fraction increased at the expense of label in the organic acid fraction. The increased labeling of the amino acid fraction with increasing aspartate concentration probably represents an increase in the label in aspartate as was found with the pyruvate + aspartate induction with *D. sanguinalis* in Table II.

E. indica and *U. panicoides* had substantial labeling of aspartate when 1 mM cold aspartate was added in combination with pyruvate (Table II). No increase in the labeling of malate was detected when 1 mM unlabeled malate was added in combination with pyruvate with the three species (data not shown).

The distribution of label after 2 sec of $^{14}\text{CO}_2$ fixation was determined with leaves of the three species. The product distribution was strongly affected by the concentration of CO_2 (Table III). While the relative distribution of label between malate and aspartate remained about the same for each species at the CO_2 levels, the absolute amount of label in OAA was severalfold higher at 0.5% CO_2 than 0.045% CO_2 as OAA became the major labeled product. After 2 sec of fixation at 0.045% CO_2 , malate

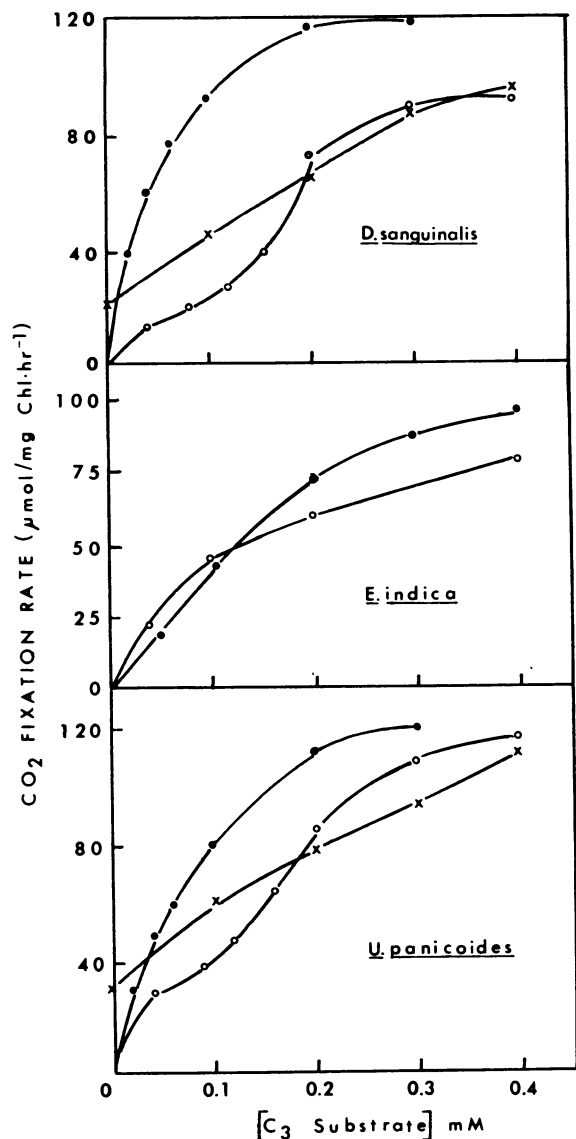


FIG. 1. CO₂ fixation rates of mesophyll protoplast extracts of *D. sanguinalis*, *E. indica*, and *U. panicoides* with variable concentrations of pyruvate (●—●), alanine + 2 mM α-ketoglutarate (○—○), or alanine + 2 mM α-ketoglutarate + 0.02 mM pyruvate (×—×). All rates were calculated after 5 min of fixation.

was the major product with *D. sanguinalis*, whereas aspartate was the major product with *E. indica* and *U. panicoides* (Table III).

C₄ Acid Inhibition of CO₂ Fixation. With *D. sanguinalis*, aspartate was more effective than malate as an inhibitor of CO₂ fixation with 50% inhibition at roughly 0.6 mM (Fig. 3). With *E. indica*, malate was a more effective inhibitor than aspartate with 50% inhibition at roughly 0.6 mM. Inhibition by malate and aspartate with *E. indica* was similar when alanine + α-ketoglutarate were used as inducing substrates (data not shown). With *U. panicoides*, neither aspartate nor malate caused more than 20% inhibition of the pyruvate-induced CO₂ fixation (Fig. 3).

