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Carbon Dioxide Fixation and Related Properties in Sections of the Developing Green Maize Leaf[1]

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ABSTRACT

Light and dark $^{14}CO_2$ assimilation, pulse-chase ($^{14}CO_2$ followed by $^{12}CO_2$) labeling experiments both in the light and in the dark, photorespiratory activity and some enzymes (ribulose 1,5-bisphosphate (RuBP) carboxylase, phosphoenolpyruvate (PEP) carboxylase, and NADP-malic enzyme) were followed in sections of 2.5 centimeters from the base (younger tissue) to the tip (oldest tissue) of the green maize leaf. Tissue was taken from the third leaf of 12- to 16-day-old plants consisting of sections 0 to 2.5 centimeters (base), 4.5 to 7.0 centimeters (center) and 9.0 to 11.5 centimeters (top) measured from the base. Some of these properties were also determined in the intact leaves of 4-day-old maize plants.

Electron microscopy indicated a Kranz anatomy in all sections. Differentiation into mesophyll granal chloroplasts and bundle sheath agranal chloroplasts had taken place only in the center and top pieces.

All of the sections contained PEP carboxylase, RuBP carboxylase, and NADP-malic enzyme. The ratio of PEP:RuBP carboxylase increased from 3.03 (top) to 4.66 (base) whereas the PEP carboxylase:NADP-malic enzyme ratio rose from 2.87 (top) to 9.57 (base).

Under conditions of light or dark, the majority of the newly incorporated $^{14}CO_2$ was found in malate and aspartate in all sections and in 4-day-old leaves. The ^{14}C-labeling pattern typical of C_4 plants was present in the center and top sections and to a lesser extent in the 4-day-old leaves. In the base tissue, the percentage of radioactivity in malate and aspartate remained relatively constant both during photosynthesis and pulse-chase experiments. In contrast, radioactivity in glycerate-3-phosphate decreased with time coupled to an increase in sugar phosphates. To account for the isotopic pattern in the base tissue, parallel fixation by PEP carboxylase and RuBP carboxylase was proposed with the photosynthetic carbon reduction cycle functioning to some extent independently within the bundle sheath chloroplasts. The apparent lack of cooperation between the mesophyll and bundle sheath cells may have been due to inadequate levels of NADP-malic enzyme required for shuttling carbon as CO_2 from the PEP carboxylase products to the Calvin cycle.

As monitored by isotopic distribution patterns in intact leaves (15, 21, 24), tissue age may be a factor influencing the assimilatory route of CO_2 reduction in plants usually considered C_4. In a recent short communication, Raghavendra *et al.* (21) reported a shift in the photosynthetic carbon fixation pathway from a C_3 to C_4 mode in leaves of the dicot, *Mollugo nudicaulis*, as development proceeded. This shift in carbon assimilatory pathway was stated to be accompanied by the appearance of a Kranz anatomy in the older leaf tissue. A decrease in photorespiratory activity accompanied this transition in leaf structure. Waygood and Law (24) noted briefly that in the maize plant the primary leaves utilized the Calvin cycle while the older leaves assimilated carbon by the C_4 pathway. Also with maize, Williams and Kennedy (26) did not find a difference in the distribution pattern of photosynthetic products for young, mature, and senescent leaf tissue after short exposure to labeled CO_2. In contrast to a fixed photosynthetic pattern, they reported an increase in the photorespiratory rate during leaf ontogeny. In an earlier publication Kennedy and Laetsch (15) reported that the photosynthetic products of *Portulaca oleracea* differed considerably depending on leaf age. Senescent leaves in contrast to young leaves were characterized by a shift of primary photosynthetic products toward phosphorylated sugars and PGA[3] with a concomitant reduction in label located in malate and aspartate. These authors concluded that the stage of leaf development was a decisive factor when evaluating the primary carboxylation products.

In a previous report from this laboratory (19), chloroplasts isolated from approximately 4-day-old maize plants assimilated CO_2 but not malate to starch by the Calvin cycle. This result was unexpected since maize has been classified as a C_4 plant utilizing bundle sheath chloroplastic NADP-malic enzyme as the principal means of providing CO_2 from malate for the RuBP carboxylase reaction (8). The structure of the chloroplasts was such that their origin from mesophyll or bundle sheath cells could not be distinguished by electron microscopy. The speculation was presented then that perhaps juvenile in contrast to mature tissue reduced CO_2 through the ubiquitous photosynthetic carbon reduction pathway.

Hitherto, all experiments describing the distinguishing features associated with C_3 and C_4 photosynthesis during tissue development have been made with leaves from differing positions on the same stem. Tissue maturation can also be assessed in sequential sections along a monocot leaf, as the base section contains young cells newly produced by the intercalary meristem and the tip sections contain the oldest cells. As demonstrated by Leech *et al.* (16), the plastids in the maize leaf cells are also arranged in a sequential series of differentiation from the basal cells to the tip. In addition to evaluating the photoassimilatory carbon pathways with isotopically labeled CO_2 in sections taken from the base, center and tip of the same maize leaf, dark respiration, photorespiration, and enzyme analyses are presented and discussed in relation to C_3-C_4 photosynthesis during tissue differentiation. Maize leaves taken from 4-day-old plants equivalent to those used for chloroplast isolation in a previous study (19) were also evaluated for some of these properties. A preliminary report has been presented (20).

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[3] Abbreviations: PGA: glycerate-3-phosphate; RuBP: ribulose 1,5-bisphosphate; PEP: phosphoenolpyruvate.

MATERIALS AND METHODS

Plant Material. Maize seeds (*Zea mays* var. Early Fortune) were germinated in covered plastic boxes for 5 days on moist filter paper in the dark. Following germination, plants were grown hydroponically in a growth chamber in half-strength Hoagland solution B with the iron concentration increased by a factor of ten. The photoperiod was 12 h at an intensity of 45 w/m^2 supplied by a mixture of incandescent and cool white fluorescent lamps. The temperature was 26 C.

