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K. Aoyagi
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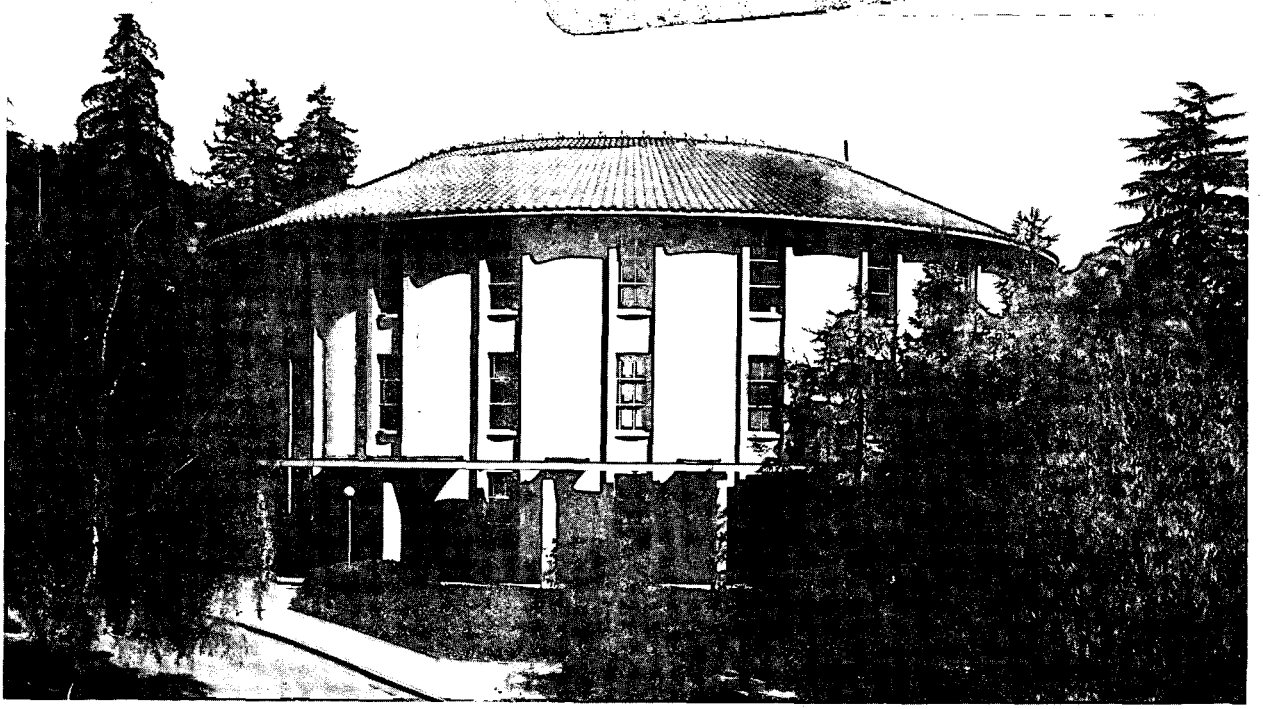

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Regulation of Pyruvate Orthophosphate Dikinase Gene Expression in C_3 Plants as Compared to that in C_4 Plants

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Ph.D. Thesis

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By

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ABSTRACT

Pyruvate orthophosphate dikinase (PPDK) was found to be more widely distributed in both leaves and seeds of C_3 and C_4 plants than previously realized. Physicochemical and enzymic properties appear to be strongly conserved over this distribution. In wheat, a C_3 plant, both in seeds and leaves, the amount of PPDK protein is developmentally regulated. In seeds the enzyme level is determined by the level of PPDK mRNA. The role of PPDK seems to be providing phosphoenolpyruvate as a substrate for amino acid interconversions during development. In leaves, the PPDK protein level increased with development. PPDK is present in chloroplasts also in C_3 plants. Even though the amount of PPDK is very small, it may be involved in 'intracellular' carbon transport.

Both in C_3 and C_4 plants, organ specific PPDK mRNA are present. A 110 kD polypeptide, precursor with a 'transit' sequence is exclusively found among the *in vitro* translation products of maize leaf mRNA whereas a 94 kD polypeptide, the normal size of the mature polypeptide is found in heterotrophic tissue . mRNA encoding the precursor form was always found in leaves and in green callus. The precursor polypeptide made by *in vitro* translation of maize leaf mRNA was taken up by spinach chloroplasts and processed into a mature polypeptide in the light.

