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Use of inhibitors to distinguish between C_4 acid decarboxylation mechanisms in bundle sheath cells of C_4 plants

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Both malate and aspartate were decarboxylated at the 4-carbon position by isolated bundle sheath strands of C_4 plants but to different extents depending upon the species. In *Digitaria sanguinalis*, an NADP-malic enzyme (NADP-ME) species, 100 μM oxalic acid blocked malate decarboxylation through NADP-ME without affecting aspartate decarboxylation which apparently occurs through NAD-ME. In several phosphoenolpyruvate carboxykinase (PEP-CK) type C_4 species, 200 μM 3-mercaptopicolinic acid (3-MPA), an inhibitor of PEP-CK, specifically inhibited the malate decarboxylation and partially inhibited aspartate decarboxylation. The aspartate decarboxylation insensitive to 3-MPA may occur through NAD-ME. Neither inhibitor prevented C_4 acid decarboxylation in bundle sheath cells of NAD-ME species. The inhibitors thus served to differentiate between the decarboxylation of C_4 acids in PEP-CK and NADP-ME type C_4 species through their major decarboxylase from that of their less active decarboxylation through NAD-ME.

In the C_4 pathway of photosynthesis the decarboxylation of C_4-dicarboxylic acids in leaf bundle sheath cells is facilitated, depending upon the species, by specific decarboxylases: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEP-CK) (*6*, *13*). Although C_4 plants have been subclassified, based on their major decarboxylase, into NADP-ME type, NAD-ME type and PEP-CK type species (*3*, *4*, *7*), we detected substantial amounts of NAD-ME in all the C_4 types, the activity being highest in the NAD-ME species (*3*, *4*, *10*). It is still uncertain as to whether the decarboxylation of C_4 acids in C_4 plants is carried solely through their major decarboxylase or in combination with NAD-ME (*10*). C_4 plants preferentially synthesize either malate or aspartate. Whether only the major C_4 acid formed or both malate and aspartate are transported from mesophyll to bundle sheath cells and decarboxylated to serve as carboxyl donors to the Calvin pathway in bundle sheath cells in a given C_4 species needs to also be considered (*1*, *10*). Presently we are concerned with elucidating specific decarboxylase systems operative in plants representing the

Abbreviations: 3-MPA, 3-mercaptopicolinic acid; DCMU, 3-(3,4-dichlorophenyl) 1,1-dimethylurea; CK, carboxykinase; ME, malic enzyme; PEP, phosphoenolpyruvate.

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three C_4 subgroups (*10*, *12*, *13*). Inhibitors of the C_4 acid decarboxylases are used in the present study as a means to identify and differentiate between decarboxylases in C_4 plants.

Materials and methods

Plants used in the present study included *Digitaria sanguinalis* (NADP-ME type), *Panicum miliaceum* (NAD-ME type), *Brachiaria xantholeuca, Eriochloa borumensis, Panicum molle* and *Urochloa bulbodes* (all PEP-CK type) (*3*, *4*). Bundle sheath strands were isolated by pectinase-cellulase digestion of leaf segments (*8*, *12*), and the chloroplasts were isolated by disrupting the bundle sheath strands in a ground glass homogenizer as described previously (*10*, *12*).

All assays were run in a standard reaction mixture consisting of 0.3 M sorbitol, 50 mM N-tris (hydroxymethyl)methylglycine at pH 8.5, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM KH_2PO_4 (except 0.2 mM KH_2PO_4 for chloroplasts) and 3 mM sodium isoascorbate. Other additions were as indicated for the individual experiments. The reactions were run at 37°C in a final volume of 0.15 ml for 10 min. All reactions were run in the light at intensities at the surface of the vials of 80 $nE \cdot cm^{-2} \cdot sec^{-1}$ (400–700 nm). The $^{14}CO_2$ fixation by bundle sheath strands was assayed as previously described (*11*) in the presence of 6 mM $NaH^{14}CO_2$.