C₄ Acid Inhibition of PEP-carboxylase Activity. The effect of 1 mM malate or 1 mM aspartate on PEP-carboxylase activity in crude leaf extracts of *D. sanguinalis*, *E. indica*, and *U. panicoides* was determined (Table IV). A rate-limiting concentration of PEP (0.3 mM) was used in the assay in order to induce rates

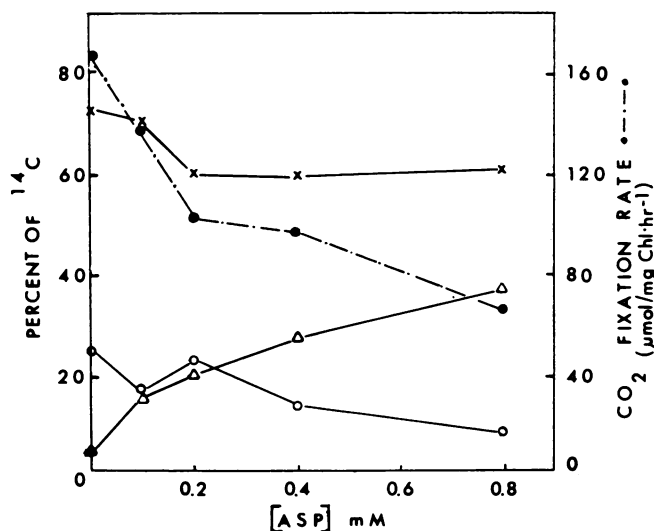


FIG. 2. Effect of aspartate concentration on the rate (●—●) of pyruvate-induced ¹⁴CO₂ fixation in mesophyll protoplast extracts of *D. sanguinalis* and the distribution of label: heat-labile fraction (○—○); heat-stable compounds in the organic acid fraction (×—×); and amino acid fraction (△—△).

Table III. Product Distribution after 2 Sec of Whole Leaf ¹⁴CO₂ Fixation

Species	Aspartate	Malate	Oxaloacetate	Others
	%			
0.045% CO ₂				
<i>Digitaria sanguinalis</i>	33	57	8	2
<i>Eleusine indica</i>	73	20	5	2
<i>Urochloa panicoides</i>	76	9	14	1
0.5% CO ₂				
<i>Digitaria sanguinalis</i>	7	21	66	6
<i>Eleusine indica</i>	35	15	46	4
<i>Urochloa panicoides</i>	34	12	50	4

and conditions comparable to the CO₂ fixation experiments. Using a reaction mixture similar to the CO₂ fixation studies, aspartate was the more potent inhibitor with PEP-carboxylase from *D. sanguinalis*, while malate inhibited *E. indica* PEP-carboxylase more than aspartate. In contrast, inhibition of PEP-carboxylase from *U. panicoides* by malate and aspartate was comparable but low.

Enzymes in C₄ Mesophyll Cells. Table V lists the levels of several enzymes from mesophyll extracts which may be involved in the metabolism of C₃ precursors to C₄ acids in C₄ mesophyll cells. Levels of PEP-carboxylase, aspartate aminotransferase and alanine aminotransferase were relatively high in mesophyll extracts of all three species. Phosphoglyceromutase and enolase activity was higher in *E. indica* and *U. panicoides* than in *D. sanguinalis* mesophyll extracts, while NADP-malate dehydrogenase was higher in extracts of *D. sanguinalis* than in *E. indica* or *U. panicoides*. The activity of pyruvate Pi dikinase was relatively low in the three species, much less than the rates of pyruvate-induced CO₂ fixation.

After differential centrifugation of the protoplast extracts, pyruvate Pi dikinase and NADP-malate dehydrogenase were predominantly in the 400g chloroplast pellet. In *D. sanguinalis* aspartate aminotransferase was associated with the chloroplast

