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Photosynthetic and Carbohydrate Metabolism in Isolated Leaf Cells of *Digitaria pentzii*[1, 2]

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ABSTRACT

Mesophyll cells and bundle sheath strands were isolated rapidly from leaves of the C_4 species *Digitaria pentzii* Stent. (slenderstem digitgrass) by a chopping and differential filtration technique. Rates of CO_2 fixation in the light by mesophyll and bundle sheath cells without added exogenous substrates were 6.3 and 54.2 micromoles of CO_2 per milligram of chlorophyll per hour, respectively. The addition of pyruvate or phosphoenolpyruvate to the mesophyll cells increased the rates to 15.2 and 824.6 micromoles of CO_2 per milligram of chlorophyll per hour, respectively. The addition of ribose 5-phosphate increased the rate for bundle sheath cells to 106.8 micromoles of CO_2 per milligram of chlorophyll per hour. These rates are comparable to those reported for cells isolated by other methods. The $K_m(HCO_3^-)$ for mesophyll cells was 0.9 mM; for bundle sheath cells it was 1.3 mM at low, and 40 mM at higher HCO_3^- concentrations. After 2 hours of photosynthesis by mesophyll cells in $^{14}CO_2$ and phosphoenolpyruvate, 88% of the incorporated ^{14}C was found in organic acids and 0.8% in carbohydrates; for bundle sheath cells incubated in ribose 5-phosphate and ATP, more than 58% of incorporated ^{14}C was found in carbohydrates, mainly starch, and 32% in organic acids. These findings, together with the stimulation of CO_2 fixation by phosphoenolpyruvate for mesophyll cells and by ribose 5-phosphate plus ATP for bundle sheath cells, and the location of phosphoenolpyruvate and ribulose bisphosphate carboxylases in mesophyll and bundle sheath cells, respectively, are in accord with the scheme of C_4 photosynthesis which places the Calvin cycle in the bundle sheath and C_4 acid formation in mesophyll cells.

Starch and reducing sugars were present in both mesophyll and bundle sheath cells following a period of photosynthesis by whole leaves. However, when isolated cells were exposed to $^{14}CO_2$ in the light, even with appropriate exogenous substrates, only bundle sheath cells accumulated appreciable amounts of labeled carbohydrates. Incubation of mesophyll cells in the light with ATP and either pyruvate and inorganic phosphate, or phosphoenolpyruvate, or 3-phosphoglycerate resulted in large increases in total carbohydrates. The 3-phosphoglycerate treatment produced the greatest increase. These results could not be explained on the basis of increased CO_2 fixation. They suggest that mesophyll cells are able to metabolize exogenously supplied 3-carbon compounds to carbohydrates, despite the apparent inability of these cells to utilize CO_2 for this purpose, and support the view that in the whole leaf 3-phosphoglycerate is transported from bundle sheath to mesophyll cells, where it is reduced to carbohydrate.

Sucrose and sucrose-phosphate synthetases and invertase were localized mainly in bundle sheath cells. ADP-Glucose starch synthetase and amylase were present mainly in bundle sheath cells whereas starch phosphorylase was present mainly in mesophyll cells.

It is now generally accepted that a major feature of C_4 photosynthesis is a compartmentation of carbon metabolism between mesophyll and bundle sheath cells (3, 7). The initial fixation of atmospheric CO_2 in leaves of C_4 plants occurs in mesophyll cells; PEP[5] is carboxylated by PEP carboxylase, and OAA thus formed is aminated to asparate or reduced to malate. These C_4 dicarboxylic acids are transported to adjoining bundle sheath cells, where decarboxylation occurs and the released CO_2 is refixed by RuBP carboxylase in the reductive pentose phosphate pathway. This compartmentalization between mesophyll and bundle sheath cells has led to the proposal (17) that the reactions of the C_4 pathway serve to concentrate CO_2 in bundle sheath cells.

The foregoing model emphasizes that mesophyll cells in C_4 plants function as a biochemical pump, shuttling CO_2 from the external atmosphere to bundle sheath cells, where it is reduced to carbohydrate. It is also known that mesophyll cells of some C_4 species can accumulate starch, the usual end product of photosynthetic carbon reduction. Whereas C_4 species such as *Zea mays* (9, 11), *Sorghum sudanense* (2, 11), *Chloris gayana* (11), and *Aristida adscensionis* (2) have been reported to accumulate starch in bundle sheath rather than mesophyll cells, other C_4 species including *Digitaria decumbens* (18), *D. sanguinalis* and *Cynodon dactylon* (4), *Muhlenbergia racemosa* (21), and *Cyperus albomaeginatus* (11) reportedly accumulate starch in both bundle sheath and mesophyll cells. The details by which carbon fixed initially into C_4 acids is metabolized into carbohydrates in mesophyll cells have yet to be elucidated. In this paper we present comparative data on the CO_2 fixation characteristics and carbohydrate metabolism of isolated mesophyll and bundle sheath cells from *Digitaria pentzii*, a species which is capable of accumulating starch in both cell types.