Plants were used at 12–16 days of age. Tissue from 2 to 2.5 cm, 4.5 to 7.0 cm, and 9.0 to 11.5 cm from the top of the mesocotyl of the third leaf was designated the base, center, and top, respectively. In some experiments, intact leaf tissue was used at 4 days of age. This material is referred to as 4-day-old leaves and was taken from plants grown on Vermiculite in the light in the same chamber at a light intensity of 5.5 w/m^2. Leaf tissue was obtained by removing the coleoptiles.

Reagents. All biochemicals were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

Electron Microscopy. For electron microscopy 1-mm slices of tissue were removed from the center of each of the maize leaf sections. Each piece was fixed as described by Leech *et al.* (16).

CO_2 Fixation. Tissue pieces (at least four or five) were cut and placed across glass microscope slides where they were held by two rubber bands running the length of the slides. The slides were inserted into 50-ml glass test tubes containing 1 ml of distilled H_2O. The tubes were capped with rubber serum stoppers and were placed in a water bath at 30 C. Hypodermic needles were inserted, and the tubes flushed with humidified CO_2-free air to open the stomates monitored by the silicon rubber technique of Zelitch (30). Illumination was supplied by a 150-w flood lamp. Tubes were transferred to a second water bath, also at 30 C where light at the desired intensity for CO_2 fixation was supplied by a locomotive headlamp (General Electric No. 100 PAR). The tubes were flushed for an additional 2 min with the humidified gas mixture being tested. At the end of this gassing period, 5 ml of gas was withdrawn and unless stated to the contrary, 5 ml containing 4 μmol $^{14}CO_2$ (32 μCi) was injected (final concentration 1,840 μl/l). At the end of the fixation period the serum stopper was removed and the slide and tissue was dropped into liquid N_2. The tissue was ground under liquid N_2 and the powder was poured into a centrifuge tube. The powdered material was extracted at 70 C for 30 min with 80% (v/v) ethanol. Following centrifugation, the supernatant fluid was analyzed for radioactivity and Chl (1, 28). Another portion was reduced in volume under vacuum for product determination by paper chromatography. For products formed during photosynthesis by the top and center sections, individual points at each time in the figures represent one extract at 1 and 2 min and two or three combined extracts at 10 and 30 s. Products produced by base and 4-day-old leaf tissue represent two or three combined extracts at 1 and 2 min and up to five combined extracts at 10 and 30 s. In the dark and pulse-chase experiments each time point in the top and center represents two or three combined extracts while in the base and 4-day-old leaves up to five extracts were combined to obtain sufficient radioactivity to expose the film.

Chromatography of Products. Two-dimensional descending paper chromatography (Whatman No. 1) was used for the separation and identification of products. Papers were developed once with the semi-stench solvent of Crowley *et al.* (5) and twice in the second dimension using the GW3 solvent of Wood (29). Radioactive spots were located by exposure of papers to Kodak No-screen x-ray film. Compounds were identified by co-chromatography with known labeled compounds or unlabeled standards which were subsequently located using various indicator sprays.

Photorespiration. Photorespiration was measured by the light-dark ^{14}C assay developed by Zelitch (30).

Enzyme Assays. Maize leaf sections (0.4 g) were ground in a chilled mortar for 1 min with sand and 2 ml of a solution containing 0.33 M sorbitol; 50 mM Hepes (pH 6.8); 0.25 mM Na-phosphate; 4 mM DTT, and 5% (w/v) PVP-40. The mixture was filtered through two layers of Miracloth and the resulting solution was used for enzyme assays.

RuBP carboxylase was assayed at 30 C by adding 0.1 ml of plant extract to 1.1 ml of a reaction mixture containing 50 mM Hepes-HCl (pH 7.8), 20 mM $MgCl_2$, 1 mM $Na_4P_2O_7$, 2 mM Na_2EDTA, 4 mM DTT, and 5 mM $NaH^{14}CO_3$ (10 μCi/μmol). After 5 min, 50 μmol RuBP was added. A sample was removed after 30 s and its radioactivity determined following addition of HCl. A control was run without RuBP.

PEP carboxylase was determined at 30 C by adding 0.1 ml extract to 0.2 ml of a reaction mixture modified from Kennedy (14) which contained 170 mM Tricine-NaOH (pH 8.0), 57 mM $MgCl_2$, 5.7 mM L-glutamate, 4 mM DTT, 3.2 mM PEP, and 32 mM $NaH^{14}CO_3$ (1 μCi/μmol). A sample was removed after 1 min and placed in a scintillation vial containing 2,4-dinitrophenylhydrazine (25 mg/ml 6 N HCl). Following addition of scintillation fluid, radioactivity was determined with a Beckman instrument LS type 150.

Malic enzyme (NADP) activity was determined spectrophotometrically at 340 nm using the method of Johnson and Hatch (10). The final reaction mixture contained 25 mM Tricine-NaOH (pH 8.5), 0.5 mM EDTA, 2.4 mM L-malate, 0.25 mM NADP, and 5 mM $MgCl_2$. With the exception of NADP and L-malate, all components including 0.1 ml crude extract were combined and centrifuged in a clinical centrifuge for 5 min at top speed. This served to remove any suspended particles. The reaction was started by addition of L-malate and NADP.

Compensation Points. CO_2 compensation points were determined using an open system IR gas analysis system (17).

RESULTS

Tissue Characteristics. The base tissue was tightly rolled and was surrounded by the primary and secondary leaves. Rudimentary Kranz anatomy was present in cross-sections of the base tissue (Fig. 1). At higher magnification (34,000-fold) bundle sheath and mesophyll chloroplasts were almost indistinguishable (illustrative material is not shown since the plastids resemble those in ref. 16). Chloroplasts from neither cell type contained starch. Occasional overlapping was seen in bundle sheath chloroplasts while a few mesophyll plastids contained stacks of two to four grana. No mature stomates were observed. Tissue from just above the mesocotyl showed no prolamellar bodies as seen in the pictures of Leech *et al.* (16).

The center sections was distinguished by the appearance of grana stacks of 8 to 10 lamellae in the mesophyll chloroplasts, but no starch. The bundle sheath chloroplasts only rarely had grana stacks and then of only 2 to 3 lamellae. Some starch grains were evident. There was little change seen in the morphology of the top section when compared to the center. Plasmodesmata were evident in all sections.