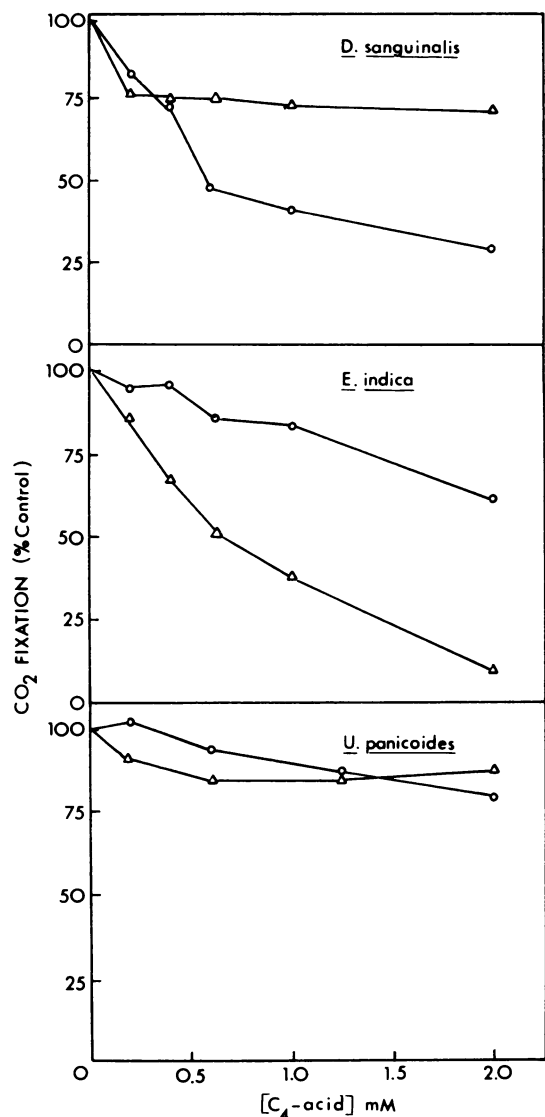


FIG. 3. Effect of the concentration of aspartate (○—○) and malate (△—△) on the rate of pyruvate-induced CO₂ fixation with mesophyll protoplast extracts of *D. sanguinalis*, *E. indica*, and *U. panicoides*. Control rates were 140, 98, and 120, respectively.

Table IV. Inhibition of PEP-carboxylase from C₄ Leaf Extracts by Malate and Aspartate

PEP-carboxylase was assayed in whole leaf extracts of the three species. The reaction mixture contained 50 mM Tricine-KOH (pH 7.8), 1 mM MgCl₂, 1 mM KH₂PO₄, 0.3 mM PEP, and 3 mM NaH¹⁴CO₃. Rates were calculated from the linear phase of CO₂ fixation. Numbers in parentheses are percentages of control rate.

Treatment	<i>D. sanguinalis</i>	<i>U. panicoides</i>	<i>E. indica</i>
	μmole/mg Chl·hr ⁻¹		
Control	392	425	290
1 mM aspartate	118 (30%)	306 (72%)	246 (85%)
1 mM malate	255 (65%)	336 (79%)	151 (52%)

pellet, whereas alanine aminotransferase was extrachloroplastic; in *E. indica* and *U. panicoides* both aminotransferases were primarily extrachloroplastic. In all three species, PEP-carboxylase appeared to be extrachloroplastic, while phosphoglyceromutase

Table V. Enzyme Activity and Separation by Differential Centrifugation in C₄ Mesophyll Protoplast Extracts of *D. sanguinalis*, *E. indica*, and *U. panicoides*

Calculations on per cent distribution of enzymes were made taking the sum of the activity in the 400g pellet and supernatant as 100%, which corresponded closely to the total activity in the mesophyll protoplast extract.

Enzyme	<i>D. sanguinalis</i>		<i>E. indica</i> ¹		<i>U. panicoides</i>	
	Activity	%400g pellet	Activity	%400g pellet	Activity	%400g pellet
	μmoles/mg Chl·hr ⁻¹		μmoles/mg Chl·hr ⁻¹		μmoles/mg Chl·hr ⁻¹	
PEP-carboxylase	2360	5	1981	4	1629	3
Pyruvate Pi dikinase	114	93	82	69	51	87
Alanine aminotransferase	818	2	796	10	917	3
Aspartate aminotransferase	315	90	1302	7	1229	3
NADP-malate dehydrogenase	862	89	174	77	89	55
NAD-malate dehydrogenase			1881	15	2048	9
Phosphoglyceromutase	122	24	352	8	453	12
Enolase	136	24	219	6	339	7
Cyt c oxidase			50	3		
Chl ²		100		100		100

¹ Data in part from ref. 12.