Much of the recent knowledge about the mechanism of C_4 photosynthesis is based on studies with mesophyll protoplasts and bundle sheath cells isolated by enzymic methods (16, 19, 27). In this study we used a rapid chopping technique for isolation of mesophyll cells and bundle sheath strands without the need for enzyme digestion, a procedure which retains intact the cell walls in both types of cells.

MATERIALS AND METHODS

Plant Material. The perennial tropical forage grass *D. pentzii* Stent. (slenderstem digitgrass) was grown in soil in a greenhouse and also in a growth chamber at 32 C under a 17 hr day. Plant tops were cut back periodically. Only the terminal 16 cm of fully developed leaves was used as experimental material. Greenhouse plants were used for the CO_2 fixation experiments. In studies of carbohydrate metabolism, growth chamber plants were harvested either at the end of the 17-hr light period (light-treated plants) or

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[5] Abbreviations: ADPG: ADP-glucose: DCPIP: dichlorophenolindophenol; OAA: oxaloacetate; PEP: phosphoenolpyruvate; PGA: 3-phosphoglycerate; R5P: ribose 5-phosphate; RuBP: ribulose 1,5-bisphosphate; UDPG: UDP-glucose.

after 24-hr darkness (dark-treated plants).

Mesophyll cells also were isolated from leaves of 3- to 5-week-old soybean (*Glycine max* L., var. Hardee) grown under the same conditions as those used for *D. pentzii*.

Cell Isolation. Approximately 8 g of leaves was rinsed with distilled H_2O and cut transversely in 4-cm segments. The segments were maintained at 4 C, and washed with 40 ml of ice-cold isolation medium while being chopped at 650 strokes/min for 5 min with a plant tissue chopper consisting of a razor blade secured to the end of a motor-driven vertical arm (YEDA Scientific Instrument Co., Rehovot, Israel). The isolation medium at pH 8 contained 0.33 M D-sorbitol, 50 mM Tricine-NaOH, 2% (w/v) PVP-40, 2 mM Na_2EDTA, 2 mM $NaNO_3$, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM K_2HPO_4, and 10 mM β-mercaptoethanol. After chopping, the unmacerated tissue was discarded and the released cells were washed through a 35-mesh stainless steel sieve with 10 ml of ice-cold isolation medium. Mesophyll and bundle sheath cells then were separated at 0 C by differential filtration (13). Mesophyll cells were collected on a 20-μm nylon screen; bundle sheath strands were collected on an 80-μm nylon screen. Both mesophyll cells and bundle sheath strands were suspended at pH 8 in ice-cold suspension medium consisting of 0.33 M D-sorbitol, 50 mM Tris-HCl, 1 mM Na_2EDTA, 3 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 1 mM K_2HPO_4, and 5 mM DTT. A light microscope was used routinely to monitor the homogeneity of the cell preparations. Chl was determined by the method of Arnon (1).

Soybean mesophyll cells were isolated from 6 g of mature leaves cut transversely into 2-cm segments using the procedure described for mesophyll cells of *D. pentzii*.

$^{14}CO_2$ Incorporation by Isolated Cells. CO_2 fixation by the isolated cell types was determined by $^{14}CO_2$ incorporation into acid-stable products in saturating light (100 $\mu E/m^2 \cdot sec$, 400–700 nm) and dark. The assay solution for mesophyll cells of *D. pentzii* contained, except as specified, in 1 ml: 50 mM Tris-HCl (pH 8), 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 12 mM PEP, and cell suspension (5–12 μg of Chl). The solutions were shaken gently for 15 min at 32 C in an illuminated water bath, and then $NaH^{14}CO_3$ (0.1 $\mu Ci/\mu mol$) was injected to initiate the reaction and to give a final concentration of 5 mM. After 6 min, 0.1 ml of 6 N HCl saturated with 2,4-dinitrophenylhydrazine was injected to halt the reaction. The solutions were flushed with air to remove unincorporated $^{14}CO_2$, aliquots were taken, and the radioactivity was determined by liquid scintillation spectroscopy. In assays of *D. pentzii* bundle sheath strands and soybean mesophyll cells, Tris and PEP in the above assay solution were replaced with 50 mM HEPES (pH 8), 5 mM R5P, and 5 mM ATP. The reaction was initiated with $NaH^{14}CO_3$ to give a final concentration of 10 mM, and terminated after 6 min with 6 N acetic acid. All other conditions were similar to those in the mesophyll cell assay.