The Chl content, fresh weight, and dark respiration rates are presented in Table I. No attempt was made to cut equilinear sections which may account for roughly equal fresh weights in the lower sections. The Chl content increased 6- to 7-fold from the base to the top of the leaf. The Chl *a*/*b* ratio was 2.5 ± 0.7, 3.1 ± 0.5, and 3.2 ± 0.5 for base, center, and top sections, respectively. The respiratory rate was highest in the base section, more so, on a Chl rather than fresh weight basis. The high respiration rate was not unexpected since cell division and major synthesis of cell components occurs in the intercalary meristem contained in the base section. Marsh and Goddard (18) reported a similar finding comparing young with old carrot leaves.

Light Saturation. Light saturation curves were measured for

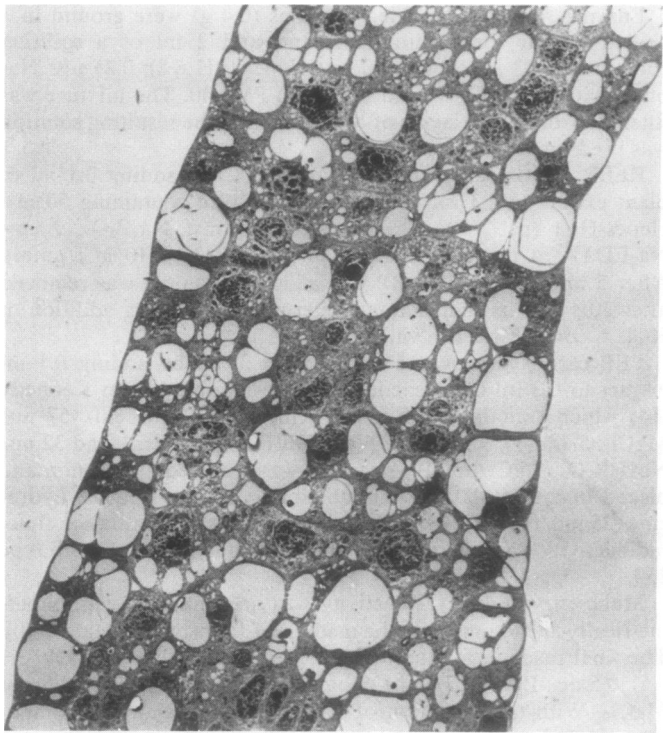

FIG. 1. Cross-section of the base section taken from the third leaf of a 12-day-old maize plant. Magnification, 1,400-fold.

Table I. *Rates of Dark Respiration in Top, Center, and Base Sections*

For each measurement two or three sections of tissue from each age were placed in a 15-ml Warburg vessel containing 1 ml water. The center wells contained 0.25 ml 1 M ethanolamine to trap CO_2. O_2 uptake was monitored in a Gilson differential respirometer at 30 C. At the end of the experiment the tissue was extracted with 80% acetone for Chl determination (1). Each value represents the average of four experiments.

Tissue Source	Fresh Weight per Section	Respiration Rate: O_2 Uptake	Chl per Section	Respiration Rate: O_2 Uptake
	mg	*μmol/g fresh wt·h*	*mg*	*μmol/mg Chl·h*
Top	36 ± 2	28 ± 2	31 ± 1	15 ± 2
Center	18 ± 4	23 ± 2	36 ± 1	27 ± 3
Base	22 ± 2	42 ± 6	5.8 ± 0.2	192 ± 37

each section and compared with the whole leaf of a 14-day-old plant. Time courses of CO_2 fixation were run at irradiances up to 1,500 w/m^2. Tubes illuminated at the desired irradiance were flushed for 2 min with compressed air prior to $^{14}CO_2$ injection. CO_2 fixation was linear throughout each experiment. Although light saturated rates were different for the three sections and whole leaves, all reached saturation at about 170 w/m^2. At this light intensity, the fixation rate in μmol CO_2/mg Chl·h was 105 (top), 121 (center), 38 (bottom), and 26 (4-day-old leaves), respectively. Light-induced CO_2 fixation was evident in all sections including the base. However, the sections prepared by Baker and Leech (2) below the 4th cm from the leaf base were reported to be photosynthetically competent with respect to O_2 evolution but not CO_2 fixation.

Photosynthetic and Dark $^{14}CO_2$ Fixation Products. A time course of the major products labeled during photosynthesis by maize sections is shown in Figure 2. In all sections about 80% of

