

# Appearance and Accumulation of C<sub>4</sub> Carbon Pathway Enzymes in Developing Maize Leaves and Differentiating Maize A188 Callus<sup>1</sup>

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## ABSTRACT

Regenerating maize A188 tissue cultures were examined for the presence of enzymes involved in C<sub>4</sub> photosynthesis, for cell morphology, and for <sup>14</sup>C labeling kinetics to study the implementation of this pathway during plant development. For comparison, sections of maize seedling leaves were examined. Protein blot analysis using antibodies to leaf enzymes showed a different profile of these enzymes during the early stages of shoot regeneration from callus from the closely-coordinated profile observed in seedling leaves. Pyruvate orthophosphate dikinase (PPDK) (EC 2.7.9.1) and phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) were found in nonchlorophyllous callus while ribulose 1,5-bisphosphate carboxylase (RuBPC, EC 4.1.1.39) and malic enzyme, NADP-specific (ME-NADP) (EC 1.3.1.37) were not detectable until later.

Enzyme activity assays showed the presence of ME-NADP as well as PEPC and PPDK in nonchlorophyllous callus. However, the activities of ME-NADP and PEPC had properties similar to those of the enzymes from C<sub>3</sub> leaves and from etiolated C<sub>4</sub> leaf tissues, but differing from the corresponding enzymes in the mature leaf.

Immunoprecipitation of *in vitro* translation products of poly(A)RNA extracted from embryoid-forming callus showed both the 110 kilodalton precursor to chloroplast PPDK and the 94 kilodalton polypeptide. Therefore, the chloroplast tye of PPDK mRNA is present prior to the appearance of leaf morphology.

Analysis of the labeled products of <sup>14</sup>CO<sub>2</sub> fixation by nonchlorophyllous calli indicated  $\beta$ -carboxylation to give acids of the tricarboxylic acid cycle, but no incorporation into phosphoglycerate. With greening of the callus, some incorporation into phosphoglycerate and sugar phosphates occurred, and this increased in shoots as they developed, although with older shoots the increase in  $\beta$ -carboxylation products was even greater. Analysis of enzyme levels in young leaf sections by protein blot and of <sup>14</sup>C-labeling patterns in the present study are in general agreement with enzyme activity determinations of previous studies, providing additional information about PPDK levels, and supporting the model proposed for developing young leaves.

These results suggest that maize leaves begin to express C<sub>4</sub> enzymes during ontogeny through several stages from greening and cell differentiation as seen in the callus and then shoot formation, and finally acquire capacity for full C<sub>4</sub> photosynthesis during leaf development concomitant with the development of Kranz anatomy and accumulation of large amounts of enzymes involved in carbon metabolism.

C<sub>4</sub> plants have unique anatomical and biochemical characteristics which are important for functioning of the C<sub>4</sub> pathway (15, 16). The significance of Kranz anatomy for C<sub>4</sub> photosynthesis has been considered frequently; however, the results are controversial. For example, some plants possessing Kranz anatomy were reported to lack functional C<sub>4</sub> photosynthesis (10, 17).

On the other hand, unorganized tissue cultures of some other C<sub>4</sub> plants were reported to have low photorespiration rates (18) and 'all elements' of the C<sub>4</sub> and C<sub>3</sub> photosynthetic carbon fixation pathways (21). After exposure of *Gisekia pharnaceoides* L. tissue culture to <sup>14</sup>CO<sub>2</sub> for a short period, 22% of label was found in malate while most of the rest was in C<sub>3</sub> cycle intermediates (29). It was concluded that the reductive pentose phosphate cycle and  $\beta$ -carboxylation were present but not the C<sub>4</sub> pathway. In pale green callus of *Amaranthus retroflexus* L., predominant products of short term photosynthesis were malate and aspartate, but there was no indication of carbon transfer to C<sub>3</sub> compounds (33). In a study involving both callus and regenerating leaf tissue, Ruzin (28) reported that C<sub>4</sub> photosynthesis did not occur in callus derived from the C<sub>4</sub> dicot *Euphorbia degeneri*, but developed concomitant with shoot regeneration.

We have reexamined the relationship between Kranz anatomy and the C<sub>4</sub> photosynthetic capacity using both a regenerating maize tissue culture system and seedlings. Tissue culture provides an excellent system to study the significance of Kranz anatomy since undifferentiated callus lack such anatomy. Furthermore, one can look at the effect of appearance of chloroplasts in greening callus which still lacks the leaf morphology. Etiolated leaves or the base section of a leaf, however, already have organized leaf structure. This is the first report to use maize tissue culture for analysis of appearance of carbon pathway enzymes during differentiation.

Amounts of RuBPC,<sup>2</sup> PEPC, PPDK and ME-NADP were determined since these enzymes play important roles in C<sub>4</sub> plants. Since RuBPC and ME are localized in bundle sheath cells whereas PEPC and PPDK are localized in mesophyll cells in C<sub>4</sub> leaves, differential expression of these enzymes accompanying cell differentiation might be expected.

Of particular interest is the timing of the appearance of the chloroplast form of PPDK, the enzyme believed to catalyze the rate-limiting step in C<sub>4</sub> carbon metabolism in leaves. The 110 kD polypeptide synthesized with maize leaf poly(A)RNA has been assumed to have a 16 kD 'leader' which facilitates entry into the chloroplasts, where it would be processed to give 94 kD

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<sup>2</sup> Abbreviations: RuBPC, ribulose 1,5-bisphosphate carboxylase; PPDK, pyruvate orthophosphate dikinase; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; ME-NADP, malic enzyme, NADP-specific; MDH-NADP, malic dehydrogenase, NADP-specific; ID, interveinal distance.

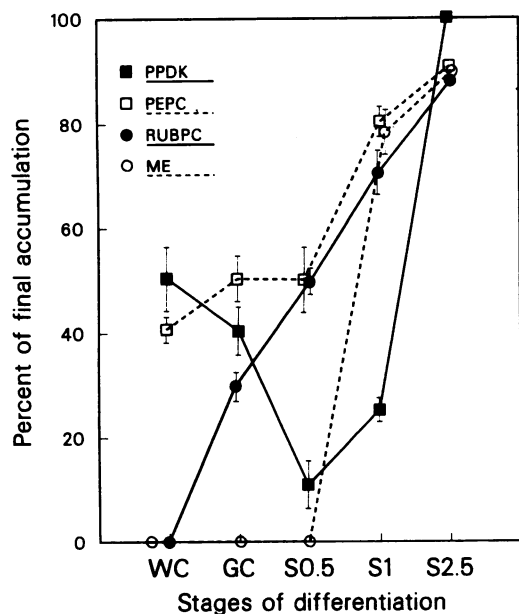


FIG. 1. Appearance and accumulation of PPDK, PEPC, RuBPC, and ME-NADP in early stages of differentiation of A188 tissue culture. The amounts of PPDK, PEPC, RuBPC, and ME-NADP were examined by protein blot analysis. The levels of enzyme polypeptides found in the 2.5 cm long maize seedling were taken as 100% and the relative amounts of enzyme protein in the sample were expressed as percentages of the final amount accumulated. WC, white callus; GC, green callus; S, shoot; the numbers adjacent to S indicate the length of the regenerated shoots in cm. For WC and GC, five replicate experiments were performed; for the other points, there were three replicate experiments. Vertical bars indicate SE.

PPDK polypeptide (12). This 110 kD polypeptide can be taken up and processed by isolated spinach chloroplasts (3). Poly(A)RNA from mature leaves of both maize and wheat gives the 110 kD *in vitro* translation product, whereas poly(A)RNA from seeds of either maize or wheat gives the 94 kD translation product (2) and we assume that *in vivo* when the 94 kD polypeptide is made in the cytoplasm (without leader) it remains there.

The properties of PEPC seem to undergo changes upon establishment of autotrophy in a  $C_4$  plant according to Deleens and Brulfert (9) who found that in 5 to 7 d old maize seedlings PEPC activity was low and had low sensitivity to glucose-6-phosphate, while after 7 d its properties shifted progressively towards those of the  $C_4$  photosynthetic type. The kinetic properties of PEPC in callus and in seedling tissue were therefore investigated in the present study.

We considered evidence for the early establishment of components of  $C_4$  metabolism based on patterns of  $^{14}C$  incorporation. The appearance of Kranz anatomy and the presence of enzymes are not sufficient criteria for the presence of complete  $C_4$  photosynthesis (10). Previous workers such as Perchorowicz and Gibbs (26) have already provided evidence, through pulse-chase labeling and enzymic studies, that complete  $C_4$  photosynthesis, involving intercellular carbon transport by  $C_4$  compounds is probably missing in the base section of young maize seedlings, appearing later in the middle and top sections of such leaves. Our emphasis was placed on the very early stages of shoot regeneration from callus, since little information is available for early transition and it is of interest to evaluate changes in enzyme presence and properties during the acquisition of autotrophy. The  $^{14}C$  tracer studies were for the purpose of following the relative importance of PEPC- and RuBPC-mediated carboxylation reactions during these early stages.

Sections of maize leaves have also been used for the study of

accumulation of carbon fixation enzymes (22, 26). It was suggested that cell differentiation may play an important role in regulating the appearance of these enzymes (22). In this study we use a similar approach to study the appearance and accumulation of enzymes in maize leaves of ordinary seedlings for comparison with the regenerated plant and to supplement previous work with respect to the appearance profile for PPDK. It has been suggested that the  $C_3$  pathway may precede the  $C_4$  pathway in the maize first leaf (8) so the effect of leaf position as well as effect of the age of the plant also was investigated.