Enzyme Assays. RuBP and PEP carboxylase activities in the whole leaf and isolated cell extracts were measured by the method of Bowes and Ogren (5), except that reactions were run at 32 C and were halted after 3 min by the injection of 6 N HCl saturated with 2,4-dinitrophenylhydrazine. Also, 0.4 mM RuBP was used in the assay for RuBP carboxylase and 5 mM PEP in the PEP carboxylase assay.

For assays of the enzymes involved in carbohydrate metabolism, cell and leaf extracts were prepared according to the methods of Downton and Hawker (9, 10). Invertase, amylase, ADPG-starch synthetase (primed activity), and starch phosphorylase were assayed by the methods of Downton and Hawker (9, 10). Assays for sucrose and sucrose-P synthetases were carried out according to the methods of Lyne and ap Rees (22).

Extraction and Identification of ^{14}C-labeled Compounds. After exposure to $NaH^{14}CO_3$, cells were extracted with hot 80% (v/v) ethanol for 20 min, centrifuged, and the supernatants decanted. For determinations of OAA, some samples were extracted with ethanol containing 2,4-dinitrophenylhydrazine. Washing with ethanol was repeated until the tissue samples were cleared of pigments. The ethanol-soluble extracts were combined, dried at 35 C, and washed with petroleum ether. The extracts were dissolved in a small volume of water, centrifuged, and 0.2 ml of 0.5 M glycine-NaOH (pH 10) containing 10 mM $MgCl_2$, 5 μl of toluene, and 8 units of alkaline phosphatase was added to the supernatants. The mixture was incubated for 24 hr at 37 C and then passed sequentially through Dowex 50-X8 (hydronium form) and Dowex 1-X8 (formate form) columns (1 × 10 cm). After eluting the sugars with water, the amino acids were eluted from Dowex 50 with 2 N NH_4OH and the organic acids were eluted from Dowex 1 with 40% formic acid. The sugar, amino acid, and organic acid eluates were dried, taken up in a small volume of water, separated by TLC (23, 28), and the individual components then were identified by co-chromatography with authenic compounds. The radioactivity in the spots was determined by liquid scintillation spectroscopy.

Plant tissue residues which remained after ethanol extraction were dried and then taken up in 0.1 M acetate buffer (pH 4.8), and homogenized. The homogenates were incubated with amyloglucosidase (Sigma) at 55 C for 90 min to convert starch to glucose. After centrifugation the radioactivity in the supernatant (glucose) and the pellet was determined. In all experiments the pellet contained less than 0.01% of the total ^{14}C incorporated.

Carbohydrate Analyses. In studies of carbohydrate metabolism by isolated mesophyll cells, the assay conditions were similar to those described for the $^{14}CO_2$ incorporation studies, except $NaH^{14}CO_3$ was omitted and the assay solutions were flushed gently with air during the course of the reaction. After 90-min incubation with various substrates, the cells were killed with hot 80% ethanol and extracted as described above. After the extracts were cleared of pigments, they were dissolved in 5 ml of water and centrifuged. The supernatants were deionized with Dowex 50-X8 (hydronium form), neutralized, and the reducing sugar content determined by the copper reagent method (26). Glucose was determined by the glucose oxidase method (Glucostat, Worthington Biochemical Corp.). Sucrose also was determined by the glucose oxidase method, by analyzing before and after a 2-hr incubation with invertase. Starch in the ethanol extracted residues was hydrolyzed to glucose as described above, and the released glucose determined by the glucose oxidase method.

RESULTS

Microscopic examination of *D. pentzii* leaf sections indicated typical C_4 anatomy. The C_4 nature of this plant was further confirmed by measurements of PEP and RuBP carboxylase activities in the whole leaf and in isolated leaf cells (Table I). PEP carboxylase activity predominated in the whole leaf, and over 92% of the activity was located in mesophyll cells. In contrast, RuBP carboxylase activity was totally confined to bundle sheath cells.

$^{14}CO_2$ Incorporation by Isolated Cells. Isolated cells were capable of substantial CO_2 fixation when supplied with substrates: PEP for mesophyll cells and R5P + ATP for bundle sheath cells (Fig. 1). Bundle sheath cells exhibited linear incorporation for over 90 min. The initial rate of incorporation with mesophyll cells was higher, but departed from linearity after only 10 to 15 min. This was not due to a lack of substrates, but may be an inhibition effect due to accumulation of C_4 acids. The pH optima for CO_2 incorporation by mesophyll and bundle sheath cells were pH 7.5 and 8.2, respectively; the curves were broad rather than sharp

Table I. PEP and RuBP Carboxylase Activity in the Whole Leaf and in Isolated Leaf Cells of D. pentzii.