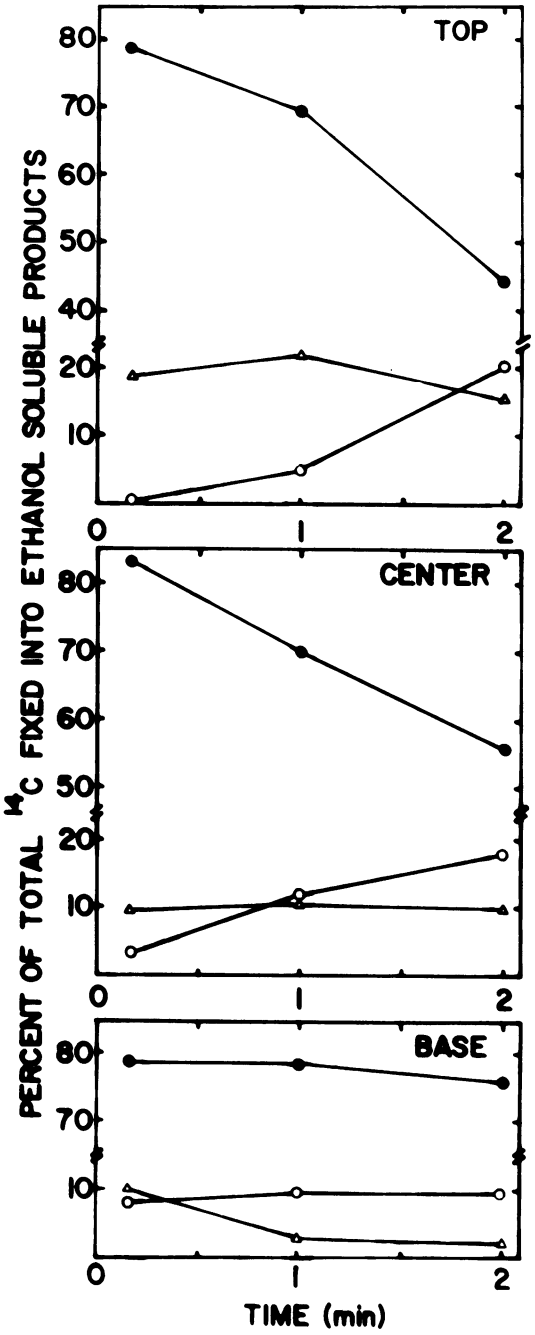

FIG. 2. Distribution of isotope in fixation products from maize leaf sections as a function of time at an irradiance of 170 w/m^2. Tubes were flushed with air for 2 min while being irradiated after which $^{14}CO_2$ was injected. Tissue was extracted at the time indicated. Sugar phosphate (fructose 1,6-bisP, fructose-6-P, glucose-6-P, RuBP) (○); PGA (△); malate plus aspartate (●). The remaining compounds (totalling less than 10%) not represented here were citric acid, alanine, and unidentified organic and amino acids.

the label fixed in 10 s was found in malate plus aspartate. In the top and center sections this dropped to roughly 50% in 2 min with a concomitant rise in sugar phosphates. Label in PGA remained constant. In contrast, label in PGA in the base dropped from an initial value of 10% to about 3% whereas that in malate plus aspartate and sugar phosphates increased slightly. In all sections, label in alanine, citrate and unidentified organic and amino acids increased very little.

CO_2 fixation at 850 w/m^2 showed approximately the same labeling pattern in the top and center sections as did fixation at an irradiance of 170 w/m^2 with the exception that label in malate plus aspartate was initially 10% lower and PGA declined slightly. In the base, the label in malate plus aspartate was also about 10–15% lower than that at 170 w/m^2 but remained at this level for the entire 2 min. This was offset by a higher level of label in sugar phosphates (data not shown).

The label in malate plus aspartate during 2 min of dark fixation remained about 85% in the top and center section whereas that in sugar phosphates, alanine, and PGA were constant at a very low level (Fig. 3). Labeling in citrate and unidentified organic and amino acids rose slowly with time. In the base, isotope in the malate plus aspartate fraction dropped rapidly from over 90% initially to 70% whereas in the same time period PGA declined from an initial value of 8% to almost zero. A concomitant rise was seen in citrate, alanine, and the remaining compounds accounting for roughly 30% of the incorporated radioactivity at the end of the experiment.

Pulse-Chase Experiments. These were formed in two ways. In the first (Fig. 4), the pulse of $^{14}CO_2$ was presented to the sections in the dark, followed by an air-flushing with the sections remaining in the dark. In the second type (Fig. 5), the plant material was illuminated at the desired irradiance in the presence of air, followed by a 10-s pulse of $^{14}CO_2$ and then an air flushing at the same light intensity.

Label in malate plus aspartate declined slightly with time during the "chase" period in the top and center sections kept under darkness (Fig. 4). Sugar phosphates, alanine, and PGA contained very little isotope and remained constant throughout whereas citrate and the remaining compounds rose slowly with time following an initial decline. In the base tissue, label in malate plus aspartate dropped with time. This decline was offset by an increase in radioactivity in citrate and unidentified amino and organic acids.

In the top and center sections irradiated at 170 w/m^2, the percentage of label in malate plus aspartate declined with moderate or little increase in the sugar phosphates and PGA (Fig. 5). The percentage of isotope in malate and aspartate remained constant in the base section but the sharp decrease in PGA was offset by an increase in the sugar phosphate fraction. In all sections, compounds other than sugar phosphates, PGA, and malate and aspartate such as alanine, citrate, and other organic and amino acids accounted for roughly 14% of the label by the termination of the experiments (60 s of the "chase" period).

A pulse-chase experiment was also carried out at the higher light intensity of 850 w/m^2 (data not shown). All sections showed a labeling pattern roughly similar to that illustrated in Figure 5 except that malate plus aspartate was about 10% lower at the end of the pulse. In the top section, PGA dropped from 18% following the pulse to 10% 10 s later. In the base, label in sugar phosphates rose from 6 to almost 20% while PGA went from 13% to less than 1%.

Pulse-chase both under darkness and in light were carried out with 4-day-old leaves. In a dark experiment similar to that described in the legend to Figure 4, malate and aspartate accounted for 80% of the radioactivity at the onset of the chase period and was little changed upon the introduction of $^{12}CO_2$. PGA initially accounting for roughly 10% dropped to nil with time and a concomitant rise in citrate and the remaining amino and organic acids was seen. During pulse-chase experiments at irradiances of 170 w/m^2 (see Fig. 5 for experimental conditions) and 850 w/m^2 responses similar to the top and center sections were recorded (data not presented). Thus malate and aspartate accounting for the bulk (70%) of the radioactivity prior to the introduction of $^{12}CO_2$ decreased during the chase and was accompanied by a decrease in PGA and a rise in the sugar phosphate fraction.