## MATERIALS AND METHODS

**Plant Material.** Maize (*Zea mays* L. cv Golden bantam or inbred B73) was grown in vermiculite in a growth chamber with a quantum flux density of  $650 \mu E m^{-2} s^{-1}$  (combined incandescent and fluorescent lamps) with a 16 h photoperiod at  $27^\circ C$ . The plants were watered with modified half-strength Hoagland solution. The first leaves of maize were harvested 7 d after planting or when the seedlings reached 8 cm in length. The second leaves were harvested 12 d after planting or when the seedlings were 14 cm long. At this stage, the third leaf is still partly rolled inside the second leaf and no ligule is formed. In this case, the first leaf was peeled away so that the second and younger leaves could be used. The third leaf was harvested when it was about 15 cm in length and the fourth leaf was still rolled inside it. The mature leaves (fourth leaf) were 50 to 60 cm in blade length when used. Only the blade was used in this case, since removing the lower leaves without tearing the sheath of the mature leaf under study was difficult.

In all cases, the sections were made from the base to tip. For the young leaves, the first and second sections were 1 cm in length and the successive sections were 2 cm except the last two sections which were 3 cm. For the mature leaves, the blade was cut into larger sections to give the same total number (6–10) so that all the sections from a given leaf could be compared on a single gel. The leaf tissue of each section was weighed and 0.5 g fresh weight tissue per section was frozen in liquid  $N_2$  and stored at  $-80^\circ C$  until used.

**Maize Tissue Culture.** The seeds of inbred line A188 were kindly supplied by Mr. C. Armstrong, University of Minnesota. Some callus cultures were the generous gift from Dr. C. Rhodes of Calgene, Davis, CA and Mr. K. Lowe of Stauffer Co., Richmond, CA.

The tissue culture was initiated and maintained in accordance with the method of Armstrong and Green (4). The maize plants were grown in a greenhouse at the Botanical Garden, UC, Berkeley. Immature embryos were aseptically removed from the endosperm and were placed on N6 medium, as modified by Armstrong and Green (4). This medium contained 25 mM proline,  $0.5 mg L^{-1}$ , 2,4-D, and 0.7% agar. After 3 to 4 weeks of incubation at  $25^\circ C$  under cool fluorescent light ( $250 \mu E m^{-2} s^{-1}$ , 14 h photoperiod) regenerable embryonic cultures were obtained. For maintenance, the tissue cultures were transferred every 3 weeks to fresh culture medium containing  $1 mg L^{-1}$  2,4-D. For shoot induction, they were transferred to media without 2,4-D.

For dark treatment, the Petri dishes containing nonchlorophyllous callus cultures were wrapped in two layers of aluminum foil. After growth in the dark for a period of 1 d to 5 weeks, the tissue was frozen in liquid  $N_2$  until used or used immediately.

**Protein Blot.** The leaf, callus, or shoot tissues were homogenized with mortar and pestle in 3 volumes of buffer containing 0.1 M Tris (pH 7.4), 10 mM  $MgCl_2$ , 18% (w/v) sucrose, 1%  $\beta$ -mercaptoethanol, and 10 mM phenylmethylsulfonyl fluoride. The homogenate was filtered through nylon cloth and centrifuged at  $12,000 g$  for 15 min. The supernatant was adjusted to 0.1 M Tris (pH 7.5), 2% SDS, 10% glycerol (v/v), and 1%  $\beta$ -mercaptoethanol, and then was heated to  $100^\circ C$  for 1 min and

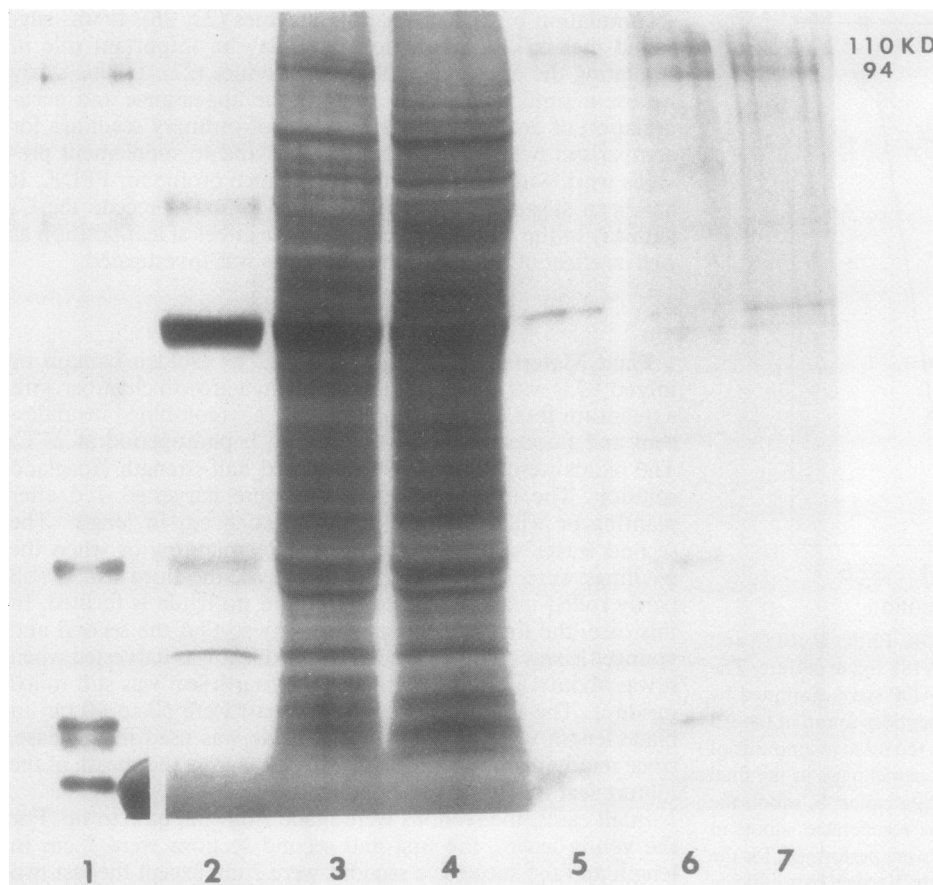


FIG. 2. *In vitro* translation of PPDK mRNA from maize leaf and chlorophyllous A188 callus. Analysis was by SDS-PAGE, followed by fluorography. Lane 1,  $^{14}\text{C}$ -labeled mol wt standards; from the top they are at 96, 69, 46, 30, and 14.3 kD, respectively (the bottom band is an artifact due to degradation); lane 2, *In vitro* translation product in the absence of mRNA; lane 3, total product of leaf poly(A)RNA; lane 4, total product of callus poly(A)RNA; lane 5, immunoprecipitation of translation product of lane 3; lane 6, immunoprecipitation of translation product of the first leaf poly(A)RNA; lane 7, immunoprecipitation of translation product of lane 4.

chilled on ice before SDS-PAGE analysis. Protein concentration was measured by the method of Bradford (6). Chl content and Chl *a*/Chl *b* ratio were determined by the method of Arnon (5).

Four identical 6.4 to 12.8% gradient SDS polyacrylamide gels were prepared. On two gels, an equal amount of soluble protein from each sample was loaded into each lane. On the other two gels, an equal volume of the plant extract was loaded into each lane. Electrophoresis was carried out at 30 mamp constant current for 3 to 4 h. After PAGE, the protein was electrophoretically transferred onto cyanogen bromide paper (1) at 1.2 amp for 90 min. The transfer paper was probed with anti-PPDK serum or anti-RuBPC serum, then with 0.5  $\mu\text{Ci}$  of  $^{125}\text{I}$ -protein A (Amersham, 36 mCi/mg) (24). Autoradiographs were prepared with Kodak AR5 X-ray film with intensifying screens at  $-80^\circ\text{C}$  overnight.

After the film was developed, the paper was probed a second time with either anti-PEPC serum or anti-ME-NADP serum. Antisera to maize leaf RuBPC and PEPC were kindly provided by Dr. W. Taylor, University of California, Berkeley (24). Antiserum to maize leaf ME-NADP was kindly provided by Dr. T. Nelson, Yale University. Antiserum to maize leaf PPDK was prepared as described previously (1).

**Dot Blot.** Plant extracts (10  $\mu\text{l}$  per dot) were blotted on nitrocellulose paper or cyanogen bromide paper using a DOT-BLOT apparatus (Bio-Rad Laboratories). Probing with antiserum was carried out by the same procedure as for the protein blot described above.

**Immunoprecipitation of PPDK Polypeptide Synthesized *In Vitro*.** Mature (fourth) leaves from 4 week old plants and young first leaves from 5 to 8 cm seedlings were harvested. Calli in which embryoids were forming were used without dissection to separate the undifferentiated part. Poly(A)RNA was extracted

from each tissue as previously described (2) and was subsequently translated *in vitro* in the presence of [ $^{35}\text{S}$ ]methionine in rabbit reticulocyte lysate (Amersham). The immunoprecipitation and fluorography were done as previously described (2).

