

Biochemical Specialization of Photosynthetic Cell Layers and Carbon Flow Paths in *Suaeda monoica*

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ABSTRACT

Suaeda monoica Forssk. ex J. F. Gmel is a C_4 plant with three different photosynthesizing cell layers. The outer chlorenchymatous layer shows a high activity of phosphoenolpyruvate (PEP) carboxylase but none of ribulose biphosphate (RuBP) carboxylase. The electrophoretic protein band of RuBP carboxylase was missing in this layer. The second chlorenchymatous cells layer shows a very high activity of RuBP carboxylase and NAD malic enzyme and only traces of activity of PEP carboxylase. The third photosynthesizing cell type is comprised of the water tissue. It has moderate activities of RuBP carboxylase and PEP carboxylase. A model for carbon flow in *Suaeda monoica* leaves is proposed.

It is commonly accepted that most of the C_4 plants are distinguished by specialized leaf anatomy and compartmentations of enzymes (2, 7, 8). The mesophyll cells show high activity of PEP-C¹ but none of RuBP-C, whereas the bundle sheath cells show high activity of RuBP-C and a decarboxylating enzyme but a very low activity of PEP-C. This arrangement permits a spatial operation of the C_4 cycle and the C_3 photosynthetic pathway (2, 5, 7-9). However, there are many C_4 plants with a different anatomy. *Suaeda monoica* Forssk ex. J. F. Gmel is among those exceptional plants having the C_4 photosynthetic pathway without possessing a typical bundle sheath (11). Three different types of photosynthesizing cells can be distinguished in the succulent leaves of *Suaeda*: (a) an outer layer of cells containing relatively small chloroplasts; (b) an inner layer of cells which are packed with large chloroplasts and mitochondria; (c) a parenchymatous water tissue with few scattered chloroplasts in its cells (11). The question whether the three cell layers are physiologically differentiated was investigated.

MATERIALS AND METHODS

S. monoica Forssk ex. J. F. Gmel was grown in a greenhouse in a sand culture and irrigated with Hoagland solution with 100 mM NaCl.

The structure of the three cell layers was investigated using an electron microscope. Leaf sections were fixed in 2% glutaraldehyde in 0.15 M phosphate buffer (pH 7.2) and postfixed in 1% OsO₄ in the same buffer. After dehydration in ethanol the sections were embedded in Epon 812. Activity and localization of the carboxylating (PEP-C and RuBP-C) and of the decarboxylating (NAD-malic enzyme) enzymes was tested in extracts of cell layers which

were mechanically separated. Green succulent mature leaves taken from 3- to 4-year-old plants were used for cell layers separation.

ENZYME EXTRACTION OF THE SEPARATE CELL LAYERS

Extraction of Enzymes of Outer Chlorenchymatous Layer. The epidermal layer of *Suaeda* leaves was stripped off. The stripping of the epidermis tore the cells of the outer chlorenchymatous layer in their middle. Such strips containing part of the injured cells' protoplasts were washed in buffer and the eluted proteins were then precipitated with ammonium sulfate. In some cases the ripped cells' strips were washed straight with specific cold enzyme reaction mixture and then the elution was analyzed for the enzyme activity.

Separation of Inner Chlorenchymatous Layer. The epidermis was stripped off the leaves, and then the remainders of the outer injured chlorenchymatous cells were removed from the stripped leaves by washing. Sections, one cell layer thick, of the inner chlorenchymatous layer were then cut, washed, and frozen on dry ice. All sections were checked microscopically to be one layer thick. PEP-C activity in this layer may partly belong to remainders of adhering broken cells.

Separation of Water Tissue. The outer and inner chlorenchymatous layers were cut off from all sides of the succulent leaves. Clean cubes of the remaining water tissue were then collected and frozen on dry ice.

Enzyme activity was assayed with extracts of these samples. Extraction procedure for enzymes was followed as described by Beer *et al.* (1). Activity of RuBP-C (EC 4.1.1.39) was assayed according to Buchanan and Schurmann (3). Activity of PEP-C (EC 4.1.1.31) was tested as described by Beer *et al.* (1). NAD-malic enzyme (EC 1.1.1.38) was extracted and assayed as described by Hatch and Kagawa (6). Mn was the only activator used.