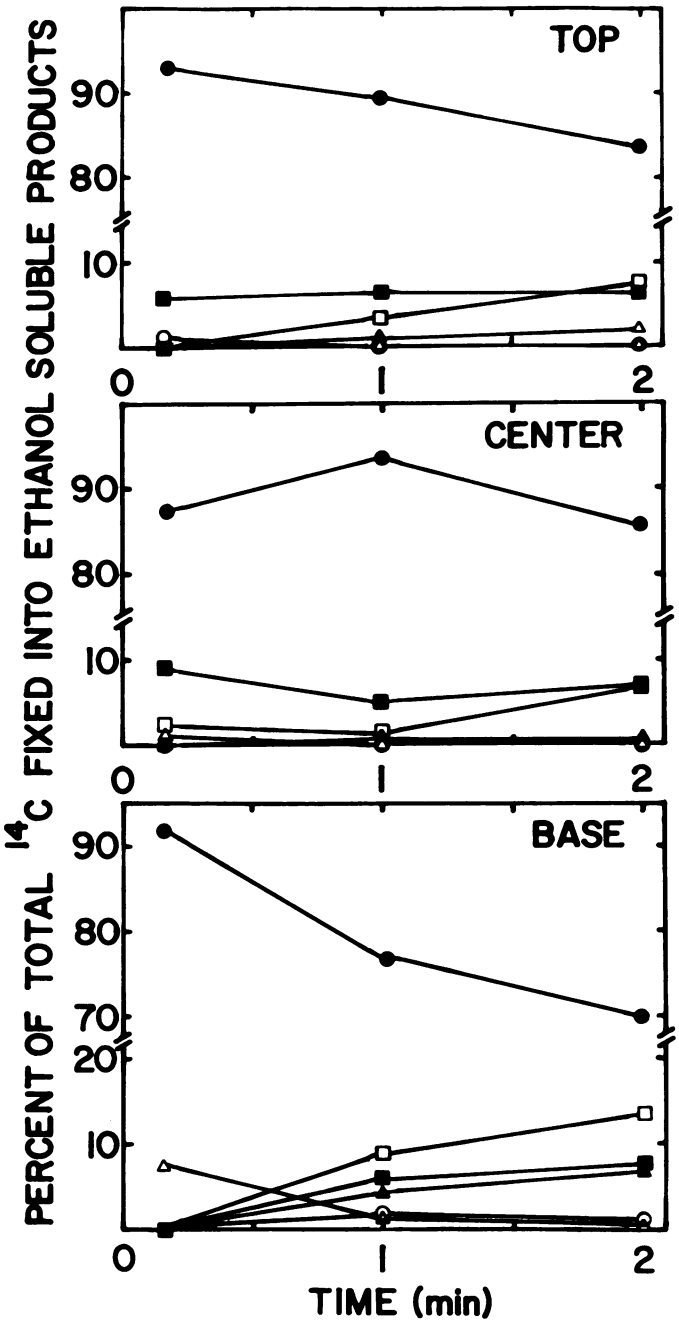

FIG. 3. Time course of isotopic distribution in dark fixation products from maize leaf sections. The tubes were flushed with air for 2 min in the dark after which $^{14}CO_2$ was injected and the tissue remained in the dark until the experiment was terminated at the times indicated. Sugar phosphates (fructose 1,6-bisP, fructose-6-P, glucose-6-P, RuBP) (○); PGA (△); malate plus aspartate (●); alanine (▲); citrate (□); remaining compounds (mainly organic and amino acids) (■).

Sensitivity of Photosynthesis to O_2. A well established photosynthetic characteristic of C_4 type plants is their relative indifference to levels of O_2 when contrasted to C_3 plants (31). A closer examination of O_2 sensitivity was undertaken by measuring the photorespiratory rate in the leaf sections, 4-day-old plants, and pea leaf discs using the technique of Zelitch (30). Of the sections photorespiration was evident only in the base as monitored by light/dark ratios of CO_2 release rates greater than one and approached that seen in pea leaf discs (Table II). The rate (slope) of

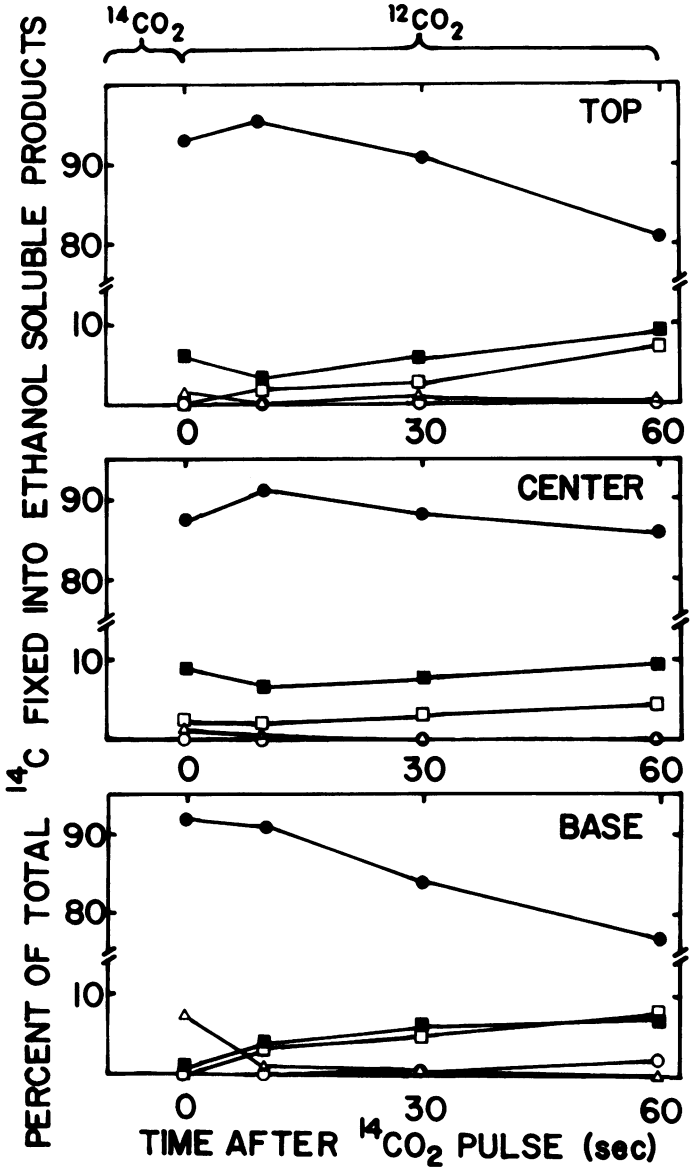

FIG. 4. Distribution of isotope in fixation products from maize leaf sections after a pulse-chase experiment carried out in the dark. Tubes were flushed with air for 2 min, after which $^{14}CO_2$ (8.6 μCi/μmol) was injected through the serum cap. After a 10-s pulse with $^{14}CO_2$, the cap was removed and the tubes were again flushed with air in the dark for the duration of the experiment. Tissue was killed and extracted at the times indicated. Sugar phosphates (fructose 1,6-bisP, fructose-6-P, glucose-6-P, RuBP) (○); PGA (△); malate plus aspartate (●); citrate (■); remaining compounds (mainly organic and amino acids) (□). Alanine was omitted since in the top and center sections, this amino acid was essentially unlabeled. In the base, the value increased from nil to roughly 5% during the chase period.

