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Photosynthetic Carbon Metabolism in the Palisade Parenchyma and Spongy Parenchyma of *Vicia faba* L.[1]

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ABSTRACT

Palisade parenchyma cells and spongy parenchyma cells were isolated separately from *Vicia faba L.* leaflets. Extracts of the cell isolates were assayed for several enzymes involved in CO_2 fixation and photorespiration. When compared on a chlorophyll basis, the levels of enzyme activities either were equal in the different cell types or were greater in the spongy parenchyma; this difference is a reflection, perhaps, of the higher protein-chlorophyll ratio in the latter tissue. The distribution of radioactivity in the products of photosynthesis by each cell type was the same at various times after exposure to $NaH^{14}CO_3$, and the kinetics of ^{14}C incorporation into these compounds was similar. However, a larger percentage of radioactivity was incorporated by the cell isolates into the 80% ethanol-insoluble fraction and correspondingly less into the neutral fraction as compared to whole leaf. It was concluded that photosynthetic CO_2 fixation is similar in the different mesophyll tissues from which these cells were derived.

Compartmentation of photosynthetic processes between the mesophyll and bundle sheath cells of C_4 plants has been partially elucidated (6, 15). C_3 mesomorphic dicotyledon leaves also have distinct photosynthetic tissues which may have different functions within the leaf. Because palisade parenchyma cells have a larger surface area and more chloroplasts than spongy parenchyma cells, and because the palisade parenchyma occupies a position near the upper surface of the leaf which intercepts the light, early anatomists (*e.g.* 12) concluded that the palisade parenchyma was the major photosynthetic tissue and suggested that the spongy parenchyma might serve as an intermediary tissue for translocating assimilates to the veins. The surface area of palisade cells is 1.6 to 3.5 times that of spongy cells on a volume basis (35), and the developmental pattern of photosynthesis is similar to that of the palisade cell surface area and Chl content (30). Leaves of some species respond readily to high light during their development by producing several layers of palisade parenchyma (14), while other species require drastic differences in light intensity to show a response (13). Some of the data of Mokronosov *et al.* (22) support the classical conclusion that the palisade parenchyma is the major photosynthetic tissue, whereas microautoradiographs of Moss and Rasmussen (23) do not.

The kinetic data of ^{14}C incorporation into glycine and serine were markedly different in extracts of shavings taken from the upper ("palisade") and lower ("spongy") surfaces of frozen coltsfoot leaf which had been exposed to $^{14}CO_2$ before freezing (22). Outlaw and Fisher (25) found that the percentage of ^{14}C in ths combined glycine and serine fraction from extracts of microtome sections of palisade parenchyma after pulse-labeling a *Vicia faba* leaf was smaller than in sections of spongy parenchyma. Moreover, differences in the internal organization of microbodies from the different mesophyll cell types have been observed (9). These observations suggest the possibility of differences in photorespiration. We have isolated palisade parenchyma cells and spongy parenchyma cells from *V. faba* leaflet, and determined levels of certain enzyme activities involved in photosynthetic carbon metabolism and the kinetics of photosynthetic ^{14}C incorporation into various compounds.

MATERIALS AND METHODS

Plant Material. *V. faba* L. plants were grown on Hoagland solution in a soil-vermiculite mixture in a growth cabinet, set on a 14-hr day at 21 C and 15 C at night. Irradiance from incandescent and fluorescent bulbs was 4000 ft-c at plant level.

Cell Preparation. Young, fully expanded leaves were used from 3- to 5-week-old plants. About 2.5 g of leaflet, from which the lower epidermis had been stripped, were placed in a 125-ml side arm flask and vacuum-infiltrated (300 mm Hg, minimum pressure) with 20 ml of maceration medium. The medium was similar to that used by others (7, 17, 32) and contained 0.8 M sorbitol, 20 mM K_2SO_4, 1 mM KNO_3, 0.2 mM KH_2PO_4, 0.2 mM $MgSO_4$, 1 mM $CaCl_2$, 1 μM KI, 0.01 μM $CuSO_4$, 300 μg/ml Cephaloradine (E. Lilly). The medium was adjusted to pH 5.8 before adding 1% (w/v) Macerase (Calbiochem). The stripped leaflets were incubated at 30 C in this solution on a shaker bath with 56 excursions of 2.5 cm each/min, while receiving 1000 ft-c of illumination from a reflector flood lamp behind a 20-cm circulating water bath. After 1.5 hr of maceration, the released cells were isolated by filtering the solution through a 295-μm nylon net (Tetko, Inc., Elmsford, N. Y.), which retained the undigested areas of the leaflet. The isolated cells were concentrated by low speed centrifugation in a clinical centrifuge for 1 min. Most chloroplasts from ruptured cells remained in the supernatant. In some preparations, the isolated cells were washed free of remaining chloroplasts on a 20-μm nylon net. By phase contrast microscopic examination, these isolated cells were primarily spongy parenchyma. Preparations were routinely monitored for purity and no spongy parenchyma cell preparation which contained more than 5% palisade parenchyma cells was used.