In maize, a C_4 plant , PPDK is not limited to mesophyll cells but also is present in bundle sheath cells, kernels and other tissues. In the leaf, the appearance and accumulation of PPDK is well coordinated with

other enzymes of carbon metabolism. In maize callus, a heterotrophic type PPDK is synthesized independently of light and is active in the dark. Shoot formation and establishment of autotrophy enhances the accumulation of the leaf-type PPDK and leaf-type isozymes of phosphoenolpyruvate carboxylase and NADP-malic enzyme. Ultrastructural changes and ^{14}C uptake patterns during regeneration of leaves from callus indicate that cell differentiation and Kranz anatomy are essential for the functional C_4 photosynthesis as well as for coordinated expression of enzymes involved in the C_4 pathway.

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ABBREVIATIONS

Chl,	chlorophyll
d,	day
h,	hour
LHCP,	light harvesting chloroplast protein
KD,	kilodalton
MDH,	malic dehydrogenase
ME,	malic enzyme
mol wt,	molecular weight
PAGE,	polyacrylamide gel electrophoresis
PEP,	phosphoenolpyruvate
PEPC,	PEP carboxylase
P,	phosphate
Pi,	orthophosphate
PMSF,	phenylmethysulfonyl fluoride
PPDK,	pyruvate orthophosphate dikinase
RPP,	reductive pentose phosphate
RuBPC,	ribulose 1,5-bisphosphate carboxylase
RuBPC-SS (-LS),	RuBPC-small subunit (large subunit)

CHAPTER I

INTRODUCTION

Purpose of this study

When the photosynthetic C_4 carbon transport pathway was discovered twenty years ago, the enzymes required for the pathway were known to have other functions in green plant cells. For a time, however, pyruvate orthophospate dikinase (PPDK, EC 2.7.9.1) was considered to be unique in green cells to the carbon transport pathway (Hatch et al 1971). Thus its presence in chloroplasts seemed to be a marker for C_4 photosynthesis and even a small amount in C_3 species might indicate the first stage of evolution towards a C_4 transport mechanism.

In spite of its importance in the C_4 pathway, studies of PPDK have been limited to its enzymic properties in the past. Information about PPDK gene expression has been limited (Hague et al 1983) as compared to that of ribulose 1,5-bisphosphate carboxylase (RuBPC, EC 4.1.1.39) (Gilham et al 1978, Viro and Kloppstech 1980, Bedbrook et al 1980). An intriguing aspect of the PPDK expression in green cells is the differential expression of PPDK and of RuBPC. Both PPDK and RuBPC-SS are nuclear encoded, synthesized on cytoplasmic ribosomes (Cashmore et al 1978, Hague et al 1983). In C_4 plants PPDK accumulates mostly in mesophyll cells while RuBPC accumulates in bundle sheath cells (Sugiyama and Hatch 1981, Huber et al 1976, Link et al 1978). This mutual exclusiveness of gene expression of these enzymes during cell differentiation is particularly interesting for investigation.

The question of the process of evolution of the C_4 transport mechanism, which has occurred independently in the plant kingdom many

times (Bjorkman 1976) is of great interest, both as a basic scientific proposition and for possible application in genetic engineering. C_4 plants have the highest efficiencies for conversion of solar energy to bio-product, under conditions of warm temperature, bright light, and limiting water, due to the virtual avoidance of photorespiration (Downton 1971).

The goal of this research was to gain some understanding of the regulation of PPDK gene expression. Wheat (C_3 plant) and a regenerating maize A188 tissue culture system were chosen because of their limited levels of PPDK and the lack of apparent C_4 photosynthesis. By comparing these plants and tissues with maize leaves where the PPDK gene(s) is expressed at high levels, one may learn more about the reasons for the big difference in regulation and events involved in increased expression.

Increased expression of a particular enzyme may be obtained by unique form of enzyme entities evolved specifically for the operation of the C_4 pathway as suggested for the C_4 form phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) (Hatch et al 1971) or by enhanced expression of genes already present in the C_3 ancestor.