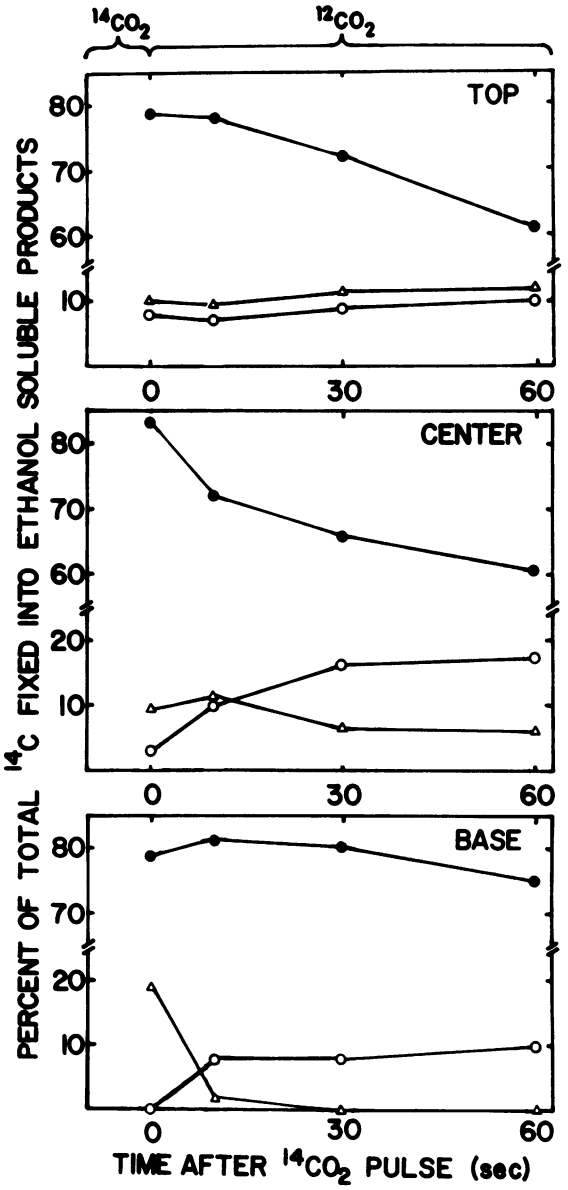

FIG. 5. Distribution of isotope in photosynthetic products from maize leaf sections after a pulse-chase experiment carried out in the light. Tubes were flushed with air for 2 min while being irradiated at 170 w/m^2 after which $^{14}CO_2$ (9.6 μCi/μmol) was injected. After 10 s, the serum cap was removed and tubes were again flushed with air in the light for the duration of the experiment. Tissue was extracted at the times indicated. Sugar phosphate (fructose 1,6-bisP, fructose-6-P, glucose-6-P, RuBP) (○); PGA (△); malate plus aspartate (●). The remaining compounds not represented here were alanine, citrate, and other organic and amino acids. After 1 min their approximate percentages were alanine, 2; citrate 3; and remaining compounds, 9, in all sections.

release by the sections in the light increased as the O_2 concentration was increased in the gas phase. The rate of release in the dark was little affected by O_2 concentration. In 4-day-old leaves the same pattern was seen as far as light/dark ratios were concerned but the rates appeared independent of O_2 concentration.

Enzyme Activities. PEP carboxylase, RuBP carboxylase, and NADP malic enzyme activities were measured in each tissue section (Table III). In all sections, PEP carboxylase had a higher activity than RuBP carboxylase or NADP-malic enzyme. The ratio increased from top to base with an intermediate value recorded for the center section. In the top and center sections, RuBP carboxylase was roughly equivalent to NADP while malic enzyme in the base this carboxylase was double that of the decarboxylating enzyme.

DISCUSSION

An attempt has been made in the last decade to relate a number of distinguishing characteristics to C_4 photosynthesis. These include Kranz anatomy, response of photosynthesis to O_2, lack of evolution of CO_2 in the light, a high level of PEP carboxylase with

Table II. *Comparison of $^{14}CO_2$ Released in Light and Dark by Maize Leaf Sections, 4-Day-Old Maize Leaves, and Pea Leaf Discs*

About 250 mg of tissue were placed into a 125-ml Warburg flask containing 2 ml distilled H_2O. The vessel was placed in a Warburg bath maintained at 30 C, shaken, and flushed with humidified CO_2-free air at an irradiance of 100 w/m^2 for 45 min to assure stomatal opening. Gas flow rate was 600 ml/min throughout the experiment. The vessel was subsequently sealed and a known amount of $^{14}CO_2$ was injected into the flask through a rubber serum stopper. The tissue was allowed to assimilate the added $^{14}CO_2$ for 45 min.

The top and center sections received 0.33 μmol of $^{14}CO_2$ (60 μCi/μmol) base and 4-day-old leaves 33 nmol (60 μCi/μmol); and pea leaf discs, 1 μmol (10 μCi/μmol). The pea leaf discs were cut with a No. 2 cork borer (4 mm diameter) from fully expanded leaves of 3-week-old plants. At the end of the assimilation period the flasks were flushed with either humidified N_2, O_2, or CO_2-free air. The gas exiting the flask was passed through 50 ml 0.1 M ethanolamine. Samples of 0.3 ml were collected at 3-min intervals for 45 min at which time the light was turned off. Sampling was continued for an additional 45 min. Rates were determined by linear regression. Each value represents the average of three determinations.

Tissue Source	Ratio of CO_2 Release Rates Light/Dark		
	100% N_2	Release into CO_2-Free Air	100% O_2
Top	0.07 ± <0.01	0.01 ± <0.01	0.04 ± 0.01
Center	0.26 ± 0.09	0.06 ± <0.01	0.06 ± <0.01
Base	1.2 ± 0.3	1.3 ± 0.1	1.8 ± 0.1
4-Day-old leaves	2.3 ± 0.4	1.8 ± 0.3	1.4 ± 0.3
Pea leaf discs	1.40 ± 0.1	2.0 ± 0.3	3.2 ± 0.7

Table III. *PEP Carboxylase, RuBP Carboxylase, and NADP Malic Enzyme Activities in Top, Center, and Base Sections of Maize*

Each value represents the average of three determinations.

