DISTRIBUTION OF CARBOXYLATION AND DECARBOXYLATION ENZYMES IN ISOLATED MESOPHYLL CELLS AND BUNDLE SHEATH STRANDS OF C_4 PLANTS

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SUMMARY: Mature leaves of *Cyperus rotundus*, *Cyperus polystachyos*, *Digitaria decumbens*, and *Digitaria sanguinalis* were separated, using pectinase and cellulase, into pure preparations of mesophyll cells and bundle sheath strands. Assays on these distinct leaf cell types show a clear compartmentation of phosphoenolpyruvate carboxylase, >98%, into mesophyll cells and of ribulose-1, 5-diphosphate carboxylase and malic enzyme, >98%, into the bundle sheath strands. The results clearly establish that the major CO_2 uptake in mesophyll cells is via a β-carboxylation and that both a decarboxylation and a carboxylation reaction occurs in the bundle sheath strands of plants using C_4-dicarboxylic acid photosynthesis.

The successful separation of leaf mesophyll cells and bundle sheath cells from crabgrass, *Digitaria sanguinalis*, has allowed incisive studies to be conducted on C_4-dicarboxylic acid (C_4) photosynthesis in these two distinct cell types found in leaves of C_4 plants (1,2). Studies with crabgrass conducted over several years comparing mesophyll cell enzyme activities with bundle sheath cell activities have shown that from 80 to 99% of the phosphoenolpyruvate (PEP) carboxylase can be localized in the mesophyll cells while 80 to 99% of the ribulose-1,5-diphosphate (RuDP) carboxylase and 95 to 99% of the malic enzyme can be localized in the bundle sheath cells. These enzyme studies, plus supporting studies on $^{14}CO_2$ fixation with these cell types, have allowed the proposal of a scheme for C_4 photosynthesis where the major pathway for CO_2 assimilation in C_4 leaves is via carboxylation of PEP in the mesophyll cells followed by the translocation of a C_4 acid to the bundle sheath cell where decarboxylation occurs. There the CO_2 is carboxylated by RuDP carboxylase and the reductive pentose cycle reduces the carbon to the level of sugars in the bundle sheath cells (2). Several groups of workers have presented a reasonably

similar scheme for C_4 photosynthesis supported by various types of experimental data using leaves of other C_4 plants (3).

Recently four laboratories have presented data from which they drew conclusions casting doubt on this compartmentation of specific portions of C_4 photosynthesis into mesophyll and bundle sheath cells. Two groups of workers have used a progressive leaf grinding technique to prepare specific cell fractions (4,5). Progressive grinding is a procedure wherein a leaf is ground with increasing force and fractions are removed at various times. The rationalization is that as increasing force is applied to a leaf, one will rupture sequentially and release the contents of epidermal cells, then mesophyll cells, and then bundle sheath cells. Based upon enzyme assays it was proposed that PEP carboxylase was in epidermal bulliform cells and in the cytoplasm of mesophyll cells while RuDP carboxylase was localized in mesophyll chloroplasts. The bundle sheath cell chloroplasts were considered not to be active photosynthetically except to synthesize starch from sucrose (4). Another worker has stated that the RuDP carboxylase is equally distributed in the bundle sheath and mesophyll cells (5). Using tissue callus cultured from stem explants of a C_4 plant, products of $^{14}CO_2$ fixation have been found which are somewhat similar to $^{14}CO_2$ uptake studies with C_4 leaves (6). This stem callus tissue culture work was extrapolated to fully-differentiated leaves of C_4 plants to state that "both carbon-fixation pathways could take place in mesophyll cells" and to conclude that the bundle sheath chloroplasts are the functional amyloplasts of leaves (6). A fourth group of workers conducted microradioautography after exposing leaves to $^{14}CO_2$ for 70 seconds. Since the majority of the silver grains seemed to be deposited in the bundle sheath cells it was stated that the C_4 pathway of "photosynthesis occurs primarily in the bundle sheath cells" (7).

The techniques for leaf cell isolation have been under examination for several years in this laboratory as have procedures for assaying leaf enzymes. This communication will present further refinements on leaf cell separation and

isolation and the results of additional improvements in enzyme assays. Enzyme activity data will be presented on four C_4 plants from which we now can obtain pure preparations of mesophyll cells and bundle sheath strands.