Tissue	PEP Carboxylase	RuBP Carboxylase
	$\mu mol\ CO_2/mg\ Chl \cdot hr$	
Whole leaf	907.1	24.9
Mesophyll cells	1386.9	0
Bundle sheath cells	122.2	127.6

peaks (data not shown). Bundle sheath cells had a temperature optimum of about 35 C. On the other hand, increasing temperatures up to the highest employed (45 C) increased the fixation rate of mesophyll cells (data not shown). Temperatures below 20 C reduced fixation by mesophyll cells to a greater extent than found for bundle sheath cells. Mesophyll cells isolated from soybean, a C_3 plant, exhibited a similar temperature response to that of the bundle sheath cells of *D. pentzii*.

Figure 2 shows double reciprocal plots of the CO_2 fixation rate *versus* $NaHCO_3$ concentration for isolated cells. The V_{max} for mesophyll cells was 475 μmol of CO_2/mg of Ch·hr; the apparent $K_m(HCO_3^-)$ was 0.9 mM, which is similar to that reported for *D. sanguinalis* mesophyll cells isolated by enzymic techniques (20). Bundle sheath cells exhibited more complex kinetics; at low HCO_3^- concentrations a V_{max} of 50 μmol of CO_2/mg of Chl·hr and an apparent $K_m(HCO_3^-)$ of 1.3 mM were found. At higher HCO_3^- levels the V_{max} extrapolated to 250 μmol of CO_2/mg of Chl·hr and the apparent $K_m(HCO_3^-)$ to 40 mM (Fig. 2). Similarly high K_m values have been reported for mechanically isolated maize bundle sheath cells (6), although Farineau (15) obtained a value as low as 0.4 mM.

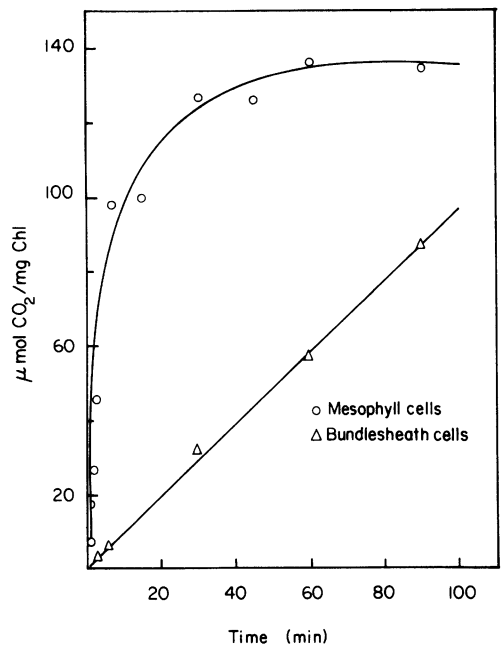

FIG. 1. Time course of $^{14}CO_2$ incorporation by isolated leaf cells of *D. pentzii*. Mesophyll cells were incubated with 10 mM PEP and bundle sheath cells with 5 mM R5P and 5 mM ATP in the light. At intervals, 0.2-ml aliquots were removed from the 2.5-ml reaction mixtures and assayed.

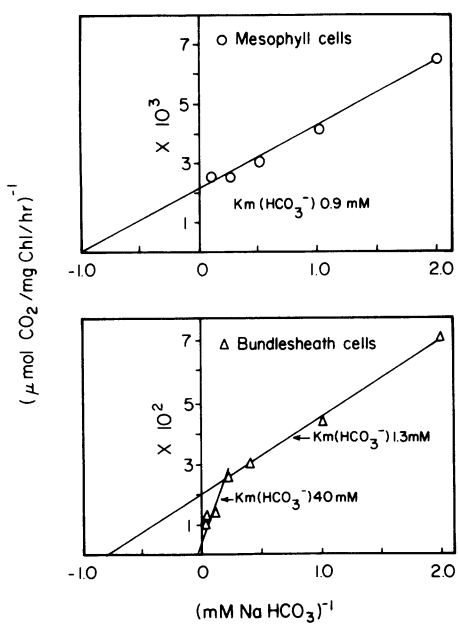

FIG. 2. Double reciprocal plots of rates of $^{14}CO_2$ incorporation *versus* bicarbonate concentration for isolated leaf cells of *D. pentzii* in the light.

Figure 3 shows the effect of increasing PEP and R5P concentrations on CO_2 fixation by mesophyll and bundle sheath cells, respectively. In the absence of PEP, fixation by mesophyll cells was low. A concentration of 10 to 12 mM PEP was required for maximum fixation. A 15-min preincubation period with PEP was found to stimulate subsequent CO_2 fixation by as much as 100%. Longer incubation times reduced the stimulation. Increasing concentrations of R5P up to 10 mM, in the presence of ATP, stimulated CO_2 fixation by bundle sheath cells (Fig. 3). Even in the absence of added R5P, bundle sheath cells exhibited substantial fixation rates.