Fresh maceration medium was added to the undigested areas of the leaflet which had remained on the 295-μm nylon set. Maceration was continued for an additional hr. Then, the leaf material was transferred to a Petri dish and the vascular nets

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were peeled off leaving only the upper epidermis attached to palisade cells. The vascular nets and released cells were discarded. The upper epidermis and adhering palisade parenchyma cells were transferred to a flask containing fresh maceration medium with 1% (w/v) Pectinase (Sigma) instead of Macerase and incubated for 1.5 hr. After this final digestion, the palisade cells were collected as described for the spongy cells, with the intact upper epidermis being retained on the 295-μ nylon net. Microscopic examinations of cell purity were always made.

Enzyme Extraction. Cells containing about 150 μg of Chl were suspended in 10 ml of grinding buffer containing 0.1 M tris-HCl (pH 8), 1% PVP 40, and 10 mM 2-mercaptoethanol (8), and immediately centrifuged at maximum speed in a clinical centrifuge for 30 sec. The top 8.5 ml of the buffer were moved with a Pasteur pipette and discarded. The cell pellet and remaining 1.5 ml of buffer were ground with sand in a mortar and pestle. A similar ratio of Chl to grinding buffer was used to extract enzymes from whole leaflet tissue. When the homogenate was used for RuBP[4] carboxylase and RuBP oxygenase assays, the grinding buffer was changed to 100 mM HEPES (pH 7.6), 4.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.5 mM ribose-5-P.

Enzyme Assays. Glycolate oxidase activity was determined by measuring glycolate-dependent O_2 uptake in 100 mM tris at pH 8. RuBP oxygenase was assayed by the rate of O_2 uptake from air (2) and RuBP carboxylase by the rate of $^{14}CO_2$ fixation (36). The carboxylase reaction was initiated by the addition of $NaH^{14}CO_3$. Phosphate release was measured in the assays for fructose-1,6-diphosphatase (3), glycerate-3-P phosphatase (26), glycolate-P phosphatase (1). References for other assays are: fructose-1,6-diP aldolase (29), except the pyridine nucleotide concentration was 6-fold lower; glyceraldehyde-3-P dehydrogenase, and glycerate-3-P kinase (18), except the ATP concentration in each was only 0.5 mM; hydroxypyruvate reductase (33) using glyoxylate as the substrate and using an absorbancy change of 1 unit/161 nmol; malate dehydrogenase (33); glutamate-glyoxylate aminotransferase (33); and aspartate aminotransferase (33). Phosphoenolpyruvate carboxylase was assayed by determining the incorporation of $H^{14}CO_3$ into acid-stable products in a reaction mixture of 50 mM tris (pH 8.3), 10 mM $MgCl_2$, 4 mM dithiothreitol, 20 mM monosodium glutamate, 0.5 unit glutamate-oxaloacetate aminotransferase, 12 mM $NaH^{14}CO_3$, and 10 mM P-enolpyruvate.

All enzyme assays were repeated on extracts from four different cell preparations from different plants. The results are expressed as an average of the two median determinations.

Protein was determined by Geiger and Bessman's modification (10) of the Lowry procedure (20). Chl was determined according to the method of Strain *et al.* (31).

Products of Photosynthesis. Three ml of a cell suspension representing about 90 μg of Chl in a buffer (10 mM TES at pH 7, 0.6 M sorbitol) were incubated for about 1 hr at room temperature and 4000 ft-c. The cells were labeled by making the suspension 2 mM in $NaH^{14}CO_3$ (shifting the media pH to 7.07) and labeling was terminated by rapidly filtering the cells on a nylon screen and freezing them in liquid N_2. About 10% of the acid-stable ^{14}C products was in the medium after 1 min of fixation under these conditions as compared with 25% with cells isolated by mechanical grinding (16). It is not clear whether this difference is a result of isolation technique, photosynthetic medium, or some other parameter.