Enzyme Source	Enzyme Activity				
	NADP Malic Enzyme	PEP Carboxylase	RuBP Carboxylase	Ratio[a]	Ratio[b]
	μmol/mg Chl·h				
Top	90 ± 6	258 ± 18	85 ± 17	3.0	2.9
Center	44 ± 4	232 ± 16	57 ± 13	4.1	5.2
Base	14 ± 3	137 ± 16	29 ± 6	4.7	9.6

[a] PEP carboxylase to RuBP carboxylase.

[b] PEP carboxylase to NADP malic enzyme.

respect to RuBP carboxylase and a flow of carbon between mesophyll and bundle sheath cells involving a shuttle mechanism linking PEP carboxylase with RuBP carboxylase. In the case of maize, the shuttle mechanism was reported to be malate-pyruvate with NADP-malic enzyme serving as the decarboxylation enzyme (8). In our study an attempt was made to determine whether some of these properties relate to tissue maturation expressed by the developing maize leaf and, in turn, to C_3-C_4 modes of photosynthesis since there have been reports of a transition in the assimilation of CO_2 during leaf maturation.

Over-all C_4 metabolism requires balanced rates of dicarboxylate production from PEP in mesophyll cells, transport of these products to the bundle sheath, decarboxylation in the bundle sheath cells, and net fixation in the Calvin cycle. Only the later autocatalytic reaction sequence is capable of increasing the plant's net fixed carbon levels (12). A relative deficiency in either the transport of malate, or in its decarboxylation in the bundle sheath cells, may account for the patterns of CO_2 fixation found here, especially in very young maize tissues.

Labeling data of early photosynthetic products showed that the bulk of the isotope was incorporated into malate and aspartate in all three sections (Fig. 2). With time, there was a decrease of labeling in the C_4 acids in the center and top sections. Coupled to a decrease of labeling in the C_4 acids was a substantial increase in the sugar phosphates. We interpret the data in Figure 2 to indicate that the C_4 pathway was present in the center and top sections. On the other hand, the pattern in the base tissue was unlike that seen in the older cells. In the base tissue the percentage of label in aspartate plus malate was virtually unchanged. The fraction of total label incorporated into PGA declined early and was offset by individual small increases in sugar phosphates, citrate, alanine, and other organic and amino acids.

To gain further insight into this difference in carbon assimilation, dark fixation (Fig. 3) and pulse-chase experiments both under darkness (Fig. 4) and in the light (Fig. 5) were carried out. Once again, the primary products of all tissues exposed to $^{14}CO_2$ for short periods both under darkness or in the light were aspartate and malate. Tissue development was an important factor in determining the subsequent fate of the newly fixed radioactivity (Fig. 2). Clearly the precursor-product relationship between malate plus aspartate and the sugar phosphates observed in the more developed tissues was not seen in the younger tissue.

A comparison of the products for dark $^{14}CO_2$ fixation in Figure 3 with that of light fixation in Figure 2 indicates that in addition to tissue age, irradiance was a crucial factor in determining isotope flow from the primary fixation products. Whereas in the light, the percent of radioactivity in the dicarboxylates remained constant in the base, in the dark, it fell from 92 to 70% in 2 min. The opposite was seen in the older tissue; in darkness malate plus aspartate tended to change relatively little.

Pulse-chase experiments carried out entirely in the dark (Fig. 4) or in the light (Fig. 5) tended to mimic the findings illustrated in Figures 3 and 2, respectively. Once again, light tended to retain the level of radioactivity in malate plus aspartate in the base accompanied by a fall in PGA and an offsetting rise in sugar phosphates. On the other hand, a light-dependent product-precursor type relation was observed between sugar phosphates and the dicarboxylates in the older tissue.

Before proposing an explanation for these findings, the distribution of the two carboxylating and the one decarboxylating enzyme followed in the sections needs consideration (Table III). Even though the absolute value of these three enzymes, determined on a Chl basis, were roughly 20–30% of those seen by others (11, 27), the relative values demonstrate the presence of the enzymes in proportion to the photosynthetic rates observed. The discrepancy in detected activity may have been caused by differences in extraction procedures resulting in only partial activity. However, the absolute values for the three enzymes and the enzymic ratios presented in Table III are similar to those published earlier (7) with extracts made with 4-day-old leaves of the same variety, Early Fortune. More pertinent were the increasing ratios of PEP carboxylase to RuBP carboxylase and to NADP-malic enzyme with respect to tissue differentiation. Nearly identical ratios for the two carboxylase activities were reported by William and Kennedy (26) for young and mature maize leaves. Our results were also consistent with the literature reports that in plants such as maize PEP carboxylase and NADP-malic enzyme are usually in excess compared to RuBP carboxylase.

It has been demonstrated in more mature tissue of maize that RuBP carboxylase located in bundle sheath cells was less readily extracted than the mesophyll-localized PEP carboxylase due to the distinctive arrangement of mesophyll and bundle sheath cells (3). The increase, of the two carboxylases, between the less differentiated base and the more mature top section, was roughly equivalent and was taken as evidence that extraction was representative. Finally, the rate of photosynthesis of 16 μmol CO_2/mg

Chl·h for 4-day-old leaves was similar to that found earlier for chloroplasts isolated from the same material and compared closely with the level of RuBP carboxylase reported then (7).

Hatch *et al.* (9) have shown that enzymes such as the NADP-malic enzyme and RuBP carboxylase in C_4 plants adjust to the increasing Chl content during greening. The base sections used here were taken from the third leaf enclosed in a sheath formed by the first and second leaves. Thus, the young tissue received less light than the top or center sections from the same leaf. This may account for very high ratios of PEP carboxylase to the other two enzymes in question. However, these enzymes assayed in extracts made from whole leaves of 4-day-old plants where shading was a lesser factor yielded similar results.