Multiple molecular forms of an enzyme in a single organism have been designated isozymes (Market and Moller 1959). Among the enzymes of the C_4 pathway carbon metabolism, PEPC is probably the most widely studied due to its key role in the initial CO_2 fixation. Multiple forms of PEPC are present in higher plants (Ting and Osmond 1973). In sugar cane, a C_4, two isozymes, PEPCI and PEPCII are present (Goatly et al 1975). PEPCI is the major form in the green leaf having a high Km (PEP)of 0.7 mM. PEPCII is the minor form in the green leaf, but is the predominant form in the etiolated leaf having a low Km(PEP) of 0.14 mM. A similar result

was reported for maize leaf PEPC (Hayakawa et al 1981). Hatch et al (1971) studied PEPC in C_3 and C_4 Atriplex species and hybrids from an A. rosea x A.patula ssp. hasta cross. Their results suggested that a unique C_4 isozyme of PEPC had to evolve rather than enhancement of the same enzyme of the C_3 ancestor.

Two forms of NADP-dependent malic enzyme (ME, EC 1.3.1.37) are present in maize leaves (Puppilo and Bossi 1979). Etiolated leaves contained isozyme typeI which is assumed to be cytosolic, while the green leaves contained typeII isozyme which is assumed to be chloroplastic. These two types differ in isoelectric point and Km (L-malate). Several C_4 species may contain only the chloroplast forms of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Herbert et al 1973). The activities of a set of cytosolic enzymes of the heterotrophic phase of maize seedlings become obscure at the onset of photosynthetic competence, since the plastid isozymes increase both in amounts and in activity to a much higher level. In spite of its important role, isozymes of PPDK in higher plants have not been studied. Therefore it was of my interest to investigate if a C_4 specific isozyme had to evolve as it is considered to be the case for PEPC.

The tissue culture system was chosen especially for examining the importance of a unique C_4 anatomy, i.e. 'Kranz' anatomy and the presence of two cell types, namely bundle sheath and mesophyll cells (Laetsch 1974) for the operation of C_4 pathway. There have been numerous reports on this subject, but the importance of Kranz anatomy for the C_4 pathway is still controversial (Kanai and Kashiwagi 1975, Laetch and Kortschak 1972, Ruzin 1984). I used the regenerating tissue culture of maize A188 in order to examine the relationship between development of Kranz anatomy and

the C_4 pathway. This was the first attempt to use the regenerating A188 tissue culture system for analysis of C^4 carbon metabolism. Regeneration of maize tissue culture has been difficult for many years. Now a method has been established to obtain regenerable tissue culture using appropriate lines of maize (Green and Rhodes 1982). A tissue culture system has some advantages over etiolated seedlings or sections of a leaf. It does not possess organized leaf structure nor two differentiated cell types (Springer et al 1979, See Fig. 10-12 of Part Four).

Wheat was chosen because of their lack of capacity for C_4 photosynthesis in spite of having PPDK, a key enzyme needed for C_4 carbon metabolism (Hatch et al 1971). Thus it is of interest to study the reason for the limited expression of C_4 enzymes in wheat from a standpoint of evolution of C_4 plants and future genetic engineering aimed at imparting C_4 characteristics to C_3 plants. Others have used C_3/C_4 intermediate plants for this purpose (Bjorkman 1976, Ku et al 1983).

The content of this dissertation is divided into four sections following MATERIALS AND METHODS. The first section includes the occurrence of PPDK in various C_3 plants, the comparison of wheat PPDK enzyme properties to those of maize leaf enzyme and the location of the enzymes in different tissues. The second section describes the discoveries of organ-specific gene expression which underlies the occurrence of PPDK in both photosynthetic and heterotrophic tissues. It also includes the regulation of PPDK polypeptide synthesis and processing into mature polypeptides. The third section includes developmental gene regulation of PPDK in leaves and seeds of wheat as compared to that of

maize. Finally the development of C4 photosynthesis in regenerating A188 tissue culture demonstrates the relationship between functional C_4 photosynthesis and biochemical and cytological factors.

A brief review of what C_4 photosynthesis is and what we know already about PPDK is necessary in order to explain why I have chosen certain methods in this research.