The cells were extracted in boiling 80% (v/v) aqueous ethanol for 45 min. The insoluble residue was collected by centrifugation; the supernatant was dried at 30 C under a mild vacuum and partitioned into water-soluble and petroleum ether-soluble fractions. The water-soluble extract in 25 ml was passed through a Dowex 50-X8 (H^+) column (15 × 1 cm) coupled to a Dowex 1-X8 (Formate) column (15 × 1 cm) (4). The effluent was designated the neutral fraction.

Basic compounds were eluted from the Dowex 50 column with 2 N NH_4OH, and were further separated by two-dimensional descending paper (Whatman No. 1) chromatography with 80% (v/v) aqueous phenol in the first direction and 1-butanol-propionic acid-water, 23:12:15 (v/v/v) in the second direction. Radioactivity was localized by autoradiography and labeled amino acids by co-chromatography and ninhydrin spray.

The acidic compounds were eluted with a formic acid gradient followed by a gradient of HCl similar to that of Atkins and Canvin (4). Compounds were identified by elution position compared with published profiles and the elution of ^{14}C-labeled authentic glycolic, malic, phosphoglyceric, fructose-1,6-diphosphoric, and ribose-5-phosphoric acids.

RESULTS AND DISCUSSION

Cell Preparation. The procedure described for the differential isolation of single cells from mesophyll tissues gave high yields. There was some variability in the rate of digestion which was attributed to differences in lots of Macerase and age of leaflets. Owing to this variability, microscopic observations were routinely used to monitor for cell type purity although significant contamination was rarely observed. In preparations of spongy parenchyma (Fig. 1) and palisade parenchyma (Fig. 2), chloroplasts remained regularly arranged along the cell periphery. Their appearance and the rate of photosynthesis of these preparations of 20 to 60 μmol CO_2/mg Chl/hr indicates that these cells have retained their organization during isolation. Up to 15% of the palisade cells appeared as protoplasts by the end of the digestion period. That these chlorphyllous protoplasts were derived from palisade cells was determined by examining the tissue prior to the last step of digestion.

Enzyme Composition. The enzyme profile from whole leaves of *V. faba* (Table I) was similar to that reported by Latzko and Gibbs (18) for spinach; in absolute values, the enzyme activities they report were similar to ours for fructose-1,6-diphosphatase, glyceraldehyde-3-P dehydrogenase (NADP), and RuBP carboxylase, and were higher for glycerate-3-P kinase and fructose-1,6-diP aldolase. When RuBP carboxylase was assayed in whole leaf extracts of *Vicia* by initiating the reaction with RuBP or with ribose-5-P (see 19), the observed rates were higher and were 110 and 440 μmol/mg Chl/hr, respectively (F. J. Ryan and N. E. Tolbert, unpublished). The RuBP oxygenase activity was similar to activity reported by Bahr and Jensen (5), but lower than that reported by Andrews *et al.* (2) who used 100% O_2. The activities of glycolate oxidase, hydroxypyruvate reductase, and NAD-malate dehydrogenase in whole leaf extracts are similar to those published for bean (34). Glycolate-P phosphatase and glycerate-3-P phosphatase were within the range of activities reported by Randall *et al.* (27). The activity of P-enolpyruvate carboxylase found for *Vicia* was about one-third that of spinach (21). The activity of aspartate aminotransferase in *Vicia* was lower than that of spinach (28). All enzyme activities were judged sufficient to account for whole leaf photosynthesis in *Vicia* (75 μmol CO_2/mg Chl/hr, unpublished).

From preparation to preparation, there was some variability in the total enzyme activities in extracts of whole leaves, palisade parenchyma, and spongy parenchyma. In general, there was no significant variation in these enzyme activities between the palisade and spongy parenchyma cells. Although more activity on a Chl basis was obtained from the whole leaf than from single cells for some enzymes, the differences were not great considering the wide variations in total activity among the enzymes, except for glyceraldehyde-3-P dehydrogenase. (The activities of nonchloroplastic enzymes might be expected to be larger in the whole leaf due to enrichment by the epidermises.) Enzymes localized

[4] Abbreviation: RuBP: ribulose bisphosphate.