² The Chl retained in the supernatant was less than 1% of the total.

and enolase appeared to be largely extrachloroplastic. Cytochrome c oxidase and NAD-malate dehydrogenase, normally considered extrachloroplastic enzymes, were found in the supernatant fractions.

DISCUSSION

In C₄ photosynthesis, it is thought that CO₂, initially fixed into the C₄ acids OAA, aspartate, and malate in mesophyll cells, is subsequently donated to the reductive pentose phosphate pathway in bundle sheath cells via C₄ acid-decarboxylating enzymes (6, 9, 10). In a shuttle of CO₂ from the atmosphere to the bundle sheath cells, it is proposed that C₄ acids are transported to bundle sheath cells and C₃ acids back to mesophyll cells. Three C₄ groups have been proposed based on the predominance of decarboxylating enzymes in bundle sheath cells: NADP-malic enzyme species, NAD-malic enzyme species, and PEP-carboxykinase species (6, 10). In any one type of C₄ species, it is not clear what the transport metabolites are in the proposed C₄ shuttle system. Although both aspartate and malate are early labeled products in C₄ plants, in a given C₄ species only one of these is generally suggested as a transport metabolite from mesophyll to bundle sheath cells. Proposed transport metabolites of the C₄ shuttle are malate and pyruvate in NADP-malic enzyme species and aspartate and alanine in NAD-malic enzyme and PEP-carboxykinase species (3, 7, 9, 10).

Substrate Induction and Products of CO₂ Fixation. One approach to studying possible transport metabolites in C₄ photosynthesis is to identify C₃ metabolites which will serve as precursors to PEP formation in C₄ mesophyll cells and characterize the regulation of malate and aspartate formation. With mesophyll preparations of species representing three groups of C₄ plants, pyruvate, alanine + α-ketoglutarate, and 3-PGA induced high rates of CO₂ fixation (Table I) and are therefore considered potential precursors for PEP formation.

Figure 4 shows suggested metabolic sequences in mesophyll cells of *D. sanguinalis*, an NADP-malic enzyme species (Fig.

4A), and *E. indica*, an NAD-malic enzyme species, and *U. panicoides*, a PEP-carboxykinase species (Fig. 4B). The proposed schemes are consistent with substrate inductions (Tables I and II), reported enzyme activities (Table IV), and compartmentation of enzymes (Table IV, ref. 7) in mesophyll preparations of the three groups. Primary differences between the metabolism of the NADP-malic enzyme species in comparison to NAD-malic enzyme and PEP-carboxykinase species are as follows. The NADP-malic enzyme species have aspartate aminotransferase chloroplastic (Table V, ref. 7), relatively low levels of aspartate aminotransferase, relatively high levels of NADP-malic dehydrogenase (Table V, ref. 7), and malate as a major product of CO₂ fixation *in vitro* (Table II). In contrast, the NAD-malic enzyme and PEP-carboxykinase species examined have relatively high levels of extra chloroplastic aspartate and alanine aminotransferases (Table V, ref. 7), relatively low levels of NADP-malic dehydrogenase (Table V, ref. 7), with aspartate as a major product *in vitro* with alanine as the C₃ precursor (Table II).

In all three groups, the logical sequence for light-dependent induction by pyruvate is conversion of pyruvate to PEP by pyruvate Pi dikinase. The high rates of CO₂ fixation induced by pyruvate with mesophyll preparations of the three species is much higher than the levels of pyruvate Pi dikinase observed in enzyme assays. The apparent lability of pyruvate Pi dikinase when extracted may be due to incomplete activation of the enzyme as Sugiyama (25) has recently demonstrated that a soluble protein factor is required for activation.

Maximum light-dependent induction by alanine requires α -ketoglutarate which suggests alanine may be converted to pyruvate through alanine aminotransferase and then to PEP by pyruvate Pi dikinase. PGA induces either in the light or dark which may be through phosphoglyceromutase and enolase in a nonenergy-requiring sequence. Both the enzyme potential for conversion of PGA to PEP through phosphoglyceromutase and enolase and the induction of CO₂ fixation with PGA were higher in *E. indica* and *U. panicoides* than in *D. sanguinalis*. Since alanine, pyruvate, and 3-PGA can serve as apparent precursors for PEP formation in C₄ mesophyll cells, all three of these substrates can be considered possible transport metabolites from bundle sheath to mesophyll cells in the C₄ shuttle.