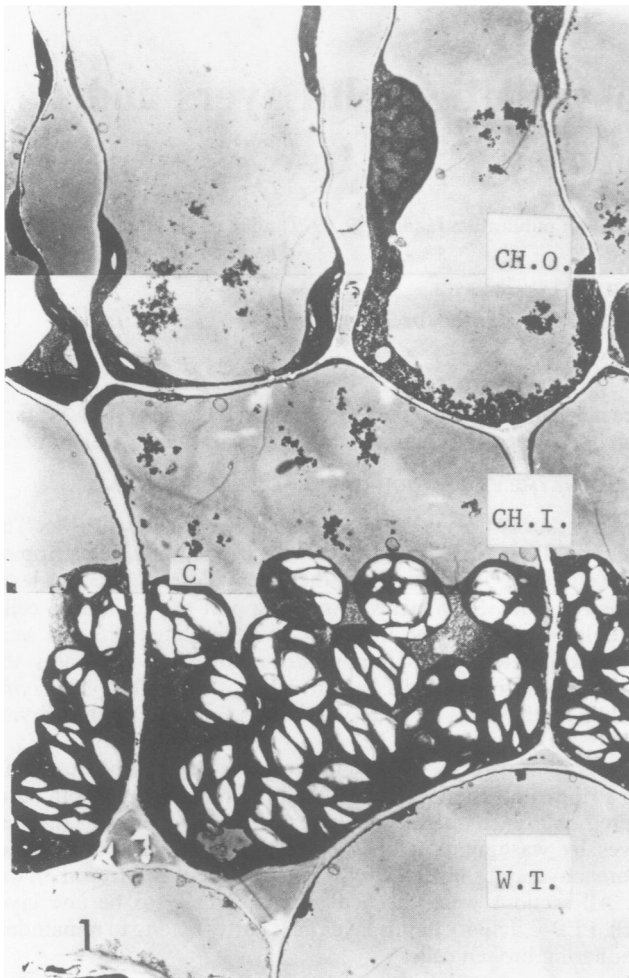
Identification of RuBP-C. Electrophoretic assays for RuBP-C were run in a Tris-glycine (pH 8.9) electrophoretic system on 5% acrylamide gel (4). An ammonium sulfate protein fraction (30-40%) was used when a whole leaf extract was assayed and a crude extract when the different cell layers were analyzed. Eluted protein bands were identified as RuBP-C by Ouchterlony immunodiffusion reaction (10) with specific prepared antibodies.

Preparation of Specific Antibodies. RuBP-C was purified by gel filtration chromatography (12), analyzed for activity and electrophoresed on 5% acrylamide gels. The pure bands were eluted and injected with Freund's adjuvant to rabbits (10).

RESULTS AND DISCUSSION

Electron micrographs show that chloroplasts of all three layers have grana and accumulated starch (Figs. 1 and 2). The relatively large mitochondria of the inner chlorenchymatous layer are rich in cristae (Fig. 3). Chloroplasts and mitochondria in the inner layer are centripetally arranged (Fig. 2). The activity and localization of the carboxylating and decarboxylating enzymes is summarized in Table I.

¹ Abbreviations: PEP-C: phosphoenolpyruvate carboxylase; RuBP-C: ribulose biphosphate carboxylase; ME: malic enzyme; PGA: phosphoglyceric acid.



FIGS. 1, 2, and 3. Electron micrographs of *S. monoica* leaf.

FIG. 1. Differentiated photosynthesizing cells; notice chloroplasts dimorphism. CH.O: outer chlorenchymatous cells layer; CH.I.: inner chlorenchymatous cells layer; W.T.: water tissue. ($\times 3,700$).

The data reveal a very high activity of PEP-C but no activity of RuBP-C in the outer chlorenchymatous layer. This is similar to the characteristics of the mesophyll cells in the classic C_4 plants (2, 7). The inner chlorenchymatous layer showed almost no activity of PEP-C but a very high activity of RuBP-C. This is similar to the characteristics of the bundle sheath cells in the classic C_4 plants (2, 7). The water tissue cells showed moderate activity of both enzymes with similar rates.

The lack of RuBP-C activity in the outer chlorenchymatous layer could have resulted either because of the absence of the enzyme or because of its inactivity *in situ* or *in vitro*. The answer to this question was found when the protein patterns of the inner and outer chlorenchymatous layers were compared.