It is appropriate to consider tissue and cellular location of PEP carboxylase, RuBP carboxylase and NADP-malic enzyme, the two carboxylating and the one decarboxylating catalysts considered necessary for a functioning C_4 pathway in maize. In maize, Kanai and Edwards (11) have presented convincing evidence that PEP carboxylase resides in the parenchyma mesophyll cells while the other carboxylase and the decarboxylating NADP-malic enzyme were localized in the bundle sheath cells. There is little doubt that RuBP carboxylase was entirely localized in the parenchyma sheath chloroplasts. Cellular location of NADP-malic enzyme needs clarification. In early studies (8, 22, 23), it was characterized as a chloroplast associated enzyme. Gibbs *et al.* (7) and Weidner *et al.* (25) presented evidence that the NADP-malic enzyme was located outside of the bundle sheath maize chloroplast. If CO_2 produced in the cytoplasm of maize was assimilated by the photosynthetic carbon reduction cycle in these cells, so should CO_2 from the atmosphere be available to RuBP carboxylase since there exists in leaves of C_4 plants free spaces through which CO_2 could diffuse to bundle sheath cells (6).

We interpret our tracer and enzyme findings to indicate that the C_4 pathway functioned in the more differentiated tissue, represented by the center and top sections, and possibly the 4-day-old leaves. We have shown in the base sections similar to the upper sections that the predominant fixation products were aspartate and malate but that isotopic transfer in terms of CO_2 was apparently rate-limiting from these compounds to RuBP possibly due to lack of availability of the C_4 acids to malic enzyme.

We were impressed by the high level of isotope remaining in the base tissue in aspartate and malate and its decreased utilization in the light (Figs. 2 and 5) in contrast to the dark (Figs. 3 and 4). Under darkness, these compounds are readily metabolized by the citric acid cycle since isotope was found in compounds such as citrate and alanine. This rapid flow into such compounds was consistent with the higher rate of respiration in the less differentiated tissue (Table I). On the other hand, light apparently impaired this route.

We have shown that the activity of PEP carboxylase with respect to RuBP carboxylase and more importantly to the NADP-malic enzyme was highest in the young tissue (Table III). This results, we believe, in an imbalance between the carboxylating and decarboxylating mechanisms leading to an excess of malate and aspartate in the young tissue. We propose that the excess of dicarboxylates are subject to conversion to PEP within the mesophyll cell. Initially malate and aspartate would be transformed to oxaloacetate by NADP-malate dehydrogenase and aspartate aminotransferase, respectively. The α-ketodicarboxylic acid would be decarboxylated to pyruvate by oxaloacetate decarboxylase. Finally, phosphorylation of pyruvate catalyzed by pyruvate Pi, dikinase would result in the formation of the CO_2 acceptor. This explanation further requires that the $^{14}CO_2$ released within the mesophyll cell be partially preferred to atmospheric CO_2 to account for our observation that the percent of isotope remained fairly constant during the pulse-chase experiments carried out in the light (Fig. 5). Further supporting evidence for a limited communication between the two carboxylases in the young tissue was the PGA-sugar phosphate patterns illustrated in Figure 5. Here PGA resulted from a direct carboxylation of RuBP primarily with atmospheric CO_2. The evidence for this arises from the fact that once $^{14}CO_2$ was replaced by $^{12}CO_2$, PGA radioactivity declined to near zero as sugar phosphates rose. Only a negligible increase in label in sugar phosphates was seen after 30 s and no label was evident in PGA (compare to the top and center sections).

It seems that the stage of tissue development may affect the expression of photosynthetic CO_2 assimilation when two distinct carboxylases are present. In the present work with maize, PEP carboxylase remained the dominant CO_2 fixing enzyme at all stages, as indicated by the bulk of newly incorporated $^{14}CO_2$ remaining in malate and aspartate. In addition, the maize vascular bundles were surrounded by sheath cells in all sections (Fig. 1). This pattern of CO_2 fixation differed in *Mollugo* (21), where only the older leaves were stated to have Kranz anatomy. The youngest sections of maize, which contained chloroplasts that had not yet differentiated into granal and agranal types, seemed either to lack a fully functional NADP-malic enzyme or malate was unavailable to the enzyme. Perhaps chloroplast differentiation and the development of a nonlimiting (fully functional) CO_2 shuttle mechanism are associated.

Response to O_2. Plants have been separated into two groups according to their photorespiratory response to various O_2 concentrations. Those plants whose photosynthetic rate responded to O_2 characteristically had higher respiratory rates when measured in terms of light to dark release ratios of $^{14}CO_2$ derived from newly assimilated carbon *i.e.* values of 3 or more (26, 30, 31). In contrast, a light to dark ratio of one or less was correlated with the "O_2-independent" plants such as maize (26) and *Portulaca* (13, 14).

The photorespiratory data presented in Table II showed that in all three gas treatments (N_2, 21% O_2, 100% O_2), when the light was turned off there was a decrease in CO_2 release by the base tissue and the 4-day-old leaves resulting in a light/dark ratio higher than 1. We interpret these ratios to indicate an altered light respiration since the slopes representing $^{14}CO_2$ evolution in the light changed with O_2 concentration while the slopes in the dark were relatively unaffected (data not shown). In the developmental sequence it was apparent that the maize tissue represented by the base section and the intact leaves taken from young plants began to resemble the response in pea leaf discs. In sharp contrast, the top and center slices always displayed dark release rates far in excess of those in the light, *i.e.* light/dark ratios much less than 1 characteristic of mature maize plants (27). These data were consistent with compensation points of 1 and 2 μl/l determined for the top and center sections, respectively, using an open system IR gas analyzer. Attempts to obtain a compensation value for base material were not successful.

We are fully aware of the claimed pitfalls of evaluating photorespiration by the light/dark ^{14}C assay (4). We are wary of the fact that the light/dark ratios in the base section and the 4-day-old leaves did not approach 3, characteristic of so-called "high photorespirers." In contrast to these plants, the undifferentiated tissues evaluated here contained fairly high levels of PEP carboxylase (Table III and ref. 7) which may account in part for the lower light/dark ratios. Nonetheless, we remain impressed by the striking difference in photorespiratory response between the less and more developed tissues represented by base and top sections. In agreement with other reports, we conclude that photorespiratory activity was affected by the developmental stage of the tissue. These workers evaluated photorespiration in intact leaves of maize (26) or *Mollugo nudicaulis* (21) taken from differing positions on the same plant rather than from successive sections of the same leaf.

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