With alanine as a precursor for PEP formation, aspartate may be a major product due to the cycling of α -ketoglutarate and glutamate through alanine and aspartate aminotransferases (Fig. 4). If 3-PGA or pyruvate is a precursor for PEP formation, malate might be a logical product unless there was reductive amination of OAA to aspartate.

Pyruvate + OAA, and pyruvate + 3-PGA induced high rates of CO₂ fixation with mesophyll preparations of *D. sanguinalis* (16, and Table II) as did alanine + α -ketoglutarate (Table II). There is a tendency for malate to be a major product and aspartate a minor product with all the substrate inductions with *D. sanguinalis*. With alanine + α -ketoglutarate only 10 to 15% of the label appeared in aspartate, and labeling in aspartate was still much lower than malate. Since the activity of alanine and aspartate aminotransferases are rather high in mesophyll cells of *D. sanguinalis* and exceed the rates of CO₂ fixation induced by alanine + α -ketoglutarate with mesophyll protoplast extracts, the enzyme levels would not appear to be limiting the potential for aspartate formation. Even with pyruvate + OAA + glutamate there was more label in malate than aspartate with mesophyll protoplast extracts of *D. sanguinalis*. This result suggests that the reduction of OAA to malate is more competitive than the transamination of OAA to aspartate in the *in vitro* system with *D. sanguinalis*.

The intracellular localization of the aspartate and alanine aminotransferases may influence the rate of aspartate formation when alanine + α -ketoglutarate serve as inducing substrates.

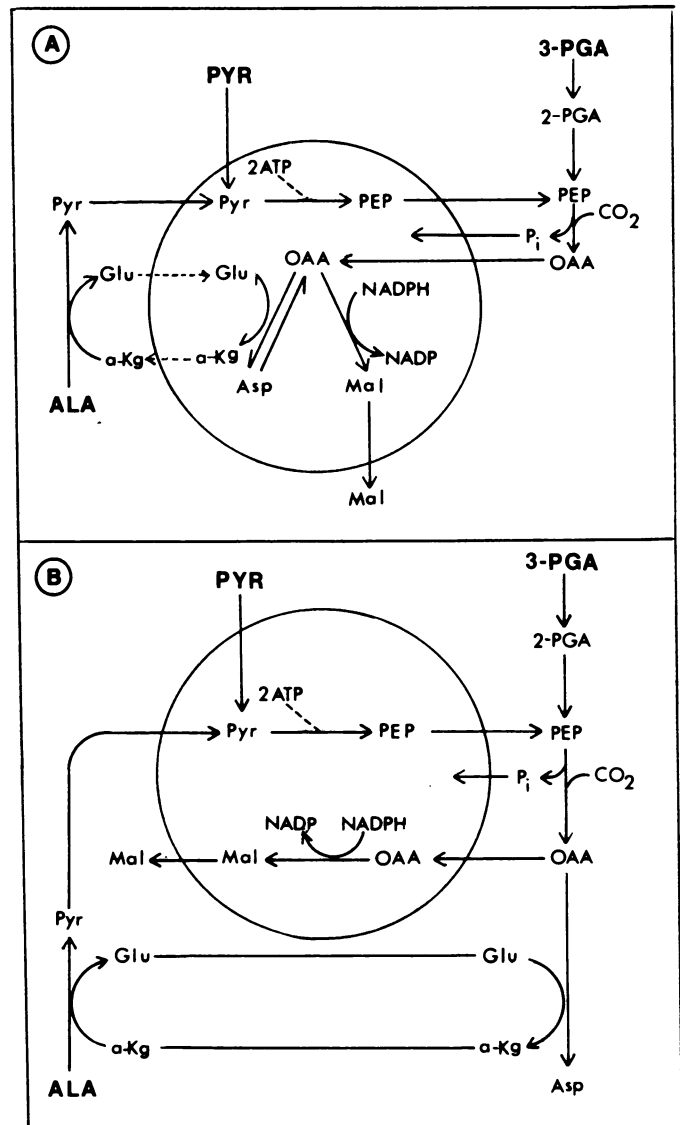


FIG. 4. Suggested intracellular metabolism of C₃ precursors (bold print) leading to aspartate and malate formation in C₄ mesophyll cells. A: *D. sanguinalis*; B: *E. indica* and *U. panicoides*.