**Enzyme Activity Assay.** Two g of maize leaves were homogenized in 3 volumes (w/v) of extraction buffer containing 0.1 M Tris (pH 7.5), 10 mM  $\text{MgCl}_2$ , 5 mM sodium pyruvate, 2 mM  $\text{K}_2\text{HPO}_4$ , 1 mM EDTA, 1% (w/v) sodium ascorbate, 5 mM DTT, and 1% (w/v) PVP. For callus, 2 g of tissue were homogenized in 1 volume (w/v) of this buffer. In either case, the homogenate was strained through Miracloth and centrifuged at 12,000g for 6 min. The supernatant was used to assay all enzymes except PPDK from callus. The activity of PPDK in the callus was too low to measure in the crude extract, given the requirement for demonstration of dependence on Pi and ATP.

For callus PPDK assay, 20 g of tissue were used. A precipitate obtained from  $(\text{NH}_4)_2\text{SO}_4$  (50–65%) fractionation of the supernatant was dissolved in 1 ml of a buffer (B) containing 0.1 M Tris (pH 7.5), 5 mM DTT, 10 mM  $\text{MgCl}_2$ , and 1 mM EDTA, and was passed through a Sephadex G-25 column preequilibrated with the same buffer. A portion was assayed for PPDK activity. For further purification of the enzyme, the eluate was next passed through a column of DEAE cellulose with a 0.1 to 0.5 M KCl gradient. About 80 fractions (1 ml each) were collected. Fractions containing active PPDK activity were pooled and the solution was brought to 70% saturation with  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate was desalted by dialysis against buffer (B) plus 2 mM  $\text{K}_2\text{HPO}_4$ .

Activities of PPDK and PEPC were assayed according to methods described previously (1). ME-NADP and ME-NAD activities were assayed by the methods of Hatch and Mau (13). Pyruvate kinase activity was assayed by following the decrease in absorption at 340 nm in an assay mixture containing 50 mM



FIG. 3. Effect of dark treatment of maize callus on PPDK content analyzed by protein blot. Each lane was loaded with 0.2 mg of soluble protein from: lane 1, chlorophyllous callus grown in the light; lane 2, white callus grown in the light; lane 3, white callus grown in the dark for 1 d; lane 4, white callus grown in the dark for 4 weeks; lane 5, white callus grown in the dark for 3 weeks followed by 1 week in the light. Small differences in intensity such as between lane 4 and lane 5 are probably due to sampling differences (since other experiments gels did not give the same differences) but the lower level of PPDK in the chlorophyllous callus grown in the light is significant.

Hepes buffer (pH 7.1), 1.6 mM ADP, 1 mM PEP, 0.1 mM NADH, 15 mM MgSO<sub>4</sub>, 50 mM KCl, and four units of lactic dehydrogenase in a total volume of 1 ml.

**Inhibition of Callus PPDK Enzyme Activity by Antibody.** PPDK antiserum was purified using protein A-Sepharose 4B column (Pharmacia Fine Chemical) according to the procedures described by the manufacturer. The final concentration of the purified IgG was 42 mg ml<sup>-1</sup>. Extract of each maize leaf or callus, containing 200 µg of soluble protein, was incubated for 30 min with 20, 40, 60, and 100 µl of purified PPDK antibody in 0.2 ml of 50 mM Tris buffer (pH 7.5) containing 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, and 1 mM DTT. After incubation, the mixture was centrifuged at 12,000 g for 40 min and an aliquot of the supernatant was assayed for PPDK activity.

**<sup>14</sup>C Incorporation Study.** Regenerating maize tissue culture (100 mg) at various stages of development was removed from the agar medium and placed in 0.5 ml of 5 mM Hepes buffer (pH 7.8) containing 3 mM NaHCO<sub>3</sub> in a glass homogenizing tube. After 2 min preillumination at 650 µE m<sup>-2</sup> s<sup>-1</sup>, 0.2 ml NaH<sup>14</sup>CO<sub>3</sub> solution was added to give a final concentration of 8.4 mM (25 mCi mmol<sup>-1</sup>) and illumination was continued for 10, 30, 60, or 120 s. Liquid N<sub>2</sub> was poured into each tube to stop the reactions. After 0.5 ml methanol was added to the frozen tissue and buffer, the mixture was homogenized as it thawed. An aliquot of this mixture was analyzed by two-dimensional paper chromatography and radioautography (25). Incorporation of <sup>14</sup>C into individual compounds was measured by cutting out each spot, eluting with 0.5 ml H<sub>2</sub>O in a counting vial, adding 20 ml of Aquassure (New England Nuclear) and counting by liquid scintillation. Chromatograms with added amino acid standards (about 50 µg each) were prepared and subsequent to radioautography were sprayed with 0.1% ninhydrin in ethanol and heated to develop color to aid identification of radioactive compounds on the chromatograms.

**Light Microscopy of Maize Tissue Section.** Callus was divided into chlorophyllous and nonchlorophyllous samples which were separately processed. Regenerated shoots of various sizes (0.5, 1–3, 8, and 15 cm) were removed from the plants regenerating from callus tissue and selected sections of these were cut into small pieces (about 5 mm × 5 mm). For example, the oldest regenerated shoot studied (15 cm) had five leaves and the tip 3 cm of the 8 cm fourth leaf was used. For comparison, a corresponding section of the third 10 cm leaf of a 3 week old maize plant grown in the growth chamber was used. Callus tissues were fixed in 4% glutaraldehyde in 0.5 mM phosphate buffer (pH 6.8), dehydrated in an ethanol series, and embedded in glycol methacrylate according to reported methods (11). These tissues were sectioned at 2 to 5 µm on a Sorval JB-4 microtome and stained with 0.05% Toluidine Blue for 5 min. Photomicrographs were taken on an Olympus microscope equipped with Microflex Model

Table I. Comparison of PEPC and PPDK Activities, Chl Content, and Chl a/Chl b Ratios in A188 Callus and Regenerated Shoots

One unit of enzyme activity is the amount of activity which catalyzes the conversion of 1 µmol of substrate per min under standard assay conditions. Enzyme activities are not expressed on an activity per unit of Chl basis, since the amount of Chl varies greatly with the samples. The values are the mean of duplicate samples.

Tissue	Total Chl	Chl a/b	Enzyme activities and K <sub>m</sub> values				PEPC/PPDK	
			PEPC	K <sub>m</sub> PEP	PPDK	K <sub>m</sub> pyr.	ratio	ratio
	µg/g fresh wt	ratio	units/g fresh wt	µM	units/g fresh wt	µM	ratio	ratio
Leaf <sup>a</sup>	780	3.2	4.4	600	0.24	250	18	
Green callus	38	2.8	0.08		0.01	80	8	
White callus	ND <sup>d</sup>	— <sup>c</sup>	0.17	50	0.04	80	5	
Regenerated leaf <sup>b</sup>	92	3.0	—	—	—	—	—	
Regenerated leaf <sup>c</sup>	1490	3.2	5.7	—	0.51	—	—	

<sup>a</sup> A second leaf of a seedling was used (for comparison, a PEPC activity of 15 units/g fresh weight was found in mature maize leaves). <sup>b</sup> A regenerated shoot (5 mm in length) was used. <sup>c</sup> A regenerated shoot (200 mm in length) was transferred from the Petri dish to a flask and was grown in atmospheric CO<sub>2</sub>. <sup>d</sup> Not detectable. <sup>e</sup> —, not measured.

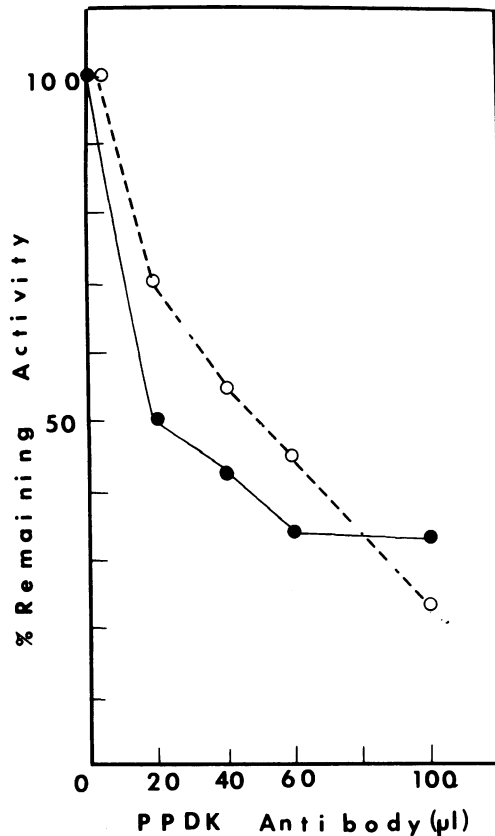


FIG. 4. Inhibition of PPDK enzyme activity by antibody. Maize leaf extract (O---O) and callus extract (●—●), each containing 200 µg of soluble protein, were incubated for 30 min with 20, 40, 60, and 100 µl of purified PPDK antibody solution (concentration of IgG = 42 mg ml<sup>-1</sup>). After incubation, PPDK activity of the supernatant was assayed. The activity was expressed as per cent of initial activity.

EFM camera with 35 mm Kodak technical pen film 2415.

Leaf tissues were fixed in 10% (v/v) acrolein overnight at 4°C, dehydrated in an ethanol series, and embedded in paraffin. Five to 10 µm thick sections were stained with Toluidine Blue for 1 min. Photomicrographs were made on a Nikon Phase contrast microscope equipped with a Nikon Polaroid camera.