Decarboxylation of C_4 acids at the 4-C position by bundle sheath strands was followed directly using 10 mM L-(4-^{14}C) aspartate in the presence of 10 mM α-ketoglutarate, and 10 mM L-(4-^{14}C) malate in the presence of 6 mM 3-phosphoglyceric acid (*12*, *13*). Decarboxylation reactions included 25 mM D,L-glyceraldehyde to prevent refixation of released $^{14}CO_2$ (for details see Ref. *13*). The reactions 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, IN, U.S.A.) with a paper wick and counted by scintillation spectroscopy.

The effect of oxalic acid on C_4 acid decarboxylation activity of bundle sheath strands was studied by incubating bundle sheath preparations with oxalic acid+ the $^{14}C_4$ acids in the light for 2 min to allow the binding of inhibitor to the decarboxylase (for details see ref. *15*). The reactions were started by adding 6 mM 3-phosphoglycerate (for L-4-^{14}C malate decarboxylation) or 10 mM α-ketoglutarate (for L-4-^{14}C asparatte decarboxylation) in the light. A control reaction (2 min preincubation with oxalic+L-4-^{14}C malate) indicated the residual malate decarboxylation in the presence of oxalate in Fig. 1 with *D. sanguinalis* was due to decarboxylation occurring before starting the reactions with 3-phosphoglycerate. In $^{14}CO_2$ fixation studies with oxalic acid the reactions were also preincubated 2 min in light before initiating the reaction with 6 mM $H^{14}CO_3^-$. With 3-mercaptopicolinic acid reactions were initiated by adding the labeled C_4 acids or $H^{14}CO_3^-$.

Chlorophyll was determined according to Wintermans and De Mots (*16*).

Results and discussion

Distinction between different decarboxylase systems was made in the present study by the use of specific inhibitors of the three known C_4 acid decarboxylases in C_4 plants.

Table 1 *Effect of oxalate on $^{14}CO_2$ fixation and C_4 acid decarboxylation activities of bundle sheath strands of C_4 plants*[a]

Group and species	$^{14}CO_2$ fixation		Malate decarboxylation		Aspartate decarboxylation	
	Control	+Oxalate	Control	+Oxalate	Control	+Oxalate
NADP-ME Type						
Digitaria sanguinalis	40	42	294	40	79	74
NAD-ME Type						
Panicum miliaceum	177	173	176	173	342	362
PEP-CK Type						
Eriochloa borumensis	203	195	405	413	256	249

[a] Experimental conditions and substrate concentrations used were as described in **Materials and methods.** Oxalate, 0.4 mM.
The rates are expressed as μmoles $^{14}CO_2$ fixed or C_4 acid decarboxylated/mg chl·hr.
See Ref. *3*, *4* for the basis of subdividing the C_4 species into three groups.

Oxalate has been shown to be a potent inhibitor of NADP-ME in potato tubers (*15*). Fig. 1 shows that oxalate at a concentration of 100 μM almost completely inhibited malate decarboxylation by isolated bundle sheath strands of *D. sanguinalis*, an NADP-ME type species; while it had no effect on the $^{14}CO_2$ fixation and aspartate decarboxylation activities. Also, oxalate at 100 μM had no effect on the $^{14}CO_2$ fixation and C_4 acid decarboxylation activities of NAD-ME and PEP-CK species (Table 1). Based on enzyme localization and C_4 acid decarboxylation by cells and chloroplasts we previously proposed that in NADP-ME species, malate is decarboxylated through NADP-ME and aspartate decarboxylation is carried through NAD-ME (*10*, *13*). This suggests that oxalate specifically blocks malate decarboxylation in *D. sanguinalis* through NADP-ME. The lack of oxalate inhibition of aspartate decarboxylation in *D. sanguinalis* (Fig. 1 and Table 1) further suggests the decarboxylation of aspartate through a decarboxylase other than NADP-ME, possibly NAD-ME. Mesotartaric acid at concentrations of 30 mM