Cells were routinely isolated at ice bath temperature. Table II shows that mesophyll cells of *D. pentzii* generally exhibited a greater capacity for CO_2 fixation in both the light and dark when isolated at low temperature and also shows the effect on CO_2 fixation of supplying mesophyll cells with various substrates in addition to $NaHCO_3$. The addition of pyruvate stimulated CO_2 fixation, especially in the light, and this was more than doubled for cells isolated at 4 C by adding Pi as well. The addition of ATP produced a further rate increase. PEP alone was the most effective in stimulating CO_2 fixation. (The explanation for the fact that the addition of pyruvate + Pi + ATP, the substrates of pyruvate Pi

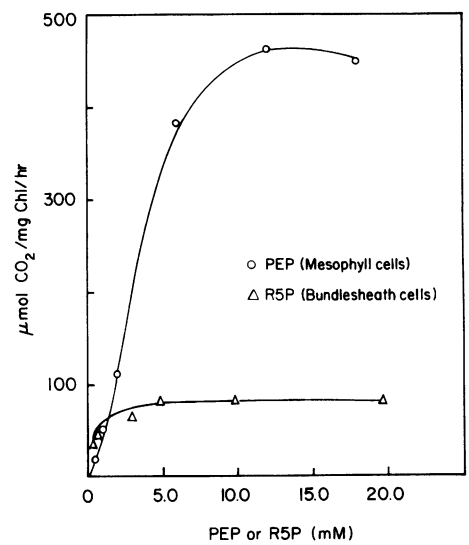

FIG. 3. Effect of increasing PEP and R5P concentrations in the light on CO_2 fixation rates of mesophyll and bundle sheath cells respectively, isolated from *D. pentzii*.

Table II. The Effects of Temperature During Isolation, and the Addition of Various Metabolites on CO_2 Fixation by D. pentzii Mesophyll Cells in the Light and Dark.

The metabolites were added at a concentration of 5 mM.

Metabolite	Isolated at 4 C		Isolated at 25 C	
	Light	Dark	Light	Dark
	μmol CO_2/mg Chl·hr			
None	1.3	0.6	0.9	0.4
Pyruvate	7.7	1.9	3.1	1.3
Pyruvate + Pi	16.1	1.2	3.8	1.0
Pyruvate + Pi + ATP	26.6	4.8	31.1	19.6
PEP	381.1	329.5	326.0	272.0

dikinase, was not as effective as PEP is not readily apparent but may hinge on a relatively low permeability of mesophyll cells to Pi and/or pyruvate and also on high PEP carboxylase activity.) In all instances, CO_2 fixation in the light was substantially greater than in the dark, even when an exogenous supply of ATP was present.

Table III shows the effect of various metabolites on the CO_2 fixation rates of isolated mesophyll and bundle sheath cells. For comparison the response of soybean cells also is included. Mesophyll cells of *D. pentzii* had low fixation rates in the light and dark in the absence of exogenous substrates, whereas bundle sheath cells had comparatively high rates in the light. R5P was especially effective in stimulating fixation by bundle sheath cells but had little effect on *D. pentzii* mesophyll cells. In the dark, R5P alone had little effect on CO_2 fixation by bundle sheath cells but with ATP added, considerable fixation resulted. The addition of PEP increased the rate approximately 50% in the light and severalfold in the dark. This increase by PEP may reflect the small amount of PEP carboxylase activity associated with bundle sheath cells (Table I). Pyruvate had no stimulatory effect. Soybean mesophyll cells showed similar responses to *D. pentzii* bundle sheath cells, although the degree of stimulation by R5P and R5P + ATP was greater.

Carbohydrate Metabolism Studies. Microscopic examination of *D. pentzii* leaf sections with I_2/KI solution indicated that both bundle sheath and mesophyll cells were capable of accumulating starch. These initial observations were confirmed by carbohydrate analyses (Table IV) of whole leaves and isolated cells obtained from plants subjected to either a 17-hr light period (light-treated) or a 24-hr dark period (dark-treated). Although much of the carbohydrate was located in bundle sheath cells, mesophyll cells of light-treated plants also contained appreciable quantities of carbohydrate. The dark treatment reduced the starch content of both cell types by more than 95% but the levels of total reducing sugars were somewhat increased (Table IV). The sucrose content of the isolated cells and whole leaves was below the level of detection, possibly because of the relatively low light intensity used in the growth chamber (240 $\mu E/m^2 \cdot sec$, 400–700 nm). In the following experiments, cells were isolated from plants exposed to either the light or dark treatment described above.