## RESULTS

**Protein Blot Analysis of Enzymes of Carbon Metabolism during Early Stages of Development in A188 Tissue Cultures.** PPDK and PEPC were already present in white, nonchlorophyllous callus at about 50 and 40%, respectively, of the levels (w/w soluble protein) found in the young seedling (2.5 cm in size), whereas ME-NADP and RuBPC were not detectable at this stage, even though ME-NADP activity was detected at this stage (see below). According to a test performed on cross-reactivity, antibody raised against maize leaf ME-NADP reacts much less efficiently against the callus enzyme than against the leaf enzyme (relative reactivity about 1:3), and the level of ME-NADP in callus tissue must be less than 0.5% of the total soluble protein, so that with the maximum protein loading of the SDS gel there is not enough to detect.

RuBPC appeared upon greening of the callus and gradually increased in amount with shoot regeneration. The appearance of ME-NADP, as detected by protein blot, lagged behind that of the other three enzymes as it was observed for the first time only after the shoot had reached about 1 cm. The level of PPDK decreased once during the early stage of shoot formation, then

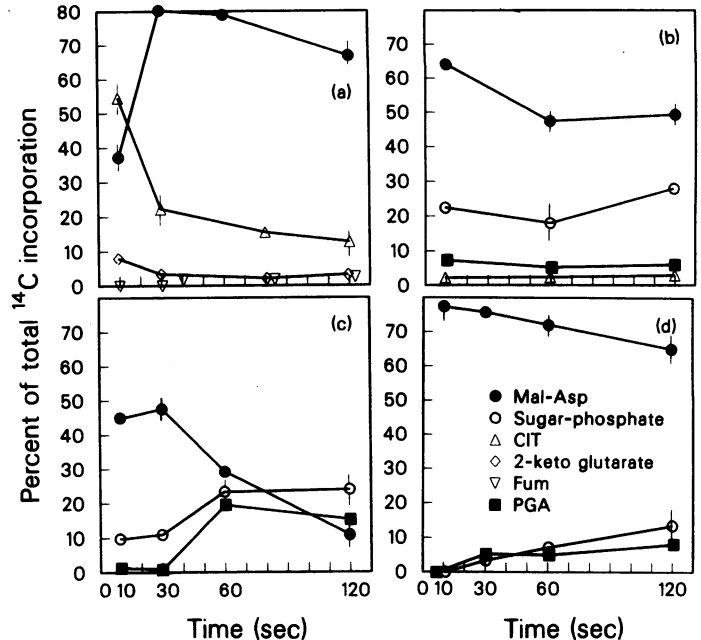


FIG. 5. Incorporation of <sup>14</sup>C into C<sub>4</sub> acids after exposure to [<sup>14</sup>C] bicarbonate in regenerating maize plant. The results are the mean of 2 or 3 replicates. Vertical bars indicate SE. Total incorporation of <sup>14</sup>C in dpm per g fresh weight in each case was as follows: a, nonchlorophyllous callus (6.0 × 10<sup>5</sup>); b, chlorophyllous callus (6.4 × 10<sup>5</sup>); c, 3 cm regenerated shoot (21 × 10<sup>5</sup>); d, 8 cm regenerated shoot (78 × 10<sup>5</sup>).

increased as the shoot developed (Fig. 1).

**PPDK Polypeptide Synthesized *In Vitro* from Seedling and Callus Poly(A)RNA.** When poly(A)RNA extracted from mature leaves (expanded second or later leaves) was translated *in vitro*, a 110 kD PPDK polypeptide was observed when reacted with maize leaf PPDK antibody (Fig. 2, lane 5). With either embryoid-forming callus or with young first leaves, however, two polypeptides with mol wt of 110 and 94 kD, respectively, were observed (Fig. 2, lanes 6 and 7).

**Light Independent Biosynthesis of PPDK in Callus.** In white callus grown in the dark for 1 d to 4 weeks there was no significant change in PPDK level even after 4 weeks (Fig. 3). When the callus was grown in the dark for 3 weeks and then was transferred to light and grown for 1 week, the level of enzyme did not increase; the level of PPDK polypeptide in white callus thus seems to be independent of illumination. Regulation of the synthesis of PPDK in callus differs from regulation in leaf tissue. In leaves, the synthesis of PPDK mRNA (12) and of PPDK polypeptides (14) are regulated quantitatively by light illumination whereas the level of PPDK in callus seems to be independent of light. Moreover, although the concentration of PPDK in the callus tissue is much less than in leaves, the apparent *K<sub>m</sub>* (pyruvate) is only about one-third as large (Table I).

**Enzyme Activity in A188 Callus.** Callus PPDK has low activity and, as has been the case for *Kalanchoë* (30) it was difficult to demonstrate ATP and Pi dependence. After dialysis against buffer containing phosphate (see "Materials and Methods"), a preparation showing pyruvate and ATP dependence was obtained, but dependence on Pi has not been demonstrated. PPDK activity tends to decrease when the enzyme is kept without Pi. Activity was strictly dependent on pyruvate concentration, giving a *K<sub>m</sub>* (pyruvate) of about 80 µM, less than one-third that of leaf PPDK (Table I). Inhibition of enzyme activity was linear with added purified PPDK antibody (Fig. 4), whereas this addition did not cause any decrease in PEPC activity, for which the assay is similar except for the absence of added PEP and the change in

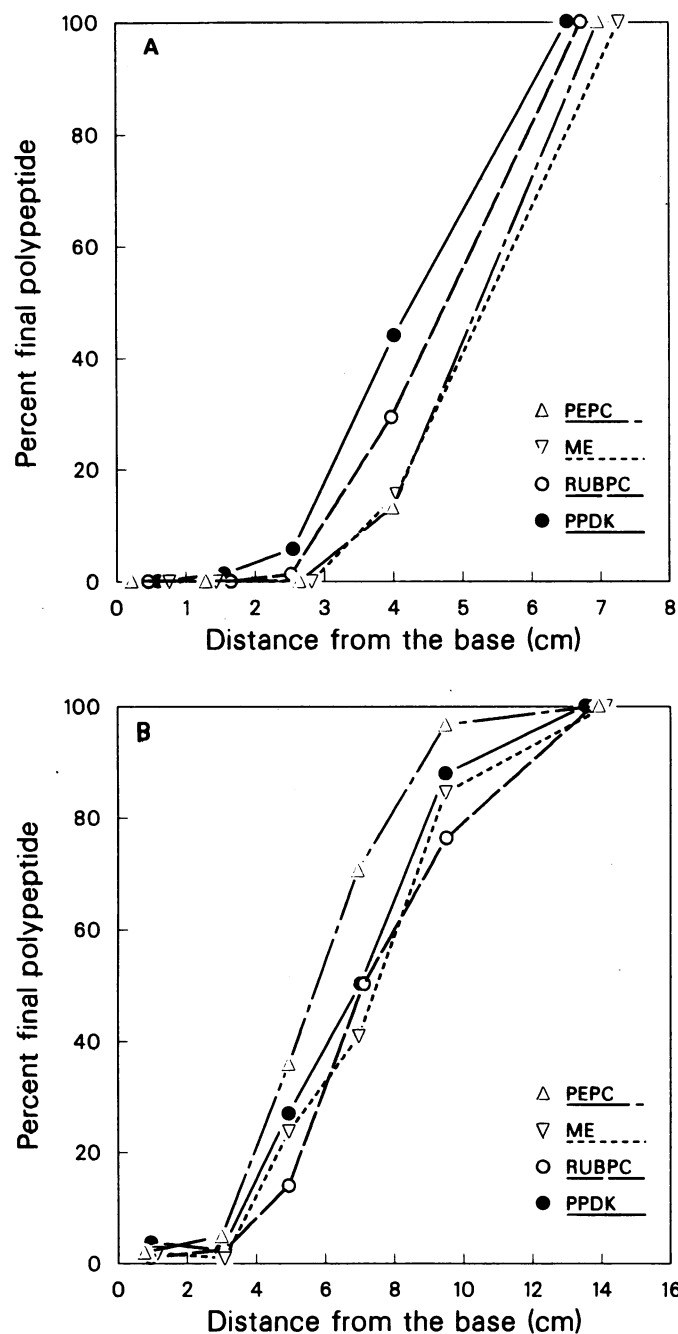


FIG. 6. Protein blot analysis of PPDK, PEPC, RuBPC, and ME-NADP in leaf sections. To each lane, extract containing 50  $\mu$ g of soluble protein was applied. After separation by SDS-PAGE and blotting, polypeptides were probed with antibodies against PPDK, PEPC, RuBPC, and ME-NADP. The relative levels of enzyme polypeptides, determined as described in "Materials and Methods," are expressed as per cent of the highest level reached, usually in the tip section of the leaf. A, The first leaf; B, the second leaf.

appropriate substrates. Incubation with Pi did not increase the PPDK activity of the callus enzyme. Since the callus tissue was grown under low light, the mechanism of activation of the callus PPDK might be different from that of the leaf enzyme and the enzyme may not be fully activated. Since any pyruvate kinase activity present in the assay mixture could interfere with the PPDK assay, a pyruvate kinase assay was performed but no pyruvate kinase activity was detected (data not shown).