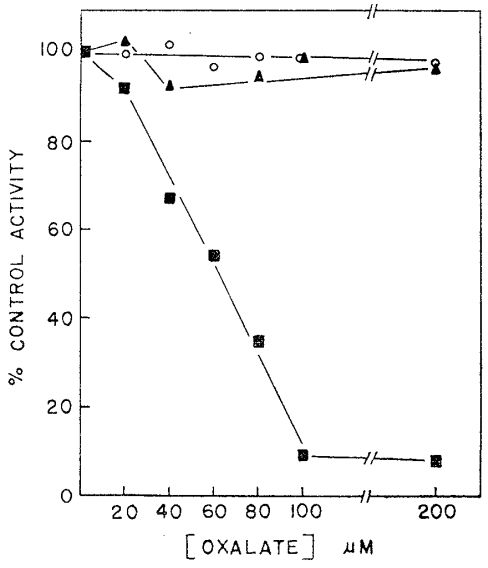

Fig. 1. *Effect of oxalate concentration on $^{14}CO_2$ fixation* (○-○), *malate* (■-■) *and aspartate* (▲-▲) *decarboxylation by bundle sheath strands of* Digitaria sanguinalis, *an NADP-ME type C_4 species.* The experimental conditions and the substrate concentrations were as described in **Materials and methods.**

Table 2 *Effect of DCMU, malate and oxalate on $^{14}CO_2$ fixation activities of* Digitaria sanguinalis *bundle sheath strands*

Conditions[a]	$^{14}CO_2$ fixation (μmoles/mg chl·hr)
a) $H^{14}CO_3^-$	40
b) $H^{14}CO_3^-$+DCMU	0
c) $H^{14}CO_3^-$+Malate	140
d) $H^{14}CO_3^-$+Malate+DCMU	100
e) $H^{14}CO_3^-$+Oxalate	41
f) $H^{14}CO_3^-$+Malate+Oxalate	44
g) $H^{14}CO_3^-$+Malate+Oxalate+DCMU	5

[a] $^{14}CO_2$ fixation was followed as described in Ref. *11*. Substrate concentrations used were 6 mM $NaH^{14}CO_3$, 10 mM malate, 1 μM DCMU, and 100 μM oxalate.

inhibited the decarboxylation of both malate and aspartate by about 50% in bundle sheath cells of *D. sanguinalis*, suggesting both NADP-ME and NAD-ME are partially inhibited. No further inhibition was apparent even when the inhibitor concentration was increased 3–4 fold.

We have further examined the interaction between DCMU and oxalic acid in inhibiting photosynthetic functions of bundle sheath cells of *D. sanguinalis*. The low CO_2 fixation capacity of the bundle sheath strands of *D. sanguinalis*, when compared to NAD-ME species, has generally been attributed to a deficient photosystem II in their chloroplasts (*5*, *6*, *9*, *13*). The malate stimulation of $^{14}CO_2$ fixation by bundle sheath strands of *D. sanguinalis* (Table 2) can be explained on the basis that the NADPH generated during malate decarboxylation by NADP-ME in the chloroplasts is utilized for the reduction of additional 3-phosphoglycerate (*2*, *6*). DCMU (1 μM) completely abolished the $^{14}CO_2$ fixation in the absence of malate apparently by inhibiting NADP reduction during non-cyclic photosynthetic

Table 3 *Effect of 3-MPA on $^{14}CO_2$ fixation and C_4 acid decarboxylation activities of bundle strands of C_4 plants*[a]

Group and species	$^{14}CO_2$ Fixation		Malate decarboxylation		Aspartate decarboxylation	
	Control	+3-MPA	Control	+3-MPA	Control	+3-MPA
NADP-ME Type						
Digitaria sanguinalis	31	31(0)	176	173(2)	56	57(0)
NAD-ME Type						
Panicum miliaceum	140	136(3)	220	223(0)	353	345(2)
PEP-CK Type						
Brachiaria xantholeuca	75	74(1)	165	0(100)	179	24(87)
Eriochloa borumensis	241	239(1)	502	0(100)	281	65(77)
Panicum molle	66	64(3)	117	0(100)	139	25(82)
Urochloa bulbodes	141	148(0)	235	0(100)	208	37(82)

[a] Experimental conditions and substrate concentrations used were as described in **Materials and methods.** 3-MPA, 0.2 mM.