C_4 photosynthesis

C_4 photosynthesis is characterized by initial CO_2 fixation by PEPC in the mesophyll cells followed by transport of C_4 acids to adjacent bundle sheath cells where CO_2 is released and reduced by the Reductive Pentose Phosphate cycle (RPP) (Fig 1). The resulting high concentration of CO_2 in the vicinity of RuBPC in bundle sheath chloroplasts has the advantage of suppressing photorespiratory loss of CO_2 due to RuBP oxygenase activity. $A^{14}C$-tracer kinetic study is often used to demonstrate the existence of C_4 photosynthesis in a plant (Hatch and Osmond 1976), and this is why I have employed this method frequently in this research.

In addition to their distinctive pathway of CO_2 assimilation, C_4 plants also differ from C_3 plants in a number of anatomical and physiological characteristics. C_4 plants are found in several thousand species in 16 or more families of angiosperms, with the specialized anatomy named Kranz (wreath or halo) (Downton 1971). The ubiquity of some form of Kranz anatomy suggests the need for spatial separation of carbon fixation enzymes. Although Kranz anatomy alone does not imply that C_4 photosynthesis is operating (Crespo et al 1979, Kanai and Kashiwagi 1975), there seems to be a correlation between the anatomical feature and the degree of C_4 photosynthetic capacity of plants (Brown et al 1983,

Laetsch 1974, Ruzin 1984). However Laetch and Korschak (1972) reported that callus of C_4 plant Froelichia gracillus possessed C_4 photosynthetic capacity. I have reinvestigated this aspect carefully by following the change in morphology using light microscopy and I found a close correlation between the anatomical feature and C_4 photosynthetic capacity.

Kranz anatomy is often seen as an adaptation resulting in high CO_2 concentration in internal tissues which inhibits photorespiration and the subsequent loss of CO_2 (Black 1973). Laetsch (1974), however has provided a different view of the functional basis for this structure. He proposes that Kranz anatomy is a structure which restricts water loss and also permits efficient CO_2 fixation. He suggests that since C_4 plants evolved in areas where rainy seasons alternated with periods of extreme aridity, and where high saline environments were common, Kranz anatomy could serve as an anion pump concentrating C_4 acids such as malate and aspartate rather than a carbon pump. These anions were used to counter balance accumulation of cations resulting from existence in a saline environments. Furthermore he suggets the evolution of structural and functional adaptations to xeric and saline environments were preadaptive with respect to the evolution of efficient assimilation of carbon.

His view is interesting considering the fact that there is no C_4 rice known even though it is a tropical grass. Rice plants have Kranz-like anatomy but the bundle sheath cells do not contain specialized chloroplasts as in the case of maize and other C_4 species. I have speculated that the absence of C_4 photosynthesis in rice species may be correlated with physiological growth conditions,

i.e. the wet environment in which rice is usually grown in contrast to the evolution of C_4 plants in arid environments. I have done some preliminary studies with the upland variety "Rikuto Norin No.24"; however, this work was not completed within the scope of this study, and I am unable, at this time to add anything to the literature concerning the functional basis of Kranz anatomy in C_4 photosynthesis. Nevertherless, since rice is an important crop, an investigation of the reasons for not having C_3/C_4 intermediate rice or C_4 rice should be worthwhile.

Another characteristic of C_4 photosynthesis is the operation of reactions in cytoplasm, mitochondria and chloroplasts in contrast to the RPP cycle where carboxylation and carbon reduction are restricted to the chloroplast. The transport of intermediates between intracellular compartments must be rapid and many enzymes are compartmentalized to achieve efficiency (Hatch and Osmond 1976). For example, enzymes involved in initial carbon fixation such as PEPC, PPDK, adenylate kinase, pyrophosphatase,and MDH are found in mesophyll cells, whereas enzymes involved in decarboxylation, refixation and carbon reduction such as ME, RuBPC, and other RPP cycle enzymes are found in bundle sheath cells (Hatch and Osmond 1976). The level of activity of these enzymes seems to be critical for the function of C_4 photosynthesis. For example, C_3/C_4 intermediate and plants possessing Kranz anatomy but lacking high activities of PEPC and PPDK did not show operation of C_4 photosynthesis (Ku et al 1983, Kanai and Kashiwagi 1975). Specific activity of C_4 type PEPC is much greater than that of those involved in C_3 type photosynthesis or heterotrophic function (Ting and Osmond 1973).