Thus when alanine serves as precursor to PEP formation in *D. sanguinalis*, an NADP-malic enzyme type, it appears that a glutamate- α -ketoglutarate shuttle across the chloroplast membrane would be required for aspartate formation (Fig. 4). In the chloroplasts, the transamination of OAA to aspartate would be dependent on the glutamate concentration, while the reduction to malate would be dependent on NADPH. It has been suggested that the membrane of C₃ chloroplasts is relatively impermeable to glutamate (17, 21). If such is the case with *D. sanguinalis* mesophyll chloroplasts, the concentration of glutamate inside the chloroplast could be limiting the synthesis of aspartate. Also, the activity of NADP-malic dehydrogenase is higher in *D. sanguinalis* than *U. panicoides* and *E. indica* which would further favor malate formation in *D. sanguinalis*.

Only with pyruvate + aspartate as substrates did aspartate become a major labeled compound (up to 53%) with mesophyll protoplast extracts of *D. sanguinalis* which suggests that aspartate can be labeled in an exchange reaction with OAA (Table II, Fig. 2). Thus at least part of the aspartate labeling *in vivo* when leaves of C₄ species are exposed to ¹⁴CO₂ could be due to

an exchange reaction of endogenous aspartate with labeled ^{14}C -OAA. Such an exchange reaction could occur without net synthesis of aspartate. In considering the percentage of label appearing in aspartate and malate when exposing leaves to $^{14}\text{CO}_2$, the results cannot be taken as an indication of the relative rate of synthesis of malate and aspartate. Hiller and Walker (15) have shown that aspartate could be labeled from CO_2 fixation in a Crassulacean acid metabolism species, *Kalanchoe crenata*, through an exchange reaction at apparent rates in excess of the maximum velocity of the aspartate aminotransferase.

The labeling of aspartate from the low rate of endogenous CO_2 fixation of *D. sanguinalis* mesophyll protoplast extracts (26% of the total) could be in part due to an exchange reaction if some endogenous aspartate was contained in the mesophyll protoplasts (Table II). Usuda *et al.* (28) found substantial labeling of aspartate from low rates of endogenous CO_2 fixation by mesophyll chloroplasts of maize. In *D. sanguinalis*, the preference for malate synthesis with the mesophyll preparations and the rapid labeling of aspartate by an exchange reaction suggest malate as the predominant transport metabolite in the C_4 shuttle in this species. Malate was the predominant initial product after the first few seconds of $^{14}\text{CO}_2$ fixation of photosynthesis in leaves of *D. sanguinalis*, although there was substantial label in aspartate (Table III).

With mesophyll preparations of *E. indica* and *U. panicoides*, there was substantial labeling in malate and OAA with little labeling in aspartate when pyruvate or 3-PGA served as substrates for induction of CO_2 fixation. However, substitution of alanine + α -ketoglutarate for pyruvate resulted in a large induction of aspartate labeling so that aspartate became the predominant ^{14}C labeled product. (Table II). In these species, the transamination of alanine to pyruvate and subsequent transamination of OAA to aspartate can all occur outside the chloroplasts without shuttling glutamate and α -ketoglutarate across the chloroplast membrane as in *D. sanguinalis* (Fig. 4). In *E. indica*, an NAD-malic enzyme species, and *U. panicoides*, a PEP carboxykinase species, when alanine is the precursor of PEP, transamination through aspartate aminotransferase in the cytoplasm may be more competitive for OAA than the reduction to malate in the chloroplasts.

Since induction by 3-PGA or pyruvate does lead to some labeled malate in *E. indica* and *U. panicoides*, malate must also be considered as a possible transport metabolite. The lack of evidence for a malate-exchange reaction comparable to the aspartate exchange further suggests that these species have the capacity for net malate synthesis. Although aspartate was a major product in whole leaf studies with *E. indica* and *U. panicoides* (Table III), part of the label in aspartate here also may be by an exchange reaction rather than net synthesis (Table II). In all three species, it is impossible to get a quantitative estimation of the relative rates of synthesis of aspartate and malate based on labeling kinetics in whole leaf studies.