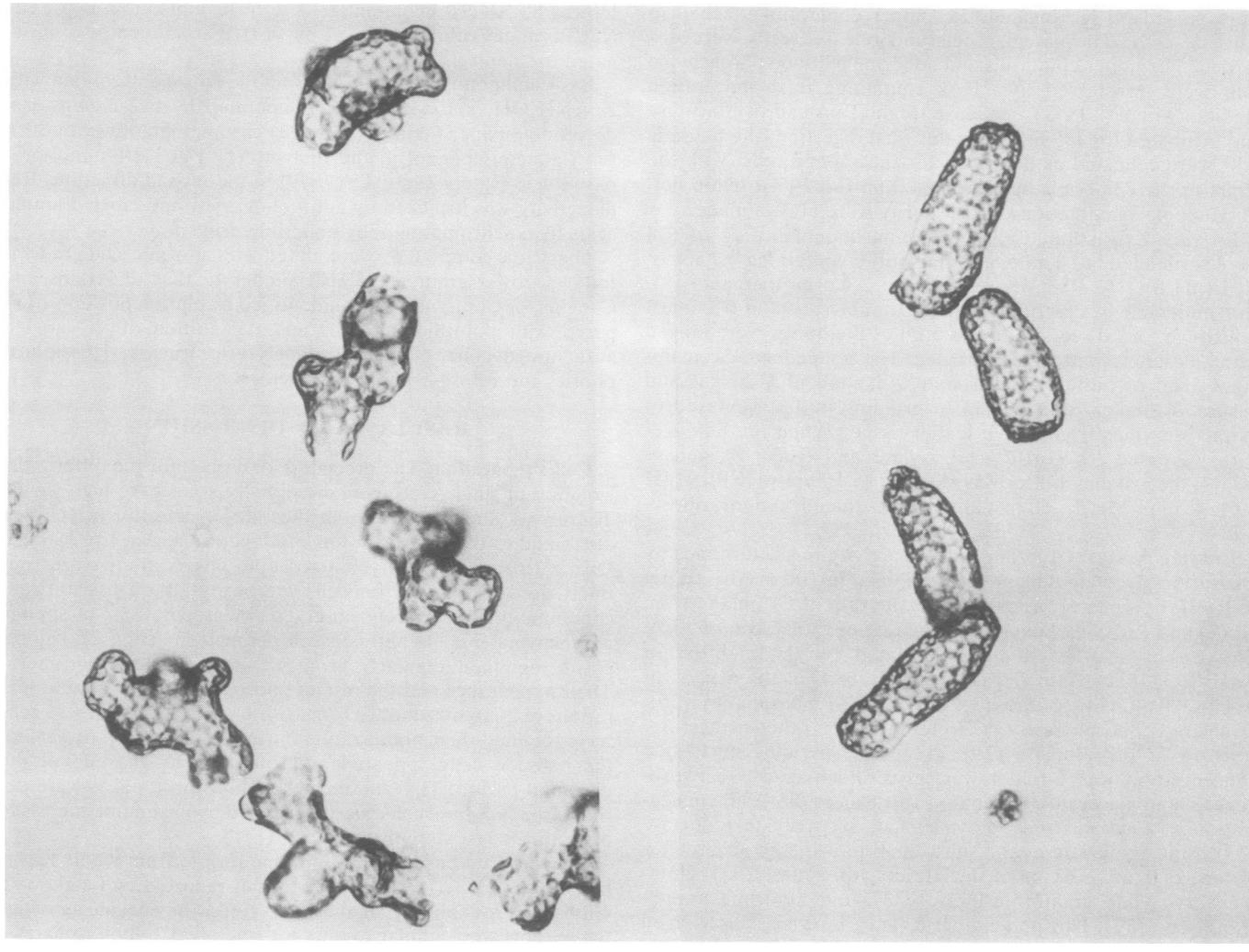

FIG. 1 (left). Typical preparation of spongy parenchyma cells. × 320.
FIG. 2 (right). Typical preparation of palisade parenchyma cells. × 320.

Table I. *Enzyme Activities in Extracts of Whole Leaves, Palisade Parenchyma Cells, and Spongy Parenchyma Cells of Vicia faba*

Enzyme	Whole Leaf	Palisade	Spongy
	μmoles/mg chl·hr		
Ribulose bisphosphate carboxylase	74	56	65
Ribulose bisphosphate oxygenase	8	6	6
Glyceraldehyde-3-P dehydrogenase (NADP)	625	242	275
Glycerate-3-P kinase	260	133	195
Fructose-1,6-diphosphatase	56	35	73
Fructose-1,6-diP aldolase	86	48	99
Phosphoglycolate phosphatase	83	65	99
Glycolate oxidase	31	28	30
Hydroxypyruvate reductase	60	69	75
3-Phosphoglycerate phosphatase	9	6	7
Malate dehydrogenase (NAD)	1592	817	1393
P-enolpyruvate carboxylase	20	17	32
Aspartate aminotransferase	31	10	93