The electrophoretic pattern exhibited by the proteins of the inner layer, *i.e.* the one with high activity of RuBP-C, reveals a thick protein band, which reacts with antibodies against RuBP-C. This protein band is missing in the electrophoretic protein pattern of the outer chlorenchymatous layer. Thus, the lack of RuBP-C activity in this outer layer is not an artifact and seems to result from the lack of the enzyme protein.

A high activity of the decarboxylating enzyme NAD-malic enzyme was found in the inner chlorenchymatous layer, a layer rich in mitochondria with very developed cristae (Fig. 3 and Table I). This is similar to the characteristics of bundle sheath cells in the classic NAD-type C_4 plants (5, 6, 9).

The water tissue cells showed a low activity of NAD-malic enzyme, similar to activity rates of this enzyme in C_3 plants (5, 6, 9).

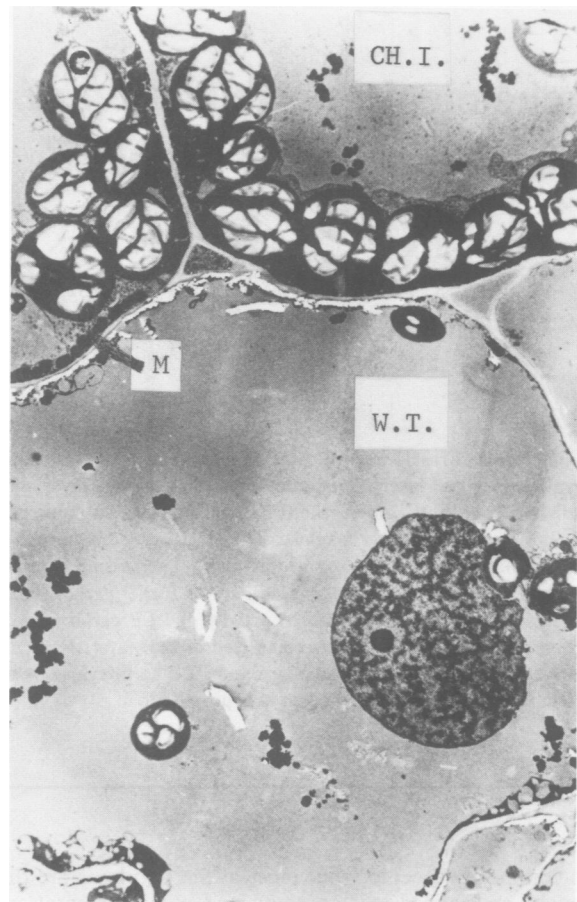


FIG. 2. Notice centripetal arrangement of chloroplasts and mitochondria in the inner chlorenchymatous layer. M: mitochondria. ($\times 1,800$).

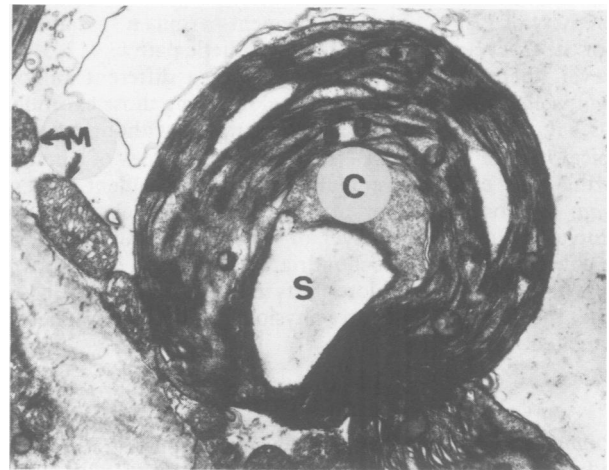


FIG. 3. Chloroplast with grana and mitochondria with developed cristae in the inner chlorenchymatous layer. M: mitochondria; C: chloroplast; S: starch. ($\times 12,000$).