The rates are expressed as μmoles $^{14}CO_2$ fixated or C_4 acid decarboxylated/mg chl·hr.

The values in parentheses indicate percent inhibition by 3-MPA.

electron transport. However, in the presence of malate, DCMU partly inhibited CO_2 fixation roughly in proportion to the rate of CO_2 fixation with bicarbonate alone. This is expected if DCMU inhibits photochemical production of NADPH but not NADPH generated from NADP-ME. Oxalic acid did not inhibit CO_2 fixation with bicarbonate alone but gave substantial inhibition of CO_2 fixation in the presence of malate (Table 2). The inhibition is proportional to the malate dependent stimulation of CO_2 fixation suggesting that oxalate inhibits by preventing malate decarboxylation as a source of reducing power. When both oxalate and DCMU were added together, conditions which should prevent NADP reduction either through NADP-ME or photochemically, there was little CO_2 fixation by the bundle sheath strands. These results suggest that the NADPH supplies by photosystem II and NADP-ME are independent and additive in their effect.

Recently evidence was presented that 3-mercaptopicolinic acid (3-MPA) is a specific inhibitor of PEP-CK in C_4 plants (*12*, *14*). Table 3 shows the effect of 3-MPA on the photosynthetic activities of the bundle sheath strands of various types of C_4 plants. 3-MPA had no effect on the light-dependent $^{14}CO_2$ fixation activities of the bundle sheath strands from species representing the three C_4 groups. 3-MPA did not affect the C_4 acid decarboxylation activities of bundle sheath strands of NADP-ME and NAD-ME type C_4 species. However, in PEP-CK species, it completely inhibited the decarboxylation of malate, while causing only a 77–87% inhibition of aspartate decarboxylation by bundle sheath strands. 3-MPA also almost completely inhibited the decarboxylation of malate by the bundle sheath chloroplasts of PEP-CK species, while it had no effect on the malate decarboxylation activity of bundle sheath chloroplasts from NADP-ME species (data not shown). There was no C_4 acid decarboxylation activity of aspartate or malate by bundle sheath chloroplasts of NAD-ME species, and there was also no aspartate decarboxylation by the bundle sheath chloroplasts of any of the species examined.

The complete inhibition of malate decarboxylation by 3-MPA in bundle sheath chloroplasts indicates that the decarboxylation was through PEP-CK only and is consistant with the chloroplastic localization of PEP-CK (*10*). A 77 to 87% inhibition by 3-MPA of aspartate decarboxylation by strands of PEP-CK species suggests that in these species from 13 to 23% of the aspartate decarboxylation is through an alternate decarboxylase, probably NAD-ME. These results support our earlier observation on 3-MPA inhibition with *E. borumensis* suggesting about 75% of the aspartate decarboxylation is through PEP-CK (*12*). Considering malate and aspartate together it follows that about 90% of the total decarboxylation capacity of bundle sheath cells in the PEP-CK species examined is through PEP-CK.

That aspartate decarboxylation by bundle sheath strands of *D. sanguinalis* and *E. borumensis* which is insensitive to 3-MPA or oxalic acid may be through another decarboxylase such as NAD-ME. Alternatively, with aspartate conversion to oxaloacetate there may be some nonenzymatic decarboxylation of oxaloacetate. To further clarify the relative roles of various decarboxylases in C_4 species a relatively specific inhibitor of NAD-ME would be quite useful.

Conclusions

Oxalate and 3-MPA appear to be specific inhibitors of NADP-ME and PEP-CK-dependent C_4 acid decarboxylations in C_4 plants respectively. The inhibitors

help to distinguish different decarboxylase systems operating in C_4 plants. It is concluded that in PEP-CK species malate decarboxylation and 75–90% of aspartate decarboxylation is through PEP-CK. About 10–25% of aspartate decarboxylation in PEP-CK species is thus suggested through their NAD-ME system. In NADP-ME species malate decarboxylation is solely through NADP-ME, while aspartate decarboxylation may proceed through the NAD-ME system. In NAD-ME type C_4 species both malate and aspartate decarboxylations may occur only through NAD-ME as no other decarboxylase has been found in this C_4 group.

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