Although OAA has been proposed as the initial product in C_4 photosynthesis, in previous studies only 1 to 10% of the initial products were found in OAA even with shortest exposures to $^{14}\text{CO}_2$. In the present study, exposing the leaves to an enriched atmosphere of $^{14}\text{CO}_2$ (0.5%, about 17 times atmospheric concentration) after preillumination under normal atmospheric conditions resulted in OAA being the major product of photosynthesis after a 2-sec exposure (46–66% of the total, Table III). Exposing leaves to a CO_2 -enriched atmosphere may alter the steady state conditions and cause at least a temporary accumulation of the initial product OAA.

Transport and Regulation in C_4 Photosynthesis. As proposed, the C_4 pathway of photosynthesis involves considerable inter- and intracellular transport of photosynthetic metabolites. Based

on the scheme of Figure 4, it can be suggested that the rate of pyruvate and Pi influx into the chloroplast and efflux of PEP from the chloroplasts would be equivalent to the rate of pyruvate-induced CO_2 fixation (roughly 200 to 400 $\mu\text{moles/mg Chl-hr}^{-1}$), which is equivalent to or higher than typical *in vivo* rates of C_4 photosynthesis (24). Although PEP is not thought to be rapidly transported across C_3 chloroplast membranes (14, 29), it appears to be a transport metabolite in C_4 mesophyll chloroplasts as PEP has been shown not to induce CO_2 fixation with washed chloroplasts of *D. sanguinalis* (16). With *D. sanguinalis*, where malate is the major labeled product (80–90%), OAA influx and malate efflux from the chloroplasts may occur at about the same rates as for pyruvate, Pi, and PEP. A system of translocators, such as the dicarboxylic acid translocator that exchanges malate and OAA in C_3 chloroplasts (13), may exist in the C_4 chloroplast membranes to facilitate such transport. Although *in vivo* concentrations of pyruvate and alanine have not been determined in C_4 species, the saturation of CO_2 fixation in mesophyll protoplast extracts at low levels of pyruvate and alanine (less than 0.5 mM, Fig. 1) demonstrates the capacity for efficient utilization of these substrates in C_4 metabolism.

The inhibition of PEP-carboxylase by aspartate and malate in crude leaf extracts of the three species paralleled the inhibition of CO_2 fixation by the mesophyll protoplast extracts. These results provide a preliminary indication that C_4 acids may be regulating CO_2 fixation by inhibition of PEP-carboxylase with a variable specificity depending on the species. Previous studies with several C_4 species reported little or no inhibition of PEP-carboxylase by low concentrations (1–2 mM) of malate or aspartate (20, 22, 26), although OAA was an effective inhibitor with maize (20). Conditions for allosteric control by malate and aspartate of PEP-carboxylase from various C_4 species will be reported later (Huber and Edwards, in preparation).

CONCLUSION

Studies on the CO_2 fixation by mesophyll protoplast extracts of *D. sanguinalis*, *E. indica*, and *U. panicoides* suggest the possibility for the following shuttles between mesophyll and bundle sheath cells: malate-pyruvate; malate-PGA (this would result in a Pi imbalance which would require Pi transport from mesophyll to bundle sheath cells); and aspartate-alanine. PGA was an effective precursor of PEP with mesophyll preparations of all three species. However, PGA would not be expected to be a major transport metabolite in the shuttle of substrates between mesophyll and bundle sheath cells unless the C_3 products of C_4 acid decarboxylation in bundle sheath cells were metabolized in part by other pathways. With *D. sanguinalis*, an NADP-malic enzyme species, malate formation and transport is favored, whereas with *E. indica*, an NAD-malic enzyme species, and *U. panicoides*, a PEP-carboxykinase species, aspartate formation and transport would be favored. Both the intracellular localization of alanine and aspartate aminotransferase in C_4 mesophyll cells and the total aminotransferase activity in mesophyll and bundle sheath cells may be important determinants of whether aspartate and alanine are transport metabolites in C_4 photosynthesis. Further insight in considering transport metabolites in C_4 photosynthesis may come from fully characterizing the C_4 acid carboxyl donations to bundle sheath preparations. The present study suggests a degree of flexibility in the possible precursors for the β -carboxylation and the subsequent formation of aspartate and/or malate in C_4 mesophyll cells.

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