only in chloroplasts and peroxisomes such as RuBP carboxylase/oxygenase, glycolate-P phosphatase, glyceraldehyde-3-P dehydrogenase (NADP), glycolate oxidase, and hydroxypyruvate reductase, were of the same level of activity in each tissue when based on Chl. Glutamate-glyoxylate aminotransferase activity (not reported) was greater in the spongy parenchyma, however. Some enzymes which are not localized in chloroplasts or peroxisomes (fructose-1,6-diphosphate aldolase, malate dehydrogenase [NAD], P-enolpyruvate carboxylase, and aspartate aminotransferase) were always of higher levels in the spongy parenchyma than in the palisade parenchyma when expressed on a Chl basis. However the ratio of protein-Chl (w/w) in the palisade parenchyma was 20:1 while it was 39:1 in the spongy parenchyma; the activities of these latter enzymes would appear more nearly equal in each tissue if expressed on a protein basis.

Products of $^{14}CO_2$ Fixation. At 15 sec, P-glycerate and sugar phosphates accounted for about 45% of the total ^{14}C incorporated into each mesophyll cell type (Fig. 3), and the per cent of ^{14}C represented by these compounds similarly declined with time. The per cent of ^{14}C in the organic acid fraction declined during the course of the experiment and was initially 22% of the total ^{14}C in the palisade parenchyma cells and only 10% in the spongy parenchyma cells. Nineteen per cent of the ^{14}C in the palisade parenchyma was in malic acid while the corresponding value for the spongy parenchyma was only 5%. It is not known how significant this may be.

Radioactivity in basic compounds increased during the course of the experiment. At 60 sec, basic compounds accounted for about 15% of the ^{14}C in each tissue. These compounds were mainly glycine, serine, and alanine. The [^{14}C]serine-[^{14}C]glycine ratio was approximately 1 and no differences between the cell types were evident. Mokronosov *et al.* (22) reported that the ratio for [^{14}C]serine-[^{14}C]glycine was 7:1 and 1:6 in the spongy parenchyma and palisade parenchyma, respectively, of a coltsfoot leaf which had been labeled for 10 sec with $^{14}CO_2$ and allowed to photosynthesize in $^{12}CO_2$ for an additional 50 sec. They report, however, that the incorporation of ^{14}C into noncarbohydrate compounds in potato and expanding tobacco leaf showed no reliable differences between the tissues.

Radioactivity accumulated rapidly in the 80% ethanol-insolu-

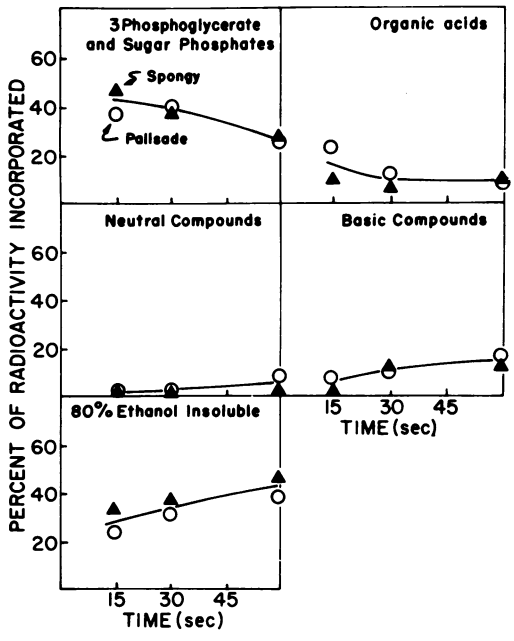

FIG. 3. Distribution of ^{14}C in various fractions. Palisade parenchyma preparations (○) and spongy parenchyma preparations (▲) were exposed to $NaH^{14}CO_3$ for periods indicated.

ble fraction in each cell type, and only very slowly in the neutral sugar fraction. On the contrary, ^{14}C accumulated slowly in the ethanol-insoluble fraction of an intact *V. leaflet* (24), while sucrose rapidly accounted for most of the water-soluble radioactivity (25). A similar difference is evident in the data for tobacco cells (7), which were prepared by as much as 3 hr of digestion time. Such cells incorporated about 33% of the radioactivity into the insoluble fraction in 2 min, while leaf discs only incorporated 8% into the insoluble fraction. However, cell suspensions prepared rapidly by mechanical isolation (11, 16), or by only 1.5 hr of enzymatic digestion (17) did not display this difference. These data might be explained by the concept that when sucrose translocation is interrupted, a feedback mechanism may inhibit its synthesis.

These data support the view (25) that there is no significant qualitative difference in the photosynthetic carbon metabolism in the different parenchyma tissues of *V. faba*.

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