I used protein blot analysis in order to study the levels of four key enzymes,i.e. RuBPC, PEPC, PPDK and ME to examine the biochemical basis for

C4 photosynthesis. The correlation between levels of these enzymes and the C_4 photosynthesis was examined by $^{14}CO_2$ kinetic studies. Furthermore I followed the changes in enzyme properties using enzyme activity assays to detect possible switches in major enzyme types during development of wheat and maize plants.

All of the methods described above are necessary, since any one of them alone cannot be used as a criterion for analyzing functional C_4 pathway.

PPDK

PPDK catalyses the formation of PEP, the substrate for PEPC as shown in the following reaction:

Pyruvate + ATP + Pi -------> PEP + AMP + PPi

In vivo the activities of RuBPC, PEPC, ME and MDH are usually in excess and it is considered that PPDK is the rate limiting enzyme in the C_4 pathway (Sugiyama et al 1984). Part of the reason for this is attributable to the strict regulation of enzyme activity under light/dark mediated regulation. This causes a rapid change (half-time of about 20 min) in enzyme activity observed both in leaves and in vitro inactivating system. In vivo this reaction is catalyzed by chloroplast protein factor, termed pyruvate, Pi dikinase regulatory protein (RDRP)(Burnell and Hatch 1983). Recently it was found that RDRP also catalyses activation/inactivation of PPDK from both maize kernel and wheat leaf (Burnell 1985). In vitro studies have provided evidence that dark/light regulation is accounted for by an ADP-dependent inactivation and a Pi-dependent activation of the enzyme (Ashton and Hatch 1983). In vivo the level of ADP as well as the ratio of pyruvate to PEP must be critical for PPDK

activity level. The decay of enzyme activity upon transferring the plants from light to darkness is characterized by a first-order reaction with a half-time of 20 min and the enzyme is rapidly reactivated upon re-exposure of the leaves to light (Hatch and Slack 1969). The amount of enzyme polypeptide is also controlled by illumination (Hatch et al 1969). When the maize plants were grown in darkness for 1 week and transferred to light for 35 h, the activity of PPDK was 10-fold greater in the illuminated plants as compared to those remaining in darkness. Such increases are also observed in the case of other C_4 enzymes such as ME and PEPC (Collins and Hague 1983, Hague and Sims 1980).

Pyruvate kinase catalyses the reverse reaction converting PEP to pyruvate (Baysdorfer and Bassham 1984). In contrast to PPDK, pyruvate kinase activity is strongly inhibited by ATP, therfore in vivo, the activities of these two enzymes may be regulated by adenylate energy charge; i.e. PPDK being active when ATP/ADP ratio is high and pyruvate kinase being active when ADP/ATP ratio is high in order to avoid a futile cycle without metabolic value.

Although I did not attempt to study regulation of PPDK enzyme activites in vivo, some of the findings from this research add insight to the subject from a different perspective.

Fig.1. C_4 photosynthetic CO_2 fixation pathway (maize)