A188 callus PPDK activity (expressed as unit/g fresh weight)

Table II. Content of Chl and of Soluble Protein in Second Maize Leaf Sections

Distance from Base	Total Chl	Chl a/b	Soluble protein
cm	$\mu$ g/g fresh wt	ratio	mg/g fresh wt
0-1	29	2.53	10
1-2	54	2.68	12
2-4	88	3.16	13
4-6	212	3.43	14
6-8	400	3.52	16
8-11	767	3.60	18
11-14	1074	3.65	22

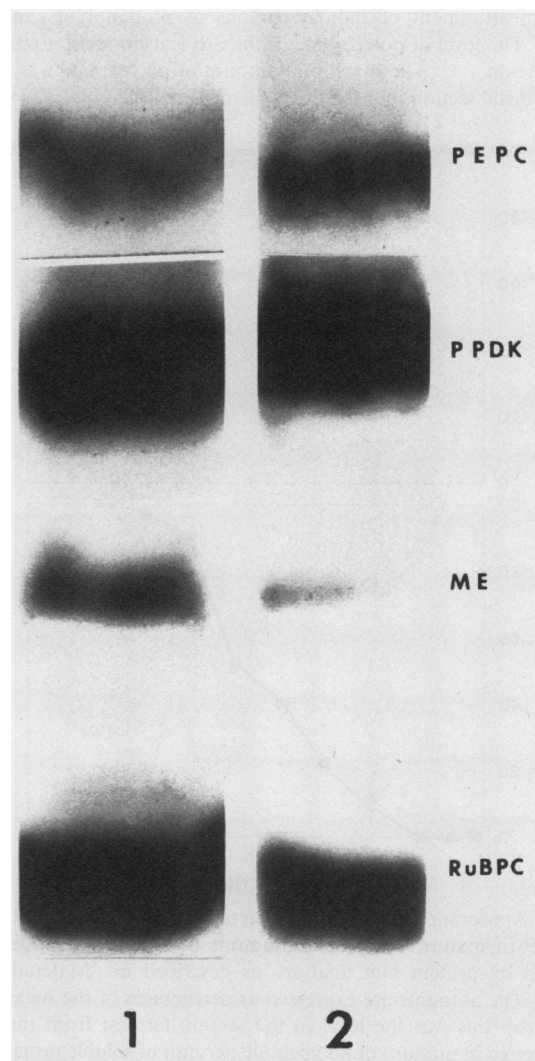


FIG. 7. Levels of PEPC, PPDK, RuBPC, and ME-NADP in the first leaf at 7 and 9 d after planting. Leaf extracts containing 50  $\mu$ g of soluble protein from each section were separated by SDS-Page, blotted, and probed with antibodies against PPDK, PEPC, RuBPC, and ME-NADP. While there was some increase in the amount of each of the polypeptides between 7 and 9 d, the relative increase in ME-NADP is by far the largest. Lane 1, The leaf was 10 cm in length and was harvested 9 d after planting; lane 2, the leaf was 7 cm in length and was harvested 7 d after planting.

was about 5 to 16% that of regenerated maize leaf while PEPC activity was 2 to 5% (Table I). The activity ratio of PEPC/PPDK in maize leaf extract was about 18 whereas this ratio in callus was 5 to 8, so that PEPC activity seems to be in great excess also in callus. For the two-step conversion of pyruvate to oxaloacetate,

Table III. Effect of Position in Maize Leaf on Levels of C<sub>4</sub> Enzymes

An amount of leaf extract containing 50  $\mu$ g of soluble protein was analyzed in each case.

Leaf Position	Enzyme Protein Level <sup>a</sup>			
	PPDK	PEPC	RuBPC	ME-NADP
	<i>relative</i>			
1st (tip)	60	60	60	45
3rd (base)	3	1	1	0.5
3rd (tip)	100 <sup>b</sup>	100	100	100
Mature (max) <sup>c</sup>	110	110	120	110

<sup>a</sup> Relative amounts of enzyme polypeptides were obtained by densitometric measurement of radioautographs of protein blots and dot blots. <sup>b</sup> The level of polypeptide in the 3rd leaf tip section was used for comparison. <sup>c</sup> The maximum amount of polypeptide was found in the less distal section, not the tip of the mature leaf.

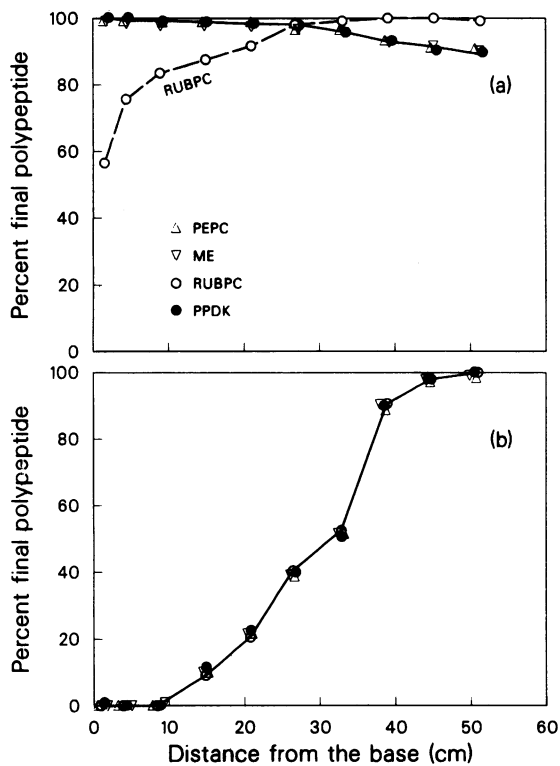


FIG. 8. Appearance and accumulation of PPDK, PEPC, RuBPC, and ME-NADP in mature leaves. The amounts of each polypeptide were determined by protein blot analysis, as described in "Materials and Methods." The amounts are expressed as percentages of the maximum level reached; this was the level in the section farthest from the base when expressed as amount of polypeptide per unit of soluble protein (b). On the basis of equal amounts of fresh weight (a), the levels of PEPC, PPDK, and ME-NADP were maximal in the base sections, RuBPC was maximal in the tip. a, An equal amount (50  $\mu$ g) of soluble protein was loaded into each lane; b, extract (20  $\mu$ l) from an equal fresh weight of leaf tissue was loaded into each lane. The amount of soluble protein per unit fresh weight increased towards the tip. The 'base' here means the base of the leaf blade next to the ligule.

the PPDK-mediated step would be rate-limiting as it is in the C<sub>4</sub> pathway in leaves (31). The  $K_m$  (PEP) of PEPC for maize leaf is 0.6 mM whereas that of callus is 0.05 mM.

The kinetic properties of maize callus PEPC are closer to the values reported by Ting and Osmond (32) for C<sub>3</sub> species than for the C<sub>4</sub> leaf type, and are consistent with those reported by Ruzin (28) for the enzyme in regenerating tissue culture of a C<sub>4</sub> dicot,

*Euphorbia degeneri*. Ruzin found that there are two isoenzymes of PEPC in leaf, but only one isoenzyme was present in callus.

ME-NADP activity was present in A188 callus with  $V_{max}$  = 0.06 unit/g fresh wt and  $K_m$  (L-malate) = 0.5 mM, similar to the value reported for ME in etiolated maize leaves (27). The activity we found in callus is one-third to one-fourth of the enzyme activity reported in etiolated maize leaves. ME-NADP polypeptide was not detected in callus by immunoassay with antibody to the maize leaf polypeptide. Differences in kinetic properties of callus ME-NADP from those of maize leaf enzyme suggest that the callus enzyme is not the one involved in malate decarboxylation in maize leaf photosynthesis. ME-NADP was not detected in callus.

<sup>14</sup>C Incorporation in Regenerating Tissue Cultures. In white, nonchlorophyllous callus, the primary labeled fixation products were malate (71%), aspartate (14%), and citrate (20%) (Fig. 5a). No discernable labeling of phosphoglycerate or other products of the reductive pentose phosphate cycle occurred. In green callus (Fig. 5b), 50 to 70% of the total <sup>14</sup>C fixed appeared in malate and aspartate, with most of the remainder in sugar phosphates and phosphoglycerate, and very little in citrate.

The total incorporation of <sup>14</sup>C per g fresh weight increased rapidly with regeneration of shoots. With 3 cm regenerated shoots (Fig. 5c), the labeling of malate and aspartate predominated at 10 s, and then decreased with time as percentage of total label, accompanied by an increase in percentage of label in P-glycerate and sugar phosphates. In the 8 cm shoot (Fig. 5d), where total fixation was 3 times greater than in the 3 cm shoot, 77% of the total label was in C<sub>4</sub> acids after 10 s, and even after 2 min it was 65%.

**Protein Blot Analysis of Enzymes of Carbon Metabolism during Seedling Development: Seedling Sections.** In maize seedling first leaves, the enzyme polypeptides for C<sub>4</sub> metabolism increased concurrently from the 3 to 5 cm section to the tip (Fig. 6A). In the basal 0 to 2 cm, RuBPC, PEPC, and ME-NADP were not detectable. In maize second leaf sections, RuBPC already was present in the basal 0 to 1 cm region at about 0.5% (w/w soluble protein) of the final level found in the distal section (Fig. 6B). The accumulation of RuBPC became noticeable in the 4 to 6 cm section where the amount reached about 10% of the final level. After reaching the maximum level in the 9 cm section, the amount of RuBPC polypeptide stayed constant or decreased slightly toward the tip. PPDK was present in the basal 0 to 1 cm region at about 0.5% of the final level and its amount increased concurrently with that of RuBPC. Increases in amounts of PEPC and of ME-NADP during development followed patterns roughly similar to those of RuBPC after 4 to 6 cm.