Table V shows the distribution of ^{14}C in organic compounds following a 2-hr exposure of isolated cells to $NaH^{14}CO_3$ in the light. For mesophyll cells, 88% of the radioactivity was in organic acids, while only 0.8% was incorporated into carbohydrates. In contrast, bundle sheath cells incorporated more than 58% of the radioactivity into carbohydrates, mainly starch, and only 32% into organic acids. A similar, lower level of ^{14}C incorporation into organic acids (as opposed to carbohydrates) also has been reported for bundle sheath cells of maize during a 5-min exposure to $^{14}CO_2$ in the light (6). It appears that although isolated bundle sheath cells can synthesize carbohydrate from CO_2, mesophyll cells of *D. pentzii* in isolation from the remainder of the leaf tissue are incapable of carbohydrate synthesis from CO_2.

Table III. The Effect of Various Metabolites on CO_2 Fixation in the Light and Dark by Isolated Leaf Cells of D. pentzii and Soybean.

The metabolites were added at a concentration of 5 mM.

Metabolite	D. pentzii				Soybean	
	Mesophyll Cells		Bundle Sheath Cells		Mesophyll Cells	
	Light	Dark	Light	Dark	Light	Dark
	μmol CO_2/mg Chl·hr					
None	6.3	6.6	54.2	3.1	67.6	3.7
Pyruvate	15.2	11.0	56.8	3.1	53.8	2.7
PEP	824.6	608.1	81.8	17.7	102.1	6.0
Ribose-5-P	7.9	6.8	106.8	4.2	164.7	10.2
Ribose-5-P + ATP	12.1	10.5	185.0	86.0	405.3	110.2

Table IV. The Carbohydrate Content of Whole Leaves and Isolated Leaf Cells from Light and Dark Treated Plants of D. pentzii.

Leaves were collected for cell isolation and carbohydrate analyses after the plants were exposed to a 17 hr light period or a 24 hr dark period.

Tissue	Light Treated			Dark Treated		
	Glucose	Total Reducing Sugars	Starch	Glucose	Total Reducing Sugars	Starch
	μmol hexose/mg Chl					
Whole leaf	27	222	214	12	303	8
Mesophyll cells	21	115	51	10	138	2
Bundle sheath cells	131	726	334	43	1110	17

Table V. Distribution of ^{14}C Incorporated into Isolated Mesophyll and Bundle Sheath Cells of D. pentzii.

The 4 ml reaction mixtures contained 10 mM $NaH^{14}CO_3$ and 10 mM PEP (mesophyll cells) or 5 mM R5P and 5 mM ATP (bundle sheath cells). A 2 hr incubation in the light was used. Leaves were collected for cell isolation from light treated plants. The product analyses and other experimental procedures were as described in Materials and Methods.

Product	Mesophyll Cells	Bundle Sheath Cells
	% of ^{14}C incorporated	
Total Organic Acids	88	31.7
Malate	41	-
Oxaloacetate	40	-
Total Amino Acids	0.07	4.0
Aspartate	0.02	-
Total Sugars	0.15	15.8
Glucose	0	-
Fructose	0.13	-
Sucrose	0.02	-
Total Carbohydrates	0.81	58.6
Starch	0.63	42.8

It has been suggested that certain metabolic intermediates produced in bundle sheath cells of C_4 plants may be transported to and reduced in mesophyll cells, eventually forming sugars and starch (3, 24). To test this hypothesis, mesophyll cells isolated from dark-treated plants were incubated in the light with several metabolic intermediates and then analyzed for carbohydrate content. CO_2 fixation rates also were determined on similar cell samples in the presence of the metabolites. The endogenous carbohydrate content of different cell preparations varied somewhat, which accounts for the differences in total carbohydrates among controls to which no metabolites were added.

Incubation of mesophyll cells with either pyruvate + Pi or PEP resulted in a slight increase in total reducing sugars, as compared to the control, but no increase in starch (Table VI, experiment A). Supplying PGA did not increase the total carbohydrate content but did produce a slight increase in starch, at the expense of total reducing sugars. When the cells were supplied with ATP, in addition to pyruvate + Pi, or PEP or PGA, there were large increases in total reducing sugars and to a lesser extent increases in starch (Table VI, experiment B). Of the metabolites added, PGA + ATP produced the greatest stimulation in total reducing sugars and starch. The addition of ATP alone only increased only slightly the total reducing sugars and starch. Concentrations of PGA above 5 mM were less effective in stimulating carbohydrate production (data not shown).

All of the metabolites which were added, except ATP alone, produced some increase in the CO_2 fixation rate. Mesophyll cells isolated from dark-treated plants generally had a higher CO_2 fixation capacity than those from light-treated plants; this accounts for the high rates of CO_2 fixation in Table VI.