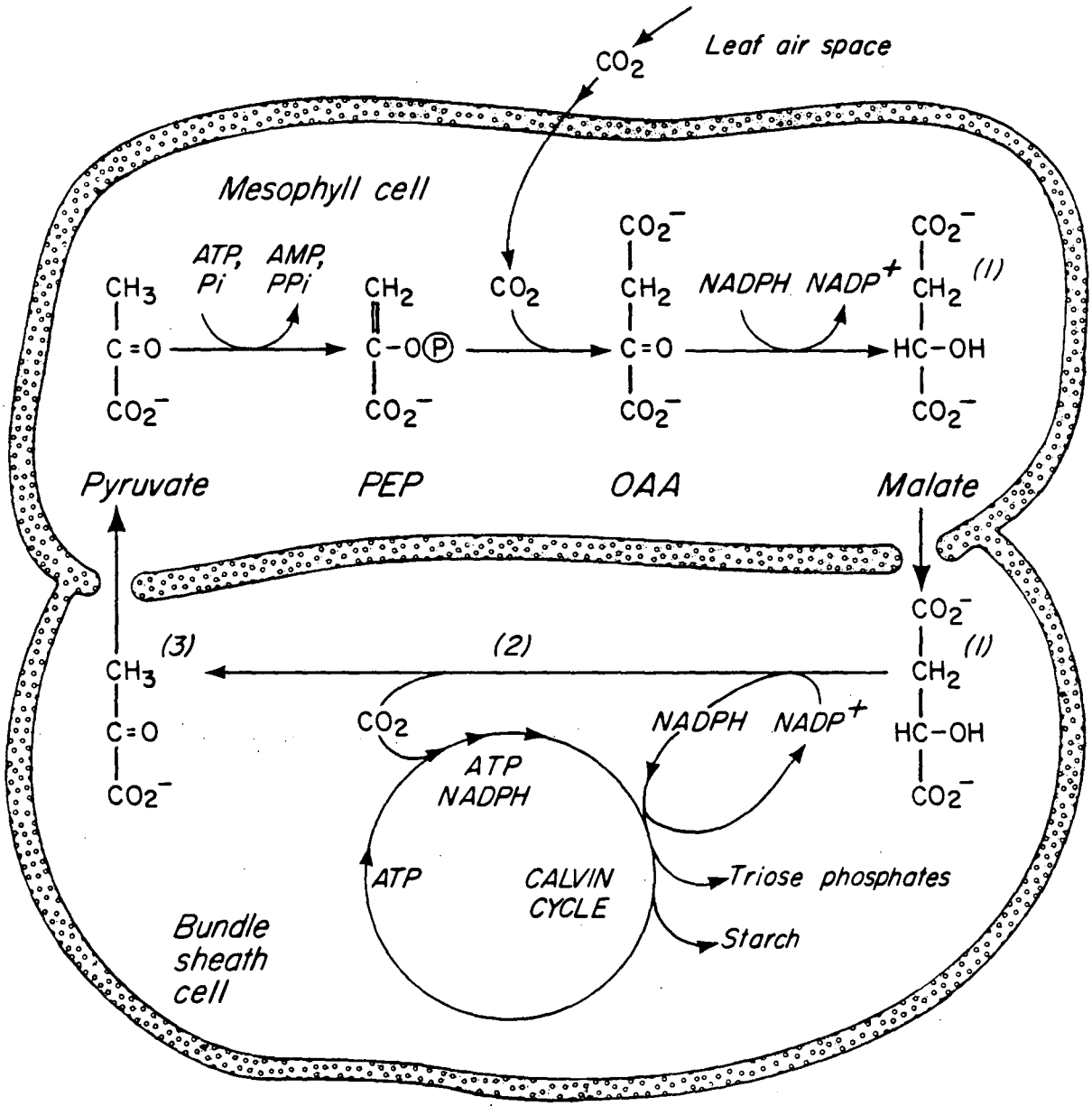

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Fig. 1

CHAPTER II

MATERIALS AND METHODS

Plant Material

Maize (Zea mays L. cv. Golden Bantam or inbred B 73) was grown in vermiculite in a growth chamber with a quantum flux density of 600 $\mu E\ m^{-2}\ s^{-1}$ with a 18 h photoperiod at 27°C. Wheat (Triticum aestivum L. cv. Cheyenne, CI 8885 or Anza), spinach (Spinacea oleracea L. cv. Highpack or 5286 America) and Tobacco (Nicotiana tabacum L.) were grown with a quantum flux density of 250 $\mu E\ m^{-2}s^{-1}$ with a photoperiod of 8 h and at 15°C. The wheat seeds were a gift of Dr. Calvin Qualset, University of California, Davis. Immature wheat seeds were harvested from 3 to 40 d past anthesis and were then frozen in liquid N_2 and stored at -80°C until used. For protein blot analysis, 0.5 g fresh weight plant tissue was frozen in the same manner. In each case the plants were fertilized with half strength Hoagland solution. Maize (Zea mays. L. inbred A188) for tissue culture was grown in a greenhouse at the Botanical Garden at U.C., Berkeley. Sunflower (Helianthus annuus L.), immature fruit of plum (Prunus tomentosa L.), rice (Oriza sativa L.) were obtained from the Botanical Garden.