MATERIALS AND METHODS: The cell isolation procedures described in detail previously (2) were used with the following modifications. The leaves were cut into 1 mm sections and vacuum infiltrated with the following medium adjusted to pH 5.0: 1% cellulysin (cellulase) and 1% macerase (pectinase, Calbiochem); 2% polyvinylpyrrolidone-40 (PVP-40, Sigma); 0.33 M sorbitol; 2 mM $NaNO_3$; 2 mM EDTA; 1 mM $MgCl_2$; and 5 mM K_2HPO_4. The infiltrated leaf sections were kept under vacuum and incubated at 37-38° C one hour, then ground gently with an ice-chilled mortar and pestle in the medium above except omitting both enzymes and adding 0.05 M tricine-NaOH, pH 8.0. Mesophyll cells and bundle sheath strands were collected and washed thoroughly on a 20 μ and 80 μ nylon screen, respectively. A Ten-Broeck glass grinder was used to remove the few mesophyll cells still attached to bundle sheath strands. The cell isolation and purity were monitored with a light microscope (2).

Preparation of crude leaf extracts and isolated cell extracts. Leaf sections, 1 to 2 mm long, were ground in an ice-chilled mortar and pestle in a medium containing 0.1 M tris-HCl, pH 8.0, together with various additives such as PVP-40, β-mercaptoethanol, thioglycolate, or dithiothreitol. Homogenates were passed through a 20 μ nylon screen to eliminate any unbroken cells and the filtrates were used as crude leaf enzyme extracts.

Isolated mesophyll cells and bundle sheath strands were suspended in 1 to 2 ml of 0.1 M tris-HCl, pH 8.0, containing 2% PVP-40, and 10 mM β-mercaptoethanol. Both types of cell were frozen in liquid nitrogen, then ground in a mortar, taken up in 2 to 5 ml of the same medium, and filtered through a 20 μ nylon screen. PEP carboxylase, RuDP carboxylase, malic enzyme and chlorophyll were assayed by established procedures (8,9), using the filtrates.

RESULTS AND DISCUSSION

A common laboratory observation with many plants is the browning of crude

TABLE I. The Effect of Polyvinylpyrrolidone and Reducing Reagents, Alone or in Combination, on Enzyme Activities of Whole Leaf or Isolated Cell Extracts from *Cyperus rotundus*.

	Treatments*	PEP Carboxylase	RuDP Carboxylase	Malic Enzyme
		μmoles/mg chl/hr		
Leaf	PVP	778	133	112
	β-ME	437	72	106
	PVP + β-ME	1275	253	175
	DTT	386	79	131
	PVP + DTT	1395	228	192
Mesophyll Cells	β-ME	1160	8	4
	PVP + β-ME	2100	5	8
Bundle Sheath Strands	β-ME	36	520	174
	PVP + βME	27	523	293

*2% PVP-40 and 10 mM reducing reagent.

leaf extracts upon leaf maceration which often results in loss of enzyme activities. Preliminary observations indicated that the inclusion of PVP or dithiothreitol in the *Cyperus rotundus* (nutsedge) leaf extracts prevented this browning for more than 24 hours at room temperatures. This result prompted us to test the effectiveness of the reducing agents β-mercaptoethanol, thioglycolate and dithiothreitol alone or in combination with PVP in overcoming the inactivation of enzymes in leaf extracts. It was found that 2% PVP-40 together with one of the reducing agents, at 10 mM, was the most effective (Table I). In the absence of these protective reagents little enzyme activity could be detected with nutsedge. The difference in this effect among the three reducing agents was small except with thioglycolate which seemed to be ineffective or inhibitory when assaying PEP carboxylase. Therefore, 2% PVP-40 and 10 mM β-mercaptoethanol were selected and routinely used in the extraction of enzymes.

TABLE II. The Distribution of Enzyme Activities in Leaves and Isolated Mesophyll Cells and Bundle Sheath Strands.