Table I. Location and Activity of Carboxylating and Decarboxylating Enzymes

Enzyme	Activity ^a			
	Whole leaf	Ch.O	Ch.I	W.T.
RuBP-C	0.6 ± 0.2^b	0	4.2 ± 0.2	0.48 ± 0.27
PEP-C	0.7 ± 0.3	0.9 ± 0.3	0.03 ± 0.02	0.45 ± 0.2
NAD-ME	5.0 ± 0.8	—	3.5 ± 0.2	0.5 ± 0.15

^a Abbreviations same as in figure legends. Activity in $\mu\text{mol mg protein}^{-1} \text{ min}^{-1}$.

^b \pm SE.

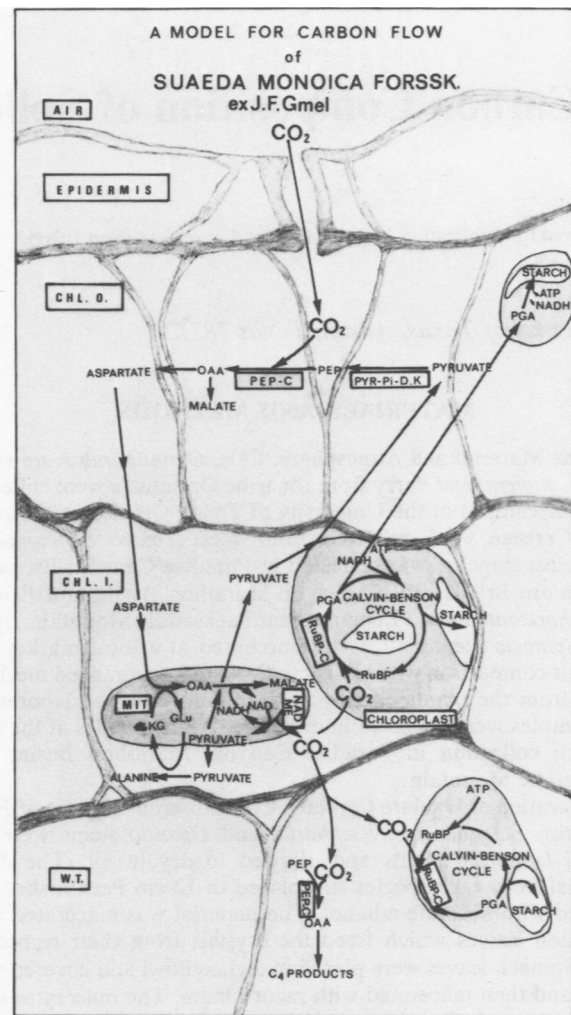


FIG. 4. A suggested model for carbon flow. PyR-Pi-D.K.: pyruvate-P-dikinase; KG: α -ketoglutarate; Glu: glutamate.

The above information enables us to suggest the following working model for carbon flow in *S. monoica* (Fig. 4).

Atmospheric CO_2 is fixed in the outer chlorenchymatous layer by PEP-C. The first major stable product in this cell layer, aspartate (11), is supposed to be transported to the inner chloren-

chymatous layer of the leaf. In the "specialized" mitochondria of that layer aspartate is transaminated and then decarboxylated by NAD-ME. Part of the released CO_2 is refixed in the chloroplasts of the same cell layer by RuBP-C and further reduced to carbohydrates by the Calvin-Benson cycle. The other part of the released CO_2 is diffusing into the water tissue. In this layer, CO_2 which originates from respiration, photorespiration, and aspartate decarboxylation, may be reassimilated either by RuBP-C in the chloroplast to PGA and further to carbohydrates, or by PEP-C to C_4 end products. The centripetal arrangement of the mitochondria in the inner chlorenchymatous layer (Fig. 2) may cause high CO_2 diffusion into the water tissue. β -Carboxylation in this water tissue may be responsible for carbon flux into organic acids, amino acids, and betaine (13) and thus contribute to the osmotic adjustment of this halophyte. Pyruvate-P-dikinase, the enzyme responsible for PEP formation in C_4 plants, should in this case be found in the outer chlorenchymatous layer.

The existence of many species of the Chenopodiaceae with anatomical features resembling those of *S. monoica* and with similar "heavy" $\delta^{13}\text{C}$ values (unpublished data) leads us to assume that a carbon flow as the one suggested for *S. monoica* is not a unique phenomenon. It seems to represent a type of C_4 plant with a characteristic anatomy, which may reflect similar biochemical specialization and carbon flow.

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