Preparation of leaf sections for developmental analysis

(A) Wheat

The primary leaves of wheat were harvested 12 d after planting or when the seedlings reached about 10-12 cm in length. The coleoptile was removed and the primary and the younger leaves were used (Dean and Leech 1982). In case of the third leaf, the primary and the second leaves

were removed as well. The sections were made from the base to tip. The first and second sections were 1 cm in length and the successive sections were 2cm, except the last two sections were 3 cm long near the tip. For the mature leaves (4th, one month after planting), only the blade was cut into larger sections: the first and second were 4 cm each and successive sections were 7 cm. In all cases, the number of sections was between 6-10 depending on the length of the leaf being studied.

(B) Maize

The first leaves were harvested 9d after planting while the second leaves are still rolled . The second leaves and the third leaves were also harvested before the formation of the ligule. The average size of these leaves were 14 cm. They were cut from the base section in the same manner as the wheat leaves. The younger leaves in the basal sections were rolled inside the leaf under study so that they were used also, but expanded part of the younger leaves were discarded. For the mature leaves (4th, 5 weeks after planting), only the blade was cut into larger sections as in the case of wheat leaves, since removing the lower leaves without tearing the sheath part of the leaf under study was difficult.

About 100 seedlings were grown in both wheat and maize cases to ensure uniformity of the sections.

Maize tissue culture

The seeds of inbred line A 188 were kindly supplied by Mr. Charles Armstrong, University of Minnesota. Some callus cultures were the generous gift from Dr. C. A. Rhodes of Calgene, Davis, CA and Mr.K.Low of Stauffer Co., Richmond, CA. The tissue culture initiation and maintenance were done according to the method of Green and Rhodes (1982).

The maize immature embryos were aseptically removed from the endosperm and were placed on N6 medium (Chu et al 1975), which was modified by Armstrong and Green (1984), contained 25 mM proline, 0.5 $mg.l^{-1}$ 2,4,-dichlorophenoxyacetic acid (2,4,-D) and 0.7% agar. After three to four weeks of incubation at 25°C under cool fluorescent light (250 $\mu E\ m^{-2}s^{-1}$, 14 h photo period) regenerable embryonic cultures were obtained. The tissue cultures were transferred every three 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 non-chlorophyllous cultures were wrapped in two layers of aluminum foil. After growing them in the dark for the specified length of time (1 to 5 weeks), the tissue was frozen in liquid N_2 until used.

Preparation of antiserum to maize PPDK

PPDK was isolated from maize leaves and purified as previously described (Sugiyama 1973). Antiserum to PPDK was prepared by injecting New Zealand white rabbits with 150 µg of the purified enzyme and Freund's complete adjuvant, followed after 26 days by a booster containing the same amount of enzyme and Freund's incomplete adjuvant and a second such booster at 40 days. The rabbits were bled at 41 days. The crude antiserum was used for the protein blot. Non-immunized serum showed no cross reaction with PPDK, and PPDK antiserum cross reacted only with PPDK (Aoyagi and Bassham 1983). This antiserum was used as a probe for the presence of PPDK in the various tissues studied. For enzyme activity assays, IgG fraction was purified using protein-Sepharose 4B column (Pharmacia Fine Chemical) according to the procedures described by the manufacturer.

Antiserum to PEPC, RuBPC and NADP-ME

Antisera to maize RuBPC and PEPC were kindly provided by Dr.William Taylor, Genetics Department, UC Berkeley. Antiserum to maize NADP dependent ME was kindly provided by Dr.Timothy Nelson, Biology Department, Yale University.

Ouchterlony two-dimensional diffusion

Small wells were cut in agar plates. One was filled with 20 µl of antiserum, second with solution containing 4µl of purified PPDK from maize, and the others with plant extract.

The precipitin band was observed after one to four days of incubation at room temperature. The reaction was stopped by addition of 0.9% (w/v) NaCl solution, and the bands were stained with Commassie Brilliant Blue (Ouchterlony 1968).