An indication of the relative photoreduction capacity of the isolated cells, without exogenous metabolites, was derived from measurements of Hill activity using DCPIP as the electron acceptor. Both mesophyll and bundle sheath cells of *D. pentzii* had rates in excess of 1,500 μmol of DCPIP reduced/mg of Chl·hr, as compared to about 1,250 for isolated soybean mesophyll cells.

Table VII shows the distribution of activity of several enzymes

Table VI. The Effect of Various Metabolites on Carbohydrate Content and CO_2 Fixation by Isolated Mesophyll Cells of D. pentzii.

Cells were incubated in the light for 90 min in 4 ml reaction mixtures with various metabolites, then extracted with 80% ethanol and analyzed for carbohydrate content. The concentration of pyruvate, PEP and Pi was 10 mM. The concentration of PGA and ATP was 5 mM. For CO_2 fixation measurements, samples of the cells were assayed with 5 mM $NaH^{14}CO_3$. Leaves were collected for cell isolation from dark treated plants. Other experimental conditions and analyses were as described in Materials and Methods. Experiments A, B, and C were performed on different cell preparations.

Metabolite	Starch	Glucose	Total Reducing Sugars	Total Carbohydrates	CO_2 Fixation
		μmol hexose/mg Chl			μmol/mg Chl·hr
Experiment A:					
None	1.8	6.0	73.7	75.5	0.9
Pyruvate + Pi	1.5	2.8	92.2	93.7	16
PEP	1.5	4.6	88.5	90.0	1842
PGA	2.6	5.5	71.9	74.5	31
Experiment B:					
None	1.5	8.6	88.5	90.0	2
Pyruvate + Pi + ATP	2.5	4.7	200.0	202.5	20
PEP + ATP	2.8	2.4	207.8	210.6	2192
PGA + ATP	5.7	3.1	270.5	276.2	11
Experiment C:					
None	2.0	7.3	143.4	145.4	0.8
ATP	1.0	18.8	152.2	153.2	0.9

Table VII. Activities of Various Enzymes Associated with Carbohydrate Metabolism in the Whole Leaf and in Isolated Leaf cells of D. pentzii.

Enzyme extracts were prepared from isolated cells and whole leaves of light treated plants. The ADPG-starch synthetase values represent primed activity.

Enzyme	Whole Leaf	Mesophyll Cells	Bundle Sheath Cells
		μmol/mg Chl·hr	
UDPG-sucrose-P synthetase	5.9	2.1	28.0
UDPG-sucrose synthetase	0.1	0	7.3
Invertase	278.5	82.4	1369
ADPG-starch synthetase	10.1	23.8	244.8
Amylase	241.0	298.8	3814
Starch phosphorylase	9.8	75.7	10.2

associated with carbohydrate metabolism in the two cell types. These results indicate that for most of the enzymes assayed more than 90% of the activity was located in bundle sheath cells. Starch phosphorylase was an exception in that this enzyme appeared to be localized mainly in mesophyll cells. The low levels of UDPG-sucrose synthetase may be a factor contributing to the low sucrose levels in the tissues.

DISCUSSION

Cell isolation by the chopping and filtration method used in this study had several advantages. The method was rapid and gave easily reproducible results; only 20 to 30 min was required to obtain relatively large quantities of both cell types from the same tissue sample; the cells were free of possible deleterious effects of maceration enzymes or impurities contained therein; cell walls were retained intact; plasmolyzing conditions were avoided. The cell preparations were homogeneous as judged by light microscopy and localization studies of the carboxylation enzymes. Rates of CO_2 fixation by both cell types, with and without exogenous substrates, were similar to or in excess of those reported for cells isolated by other methods (16, 20, 25). As has been found in other cell isolation studies (20), pyruvate-induced CO_2 fixation by the isolated mesophyll cells was low, indicating low rates of ATP production in these cells. To date, only mesophyll cell extracts appear capable of high rates of pyruvate-dependent CO_2 fixation in the absence of compounds to induce noncyclic electron transport (14, 20).

For the two cell types isolated from *D. pentzii* the different activation effects upon CO_2 fixation by exogenous organic substrates, the separate locations of the two major carboxylation enzymes, and the differences in the major products of photosynthesis are all in accord with the scheme of C_4 photosynthesis which places the Calvin cycle in bundle sheath cells and the formation of C_4 acids largely in mesophyll cells (3, 7).

CO_2 Fixation into Carbohydrates. Appreciable levels of reducing sugars and starch were found in both mesophyll and bundle sheath cells isolated from *D. pentzii* following a period of photosynthesis by whole leaves (Table IV). In contrast, when isolated cells were allowed to photosynthesize in $^{14}CO_2$, even with an appropriate exogenous substrate, only bundle sheath cells accumulated appreciable amounts of label in carbohydrates (Table V). This suggests that although leaves of *D. pentzii* are able to accumulate starch in mesophyll cells, only bundle sheath cells possess all of the components necessary to manufacture carbohydrates from CO_2.