The profile of appearance and accumulation of these enzymes we found in maize seedlings was mostly consistent with reports by Williams and Kennedy (35), Perchorowicz and Gibbs (26), and Mayfield and Taylor (22). Mayfield and Taylor, however, did not see any RuBPC, PEPC, and PPDK polypeptide in basal 0 to 2 cm sections, but observed them first in 2 to 4 cm sections. This apparent difference can be explained by the low level (0.5% of the final level) of these enzymes in the basal section. Since our objective was to determine the order of appearance of these enzymes, special efforts were made to detect small amounts of polypeptides in the youngest tissues. By loading 2 to 4 times more soluble protein in each lane for the first few sections we were able to detect polypeptides which are present in less than 1% of the amount present in the leaf tip section.

The increase of total amount of these polypeptides (per g fresh weight) was due both to the increase in their proportion of total soluble protein with development (Fig. 6) and to an increase in the amount of soluble protein per g fresh weight. Thus, the increase could be seen either on a soluble protein basis or fresh weight basis. The profile of enzyme accumulation was related to

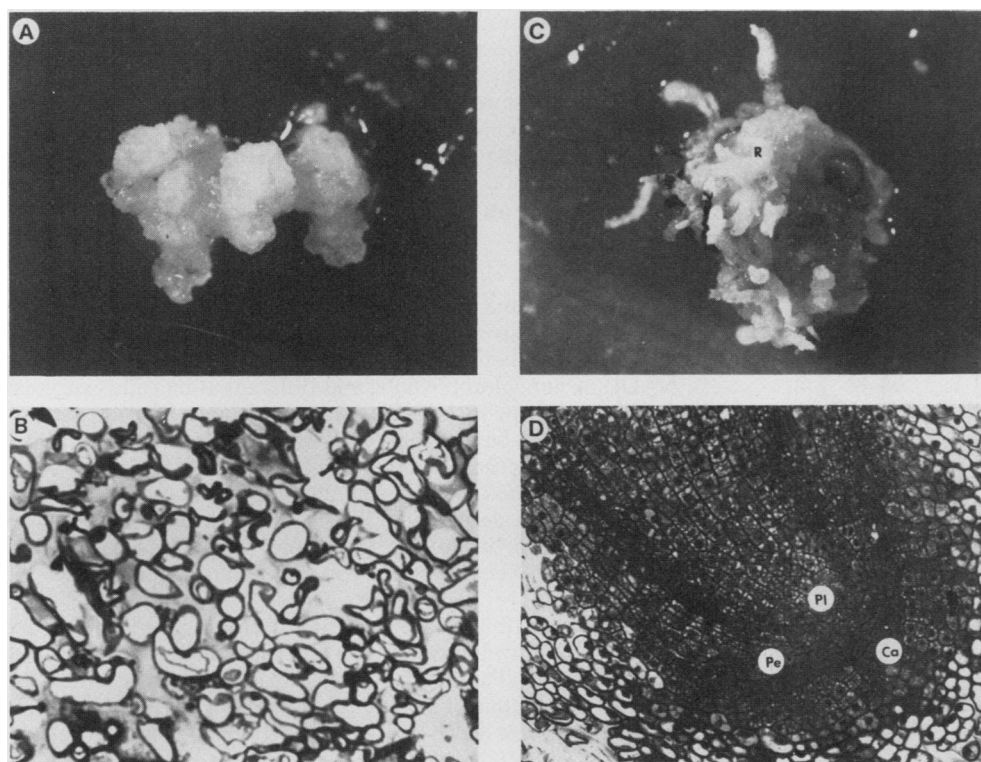


FIG. 9. Nonchlorophyllous callus. A, The callus is creamy white in color and it does not have any apparent leaf morphology; B, cross-section of the callus shown in (A) ( $\times 50$ ). The callus has loose aggregates of irregularly shaped cells; C, root-forming callus; D, root-like structure. Pl, plerome; Pe, periblem; Co, calyptrogen ( $\times 100$ ).

that of soluble protein (Table II), whereas there was less correlation with Chl content which increased more rapidly. The Chl *a*/Chl *b* ratio increased with development to a value typical for mature maize leaves (7).

**Leaf Age and Position.** First leaves harvested 7 and 9 d after planting were analyzed by protein blot, and the levels of the enzymes were compared. Of the four enzymes, RuBPC, PEPC, and PPK levels were similar at the two ages, but the level of ME-NADP was very different, increasing about several-fold from 7 to 9 d (Fig. 7). The pattern of appearance and accumulation was basically the same for upper maize leaves, but the level of enzymes was higher (Table III).

In the mature maize leaf, the portion of soluble protein allocated to these enzymes did not increase along the length of the leaf. When an equal amount of soluble protein was loaded into each lane, there was no increase in the band intensity of these enzymes (Fig. 8a). However, since equal amounts of plant extract contained increasing amounts of soluble protein toward the tip, an increase of the band intensity for each polypeptide was observed when equal amounts of extract were loaded into each lane (Fig. 8b). Compared with that in first leaves, the relative accumulation of enzymes for the mature leaf sections was less since there already were appreciable levels of these enzymes in the section next to the ligule.

**PEPC Activity of Maize Second Leaves.** Heavier loading of protein extract for the protein blot analysis of polypeptides from the first sections showed the presence of small amounts of PEPC, in contrast to previous reports (22) of the absence of PEPC polypeptide in the first 2 cm section, but in agreement with reports of its activity in basal sections (26, 35). Enzyme assays of PEPC in the tip 3 cm of maize second leaf (11 d after planting) gave 4.4 units/g fresh weight whereas that of the 0 to 3 cm basal section was only 0.13 unit/g fresh weight (Table I). When the activity is expressed as unit per mg of Chl, however, the values were 3.65 and 2.5, respectively (since the Chl content is so low in the base section).

**Light Microscopy of Callus and Regenerated Shoots.** A188

callus culture showed some degree of differentiation. Nonchlorophyllous callus tissue (Fig. 9A) varied from loose aggregates of irregularly shaped cells (Fig. 9B) to a compact tissue (meristemoids) and thence to root-like structures developing from these meristemoids. Root-like structures (Fig. 9C) apparently had the typical organization of roots (root caps, lineages of cells originating from a meristematic area) and a central vascular cylinder (Fig. 9D). These structures lacked the highly organized anatomy of a mature corn root, however. No obvious plastids were evident, but amyloplasts may have been present in the root-like structures.

Chlorophyllous tissue (Fig. 10) had groups of tracheoids associated with chlorenchyma which were several layers thick, individual cells with large vacuoles. Under direct microscopic observation, the chloroplasts appeared to be agranal and to contain starch granules (not shown).

Short shoots (Fig. 11C) developed from embryoids in the embryogenic callus (Fig. 11A). There were islands of meristematic areas and vascular elements. Chloroplasts were scattered throughout the tissue. A section of a 5 mm regenerated shoot showed an apical meristem (Fig. 11B). The shoots which were 1 to 3 cm did not have Kranz anatomy (Fig. 11D). The interveinal distance was about 270  $\mu\text{m}$  and there were 2 to 8 mesophyll cells between a pair of vascular bundles. Mesophyll cells were about 60  $\mu\text{m}$  across, were irregular in size, and contained few chloroplasts. Two types of vascular bundles, large and small, have been found in the *Z. mays* leaf (23). There was one small vascular bundle between a pair of large vascular bundles. Intercellular spaces occurred frequently.

In shoots 8 cm or more in length (Fig. 12A), the ID was 150 to 200  $\mu\text{m}$  and the number of mesophyll cells between a pair of vascular bundles was about 2 which is the typical value reported for maize leaf (23). The ID of the maize leaf obtained from a greenhouse was similar. The number of small vascular bundles between a pair of large vascular bundles increased to 8, again the typical value obtained from a maize leaf (23).

Hattersley and Watson (16) have shown that the condition where there are no cells between the metaxylem vessel elements



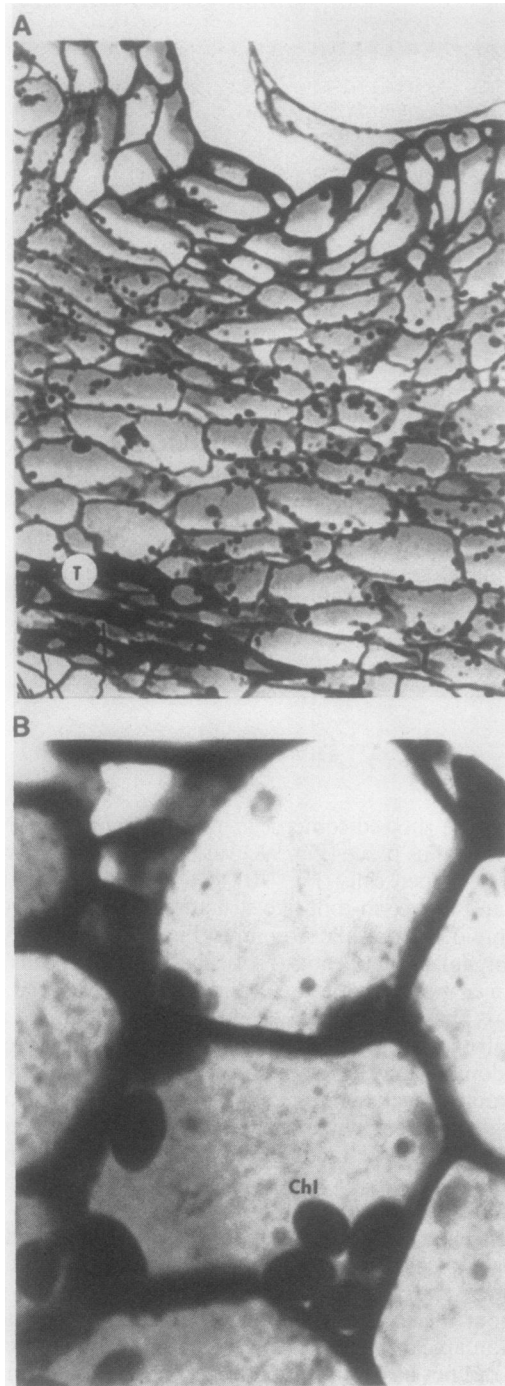


FIG. 10. Chlorophyllous callus. A, Cross-section of the callus showing cells containing chloroplasts and occasional areas of tracheoid (T) ( $\times 50$ ); B, enlarged view of the cell seen in (A) ( $\times 300$ ).