Source of Extract	PEP Carboxylase	RuDP Carboxylase	Malic Enzyme
		µmoles/mg chl/hr	
		Cyperus rotundus	
Leaf	1275	253	175
Mesophyll Cells	2100	5	8
Bundle Sheath Strands	27	523	322
		Cyperus polystachyos	
Leaf	1120	150	170
Mesophyll Cells	1600	<3	<1
Bundle Sheath Strands	38	373	370
		Digitaria sanguinalis	
Leaf	475	170	400
Mesophyll Cells	1220	24	42
Bundle Sheath Strands	22	450	845
		Digitaria decumbens	
Leaf	278	117	310
Mesophyll Cells	626*	16	22
Bundle Sheath Strands	22	560	1000

*Assayed in isolated cells.

As a refinement on the mechanical separation of leaf cells, the use of pectinase and cellulase enabled the "peeling" of epidermal strips away from other cells during a gentle grinding of leaf sections. Subsequent filtration through 20 mesh steel screen and 210 µ nylon screen eliminated practically all of the epidermal strips, therefore, epidermis contamination was almost absent. Furthermore this enzyme cell separation procedure reduced the force required to grind leaf sections and hence reduced the release of bundle sheath cells into mesophyll cell preparations. In the case of nutsedge and *Cyperus polystachyos*, the presence of a secondary colorless bundle sheath layer (10) outside

the regular green layer further eliminated such contamination. Only mesophyll cell or bundle sheath strand preparations which were judged by microscopy to be uncontaminated were used in this study to obtain the results in Table II.

In crude leaf extracts from the four plants in Table II PEP carboxylase, RuDP carboxylase, and malic enzyme are active within the approximate range of leaf net photosynthesis, about 200 to 400 μmoles of CO_2 fixed/mg chl/hr, commonly observed with these plants. However, leaf specific activities are not necessarily near cellular specific activities as seen in the enzyme activities in the separated cell types in Table II. With each of the three enzymes assayed in these C_4 plants, the cell specific activity is quite different from the crude leaf extracts. Clearly this is a reflection of the fact that the enzymes are compartmentalized into specific cell types in these fully-differentiated leaves. Hence in the leaf cell types from these four C_4 plants the PEP carboxylase is localized, >98%, in the mesophyll cells, while the RuDP carboxylase and malic enzyme are localized, >98%, in the bundle sheath cells (Table II).

Therefore we conclude that there remains little doubt that mature leaves of C_4 plants have compartmentalized their enzyme activities into specific cell types and that schemes such as those initially proposed for crabgrass and sugar cane (2,3) are correct as based upon such data as that in Table II. Also, the suggestion that enzyme activities in crabgrass cells and in other C_4 plant work have been underestimated due to improper protection from leaf inactivating compounds (4,5), can be considered incorrect. The work on progressive grinding (4,5) is quite empirical and it has not been supported by proof of chloroplast separation nor can other workers verify their findings (11). In the micro-radioautography work (7) a 70 second exposure to $^{14}CO_2$ is well within the time required for the diffusion of photosynthetic products to the bundle sheath cells. The activity of the green mesophyll cells was not even considered. Finally work with nondifferentiated stem callus tissue cultures (6) can not be extrapolated to work such as that in Table II with cells isolated from fully-differentiated leaves.

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REFERENCES:

1. Edwards, G. E., Lee, S. S., Chen, T. M., and Black, C. C., Biochem. Biophys Res. Comm. 39, 389 (1970).
2. Edwards, G. E., and Black, C. C., Plant Physiol. 47, 149 (1971).
3. Hatch, M. D., Osmond, C. B., and Slatyer, R. O., Eds. 1971. Photosynthesis and Photorespiration, Wiley-Interscience, New York.
4. Coombs, J., and Baldry, C. W., Nature 238, 268 (1972).
5. Poincelot, R. P., Plant Physiol. 50, 336 (1972).
6. Laetsch, W. M., and Kortschak, H. P., Plant Physiol. 49, 1021 (1972).
7. Bednarz, R. M., and Rasmussen, H. P., J. Expt. Bot. 23, 415 (1972).
8. Chen, T. M., Brown, R. H., and Black, C. C., Plant Physiol. 47, 199 (1971).
9. Wintermans, J. F. G. M., and De Mots, A., Biochim. Biophys. Acta 109, 448 (1965).
10. Black, C. C., and Mollenhauer, H. H., Plant Physiol. 47, 15 (1971).
11. Huang, A. H. C., and Beevers, H., Plant Physiol. 50, 242 (1972).