Metabolism of Exogenous Substrates into Carbohydrates. Incubating isolated mesophyll cells of *D. pentzii* in the light with PGA did not increase the total carbohydrate content, although pyruvate + Pi or PEP did produce a small increase (Table VI). However, the addition of ATP, which by itself had little effect on carbohydrate accumulation, in combination with pyruvate + Pi, or PEP, or PGA, resulted in large increases in the carbohydrate content of the cells. These large increases could not be explained on the basis of increased CO_2 fixation because, except for PEP, the small stimulation of CO_2 fixation brought about by the added compounds (Table VI) was insufficient to account for the amount of carbohydrate formed. In the case of PEP, the analysis of the photosynthetic products in mesophyll cells (Table V) indicated that only a small fraction of the label from $^{14}CO_2$ accumulated in carbohydrates. These results suggest that mesophyll cells of *D. pentzii* metabolized the exogenously supplied 3-carbon compounds to carbohydrates, despite the apparent inability of these cells to utilize CO_2 for this purpose.

The most effective substrate was PGA, in combination with ATP, suggesting that the carbohydrate production observed in the mesophyll cells of whole leaves (Table IV) could have resulted from the transport of some of the PGA from the bundle sheath cells and its subsequent reduction in the mesophyll cells. If substantial amounts of PGA were involved, a portion of the reduced triose would have to return to the bundle sheath in order to regenerate RuBP in the Calvin cycle. The Hill reaction activity of the mesophyll cells was found to be greater than that of isolated soybean mesophyll cells, which further supports the possibility that some reduction of PGA might occur in the mesophyll cells of *D. pentzii*. These observations also support the proposal that in those C_4 plants with low PSII activity in the bundle sheath cells, the PGA produced there may be transported and reduced in the mesophyll cells (14, 24).

The observation that 3-carbon compounds can be utilized by isolated mesophyll cells to form carbohydrates provides only circumstantial evidence for their *in vivo* involvement in the proposed shuttle between bundle sheath and mesophyll cells. The possibility exists that hexoses, for example, also may be transported for carbohydrate metabolism. In this regard, mesophyll cells of C_4 plants may be similar to leaf guard cells. The latter assimilate CO_2 into malate but cannot reduce CO_2 to Calvin cycle intermediates; instead, there is a transfer of sugars from palisade and spongy parenchyma tissues to epidermal tissue and a conversion of sugars to starch in guard cells (8).

The requirement of mesophyll cells for exogenous ATP in order to form carbohydrates, depsite the incubation of the cells in the light, is consistent with the fact that mesophyll cells isolated from C_4 plants characteristically exhibit low rates of pyruvate-dependent CO_2 fixation (20). This has been attributed to insufficient ATP production by the isolated cells for the effective operation of the pyruvate Pi dikinase reaction (20). Huber and Edwards (20) reported that OAA and PGA added in combination with pyruvate produced a substantial synergistic stimulation of CO_2 fixation in

D. sanguinalis mesophyll cells. They proposed that the addition of pyruvate + OAA induces the parallel production of ATP and NADPH (via noncyclic photophosphorylation) by enabling ADP and $NADP^+$ to be regenerated through the conversion of pyruvate to PEP and OAA to malate, respectively. Thus, it is possible that the mesophyll cells in the present study, supplied only with pyruvate + Pi, or PEP, or PGA, had low rates of noncyclic photophosphorylation and therefore did not generate sufficient ATP to support the reactions of carbohydrate metabolism that require its input. It is unclear why the addition of ATP, which in combination with pyruvate stimulated carbohydrate formation, did not under the same conditions also appreciably stimulate PEP formation and hence CO_2 fixation.

Carbohydrate Metabolism Enzymes. In general, the activities of the enzymes associated with carbohydrate metabolism were considerably higher than those reported for cells isolated from maize (9). As in maize bundle sheath cells of *D. pentzii* appear to be the major repository of enzymes of carbohydrate metabolism (Table VII). Phosphorylase in *D. pentzii* was an exception, with over 7-fold higher activity in the mesophyll cells as compared to the bundle sheath cells. In this respect *D. pentzii* differs from both maize (9) and *D. sanguinalis* (12). The activity of ADPG-starch synthetase in *D. pentzii* mesophyll cells, although less than 10% of that in the bundle sheath cells, was still higher than that reported for either cell type in maize (9) and *D. sanguinalis* (12), and together with the high phosphorylase activity could account for the starch formulation that occurs in *D. pentzii* mesophyll cells. The differences between maize and *D. pentzii* in enzyme content of isolated cells may be related to the fact that maize does not accumulate much starch in the mesophyll cells under normal daylengths (9) whereas *D. pentzii* does.

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