and the laterally adjacent sheath cells of large bundles, *i. e.* the 'Xy-Ms conditon,' is a characteristic of ME-NADP species. This condition was present in older regenerated shoots (Fig. 12B). This anatomical evidence suggests that  $C_4$  metabolism should be present.

In the regenerated maize leaf, the mesophyll cells were arranged in a less orderly way than in seedling leaves (Fig. 12C). The mesophyll consisted mostly of palisade parenchyma and was unifacial. They were 30 to 40  $\mu\text{m}$  in diameter, similar to those in the seedling leaves. Not all the mesophyll cells contained chloroplasts, so only a fraction of the mesophyll cells were

capable of photosynthesis. Occasionally, bundle sheath chloroplasts were not centrifugally arranged, however, in contrast to those of seedling leaf (Fig. 12, C and D). Sometimes chloroplasts were missing or were randomly scattered throughout the cell. The number of chloroplasts was only one-fourth to two-thirds of that found in the seedling leaf tissue. In summary, the older regenerated shoots had Kranz anatomy but lacked some structures which are present in the seedling leaves.

## DISCUSSION

**Appearance and Properties of Enzymes in A188 Callus.** Protein Blot analysis of appearance and accumulation of enzymes of carbon metabolism such as PEPC, RuBPC, PPDK, and ME-NADP in nonchlorophyllous and differentiating callus suggests that there may be an earlier stage of appearance of some parts of  $C_4$  carbon metabolism than has been seen in sections of a leaf (22). PPDK appeared at relatively high levels in the nonchlorophyllous callus before the appearance of RuBPC and ME-NADP. The temporary decrease of PPDK at the shoot-forming stage may have been due to the transition from a cytoplasm form characteristic of heterotrophic tissue to a chloroplast form found in leaves.

That a transition from cytoplasm to chloroplast PPDK occurs in embryoid-forming callus also was indicated by the presence of both a 94 and a 110 kD polypeptide among the products of *in vitro* translation of poly(A)RNA extracted from this tissue. Although mature leaves contained poly(A)RNA translating only to 110 kD PPDK polypeptide (within our limits of detection), the embryoid-forming callus contained poly(A)RNA which yielded roughly equal amounts of 110 and 94 kD PPDK polypeptides while the white callus poly(A)RNA yielded only 94 kD polypeptide. It thus appears that the change from cytoplasm to chloroplast PPDK occurred during a very early stage of leaf ontogeny, before the appearance of Kranz anatomy.

**Pattern of  $^{14}\text{C}$  Incorporation in A188 Callus and Shoots.** The  $^{14}\text{C}$  incorporation products in nonchlorophyllous callus (Fig. 5a) were as expected in tissue with an active  $\beta$ -carboxylation system (PEPC) but no RuBPC. Some of the  $C_4$  compounds are converted to citrate, 2-ketoglutarate, and fumarate via the tricarboxylic acid cycle. In the green callus tissues (Fig. 5b), some labeled P-glycerate and sugar phosphates appear as expected, given the presence of RuBPC (Fig. 1). In both kinds of callus tissues, malate and aspartate presumably may have been used for anaplerotic synthesis of other amino acids, Chl etc. Even though ME-NADP activity was found in callus tissues, its properties were like those of enzyme from etiolated tissue rather than those of enzyme from chloroplasts (see "Results") and ME-NADP polypeptide was not detected from these tissues by protein blot probed with antibody to chloroplast ME-NADP (Fig. 1). Thus, conversion of  $C_4$  acids to  $\text{CO}_2$  and pyruvate in chloroplasts was unlikely. The source of PEP for  $\beta$ -carboxylation may have been pyruvate, perhaps from alanine, since we found PPDK activity, whereas pyruvate kinase activity (indicative of glycolysis) was missing.

Our results with maize callus are consistent with those of Kennedy *et al.* (19) who did not find any net increase in end products of the  $C_3$  cycle during the pulse chase in callus of another  $C_4$  species (*Portulaca oleracea*). Considering the lack of an efficient vascular system in these tissues, translocation of the  $C_4$  acids would not be expected.

The  $^{14}\text{C}$ -labeling patterns observed in metabolites in 3 and 8 cm shoots (Fig. 5, c and d) seem possibly to be the consequences of differential rates of formation and activation of PEPC as compared to RuBPC. Substantial labeling of  $C_4$  acids was seen as the rate of  $\beta$ -carboxylation increased with shoot development. The amounts of  $^{14}\text{C}$ -labeled products such as citrate, 2-ketoglutarate, fumarate, and glutamate that were formed during ana-

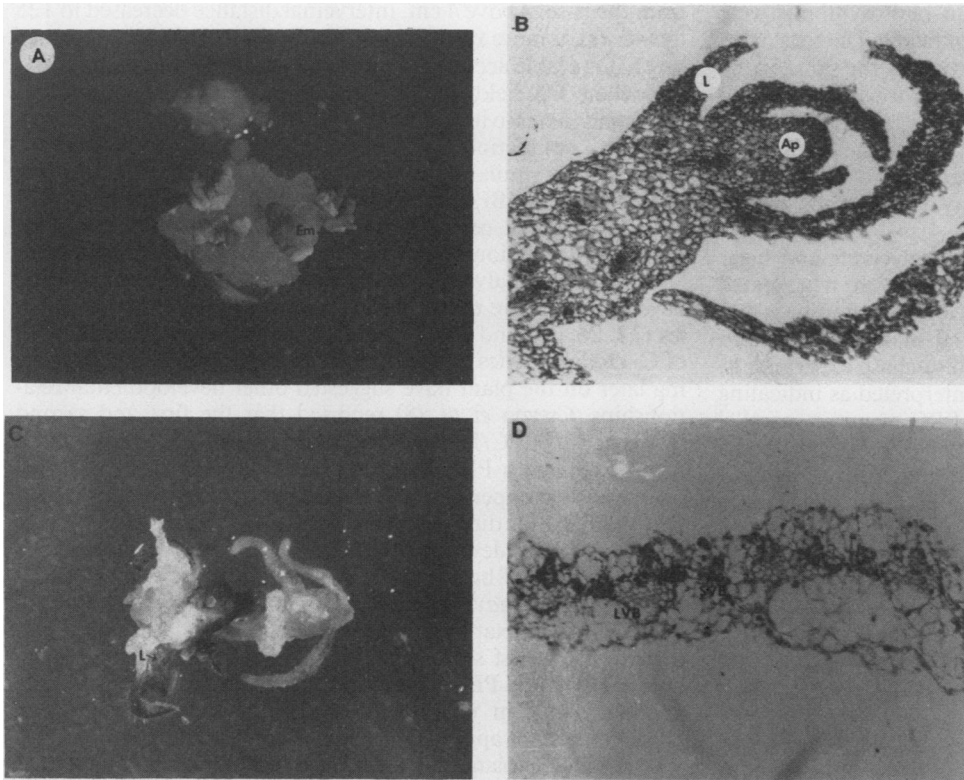


FIG. 11. Young regenerated shoot. A, Embryoid (Em)-forming callus; B, transverse section of the apical meristem (Ap) and leaf initials (L) of the 5 mm regenerated shoot ( $\times 40$ ); C, regeneration of shoots from the embryoids. As many as 7 to 8 shoots may form in 1 cm<sup>2</sup> area on callus; D, regenerated shoot (1 cm in length) transsection. The large vascular bundles (LVB) and small vascular bundles (SVB) alternate. No Kranz anatomy is present.

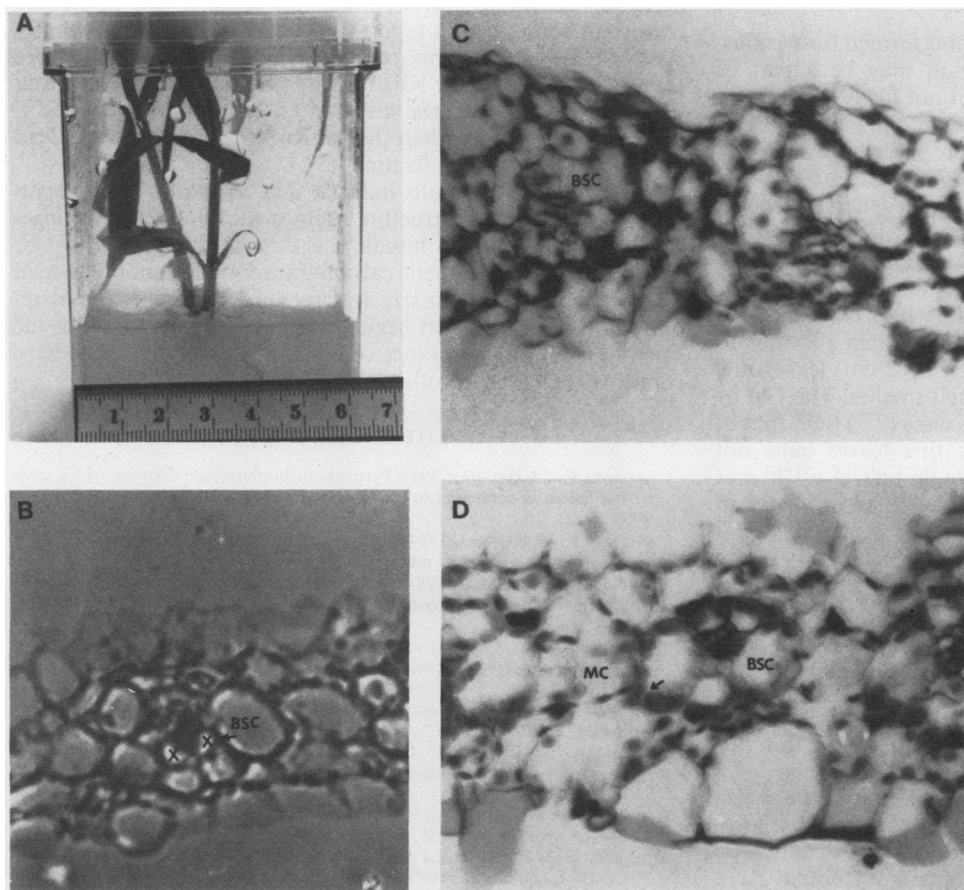


FIG. 12. Older regenerated shoot. A, Regenerated shoots with four leaf blades; B, transsection of a regenerated leaf (12 cm in length) ( $\times 80$ ). 'Xy-Ms' condition is present as indicated by the arrow. X, metaxylem; C, transsection of a regenerated shoot 7 cm in length, showing the absence of complete Kranz anatomy. Occasionally, the arrangement of bundle sheath cells (BSC) is irregular and the chloroplasts are not centrifugally arranged as they were in the seedling leaf; D, transsection of a seedling leaf ( $\times 80$ ). Large chloroplasts are centrifugally arranged in the bundle sheath cells (BSC) as indicated by the arrow. MC, mesophyll cells.

plerotic conversion of  $C_4$  acids by 2 min photosynthesis were minor and their total labeling was not significant. The total label of sugar phosphates and P-glycerate was roughly the same in the 3 and 8 cm shoots (total fixation per g fresh weight multiplied by percentage of total in these compounds). The total fixation into  $C_4$  acids was initially (30 s) much higher in the 8 cm shoot than in the 3 cm shoot. This suggests that most of the increase in fixation was due to increased capacity for  $\beta$ -carboxylation, that is, PEPC activity.

The 1 min and 2 min labeling data for P-glycerate and sugar phosphates and for malate plus aspartate in the 8 cm regenerated shoot (Fig. 5d) are similar to those reported by Perchorowicz and Gibbs (26) for the base section of young maize leaves (third leaves from 12 to 16 d old plants). In pulse-chase experiments, they obtained labeling data which they interpreted as indicating that carboxylation with PEPC and RuBPC occur in parallel without much cooperation between mesophyll cells and bundle sheath cells, that is, intercellular transport and conversion of  $C_4$  compounds. They did find a  $^{14}C$ -labeling pattern typical of  $C_4$  plants in the center and top sections of these leaves, however. They reported Kranz anatomy in all three sections, but the appearance of bundle sheath agranal chloroplasts in the center and top sections only. In the present study with 3 and 8 cm regenerated shoots, the labeling data suggest parallel carboxylation reactions but little, if any, intercellular  $C_4$  transport and conversion; however, limitations of material did not permit us to carry out pulse-chase studies.

The slowness of development of effective intercellular  $C_4$  transport in shoots even up to 8 cm (compared maize seedlings) seems likely to be a consequence of the disorderly morphological development towards Kranz anatomy and lack of correct arrangement of chloroplasts within bundle sheath cells in shoots (Fig. 12C) as compared with seedlings (Fig. 12D). As mature plants develop from regenerated shoots, these defects would be expected to disappear.

In summary, in callus tissues and in shoots formed from callus,  $C_4$  acids are formed, and their formation may be a part of anaplerotic metabolism (biosynthesis) but not likely of  $C_4$  intercellular carbon transport. The transition from the type of PPDK found in cytoplasm to that found in chloroplasts begins already in embryoid-forming chlorophyllous callus. The  $C_4$  carbon transport pathway develops slowly in the longer regenerated shoots and may be concomitant with delayed cell differentiation towards  $C_4$  leaf morphology and with the accumulation of all the enzymes of the  $C_4$  transport and decarboxylation pathway.

**Appearance and Accumulation of  $C_4$  Enzymes in Sections of Maize Seedlings.** The percentage of soluble protein allocated to PPDK is relatively higher in the youngest (0–2 cm) leaf tissue, while that allocated to ME-NADP is much smaller. The Chl *a*/Chl *b* ratio of this young first leaf tissue is less (2.7) than that of the older leaf (3.2) indicating that the first leaves have not developed sufficiently at this stage (7). We find a Chl *a*/Chl *b* of about 3.2 in expanded leaves. Since ME-NADP accumulated in bundle sheath cells, it is expected that its early appearance would be less than that of PPDK which is made predominantly in the mesophyll cells.

In maize second leaf sections, stomatal resistance in the first 0 to 2 cm region is insensitive to  $CO_2$  concentration (23), suggesting that the stomata are nonfunctional. PEPC in the basal section, therefore, must be refixing respiratory  $CO_2$  and may not be of the  $C_4$  type. The basal sections of the young seedlings thus may in this respect resemble embryoid-forming callus tissue in terms of enzyme content and metabolic function.

It is only in tissue above 3 cm from leaf base that the maize leaf exhibits high photosynthetic rates and dimorphic chloroplasts characteristic of a mature maize leaf (23). The centrifugal arrangement of bundle sheath cell chloroplasts was found 3 cm

from the base. Above 4 cm, interveinal distance decreased to 125 to 140  $\mu m$ , a more typical value for the  $C_4$  leaf. This is where we saw a noticeable accumulation of the four enzymes studied, and also where Mayfield and Taylor (22) observed their appearance. The rapid increase in amounts of RuBPC and PPDK began with the 4 to 7 cm section obtained under our maize seedling conditions. This is similar to the stage when light-independent onset of PEPC and RuBPC accumulation has been reported to occur (24). As suggested, cell differentiation may be an important factor for an accumulation of  $C_4$  carbon fixation enzymes in maize.

While these analyses of enzyme appearance in sections of the developing leaf are mostly consistent with several previous studies (22, 26, 35) and provide a clear picture of the development of  $C_4$  characteristics within a leaf, examination of leaves appearing later on the plant have suggested other developmental relationships. Crespo *et al.* (8) reported that the first and second leaves had a PEPC/RuBPC ratio of less than 1 while the third to fifth leaves had a PEPC/RuBPC ratio of greater than 1. Based on the  $CO_2$  compensation point and this ratio, they proposed that the first leaf did not have  $C_4$  photosynthetic characteristics like other leaves developed later in the ontogeny and suggested the occurrence of both  $C_3$  and  $C_4$  photosynthetic characteristics in a single *Zea mays* plant. Mayfield and Taylor (22) found no evidence for  $C_3$  fixation in sections from the third leaf. We found that from the first section on, in either the first or later leaves, both RuBPC and PEPC appear and increase in concert with one another. Thus, in maize seedlings,  $C_3$  photosynthetic carbon fixation does not appear to precede  $C_4$  photosynthesis.

A possible explanation for such discrepancies might be that Crespo *et al.* (8) used the 1st to 5th maize leaves from 3 week old plants. In our studies with maize seedlings, 1st and 2nd leaves are already becoming senescent by the 3rd week as we find both Chl content and the enzyme polypeptides as measured by protein blot, reduced to about half the levels of leaves harvested at earlier ages (see "Materials and Methods" for details). Our results are consistent with Usuda's report (34) on variations in the photosynthetic rate and activity of photosynthetic enzymes in maize leaves of different ages. Khanna and Shinha (20) reported that in old sorghum leaves the levels of  $C_4$  enzymes such as PEPC decrease more rapidly than that of RuBPC, and this could lead to more  $C_3$ -type of  $CO_2$  fixation.

In summary, our results indicate that, in maize, Kranz anatomy and certain fine structure such as the centrifugal arrangement of chloroplasts in bundle sheath cells are essential for  $C_4$  intercellular photosynthetic carbon transport, commonly termed  $C_4$  photosynthesis. In maize leaf sections, all the enzymes required for such transport begin to increase rapidly at about the same time as the appearance of Kranz anatomy and increase in coordination with each other.

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