Protein blot

Plant materials were homogenized in 3 volumes of 0.1M Tris buffer, pH 7.4 containing 10mM $MgCl_2$, 18% w/v sucrose, 1% β-mercaptoethanol and 10mM PMSF using motar and pestle. The homogenate was filtered through Miracloth and centrifuged at 12,000xg for 10 min. The total soluble protein was determined by the method of Bradford(1976). The supernatant was brought to 2% SDS, 12% glycerol, 20mM DTT and 2% bromophenol blue before electrophoresis. The samples were loaded onto 6.4-12.8% SDS polyacrylamide gradient gel. Following SDS-PAGE at 30mA constant current for 3h, the protein was electrophoretically transferred to cyanogen bromide paper prepared by the method of Clarke, et al (1979). The paper was quenched and washed as described by Nyari et al (1981). Blots were incubated with antiserum first and then with I^{125} labeled protein A (Amersham, 30mCi.mg^{-1}) according to the method of Nelson et al (1984). An autoradiograph was

prepared using Kodak AR5 X-ray film with an intensifying screen at -80 °C overnight.

Densitometry

Relative levels of PPDK in preparations from different plant samples were estimated by densimetry of the x-ray film by comparison of the peak areas. Three to four known amounts of purified PPDK were loaded on the SDS gel adjacent to the plant tissue samples each time to calibrate the amount. Exposures were chosen that remained within the linear rage of the film. Within this range,there was a linear relationship between the amount of protein loaded on the gel and the area of the densitometry peak.

Two dimensional gel electrophoresis

Procedures for sample preparation and running the first dimension isoelectric focusing gels were adapted from O'Farrell(1975). The maize leaves and kernels were frozen in liquid N_2 and were homogenized in 3 volumes of extraction buffer containing 10mM Tris(pH7.5), 10mM $MgCl_2$, 5% sucrose and 1mM DTT. The homogenate was filtered and centrifuged at 12,000xg for 10min. The supernatant containing 20µg soluble protein was lyophilized to dryness, reconstituted in lysis buffer (O'Farrell, 1975), and stored at -80 °C.

Isoelectric focusing gels were prepared and run according to O'Farrell (1975), except that the acrylamide concentration was reduced to 3% and 1% TritonX-100 replaced the 2% NP-40. The second dimension was run on gradient gels (6.4-12.8%). The results were visualized by silver staining using silver staining solution obtained from Bio-Rad Laboratories (Richmond) and autoradiography of protein blot.

Enzyme Assays

PPDK

Plant tissues were homogenized in 3 volumes of ice-cold extraction buffer containing 0.1M Tris-HCl(pH7.5), 10mM $MgCl_2$, 5mM sodium pyruvate, 2mM K_2HPO_4, 1mM EDTA, 1% (w/v) sodium ascorbate, 5mM DTT, and 1% (w/v)PVP. The homogenate was strained through Nylon cloth and centrifuged at 9,000xg for 6min. The supernatant was equilibrated to 65% $(NH_4)_2SO_4$. The precipitate was dissolved in 1ml of the above buffer minus sodium pyruvate and Pi, and passed through a Sephadex G-25 column. The complete assay mixture contained 0.1M Tris-HCl(pH7.5), 10mM $MgCl_2$, 0.1mM EDTA, 5mM $NaHCO_3$, 1.25mM sodium pyruvate, 1.25mM ATP, 5mM DTT, 2.5mM K_2HPO_4, 0.3mM NADH, 2 units of MDH, and 2 units of PEPC. The PEPC was isolated from spinach leaves, and shown to be free of interfering enzymes (Baysdorfer and Bassham1984). The enzymic reaction was observed by following the change in absorption at 340 nm.

PEPC

PEPC was assayed by coupling with MDH. Oxidation of NADH was measured at 340 nm (Lane and Maruyama 1969). Following 3 min pre-incubation of the PEPC at 25°C in 0.9ml of assay mixture containing 0.1M Tris-HCl (pH8.0), 10mM $MgCl_2$, 2mM DTT, 0.2mM NADH, 10mM $NaHCO_3$, and 4 units of MDH, the reaction was initiated by the addition of PEP (to give 2.5mM).

NADP-ME and NAD-ME

NADP-ME was assayed by following NADPH formation at 340nm in reactions containing enzyme, 50mM Tricine-KOH (pH8.3), 5mM malate, 0.5mM NADP, 0.1mM EDTA, and 2mM $MgCl_2$ (Hatch and Mau 1977). A similar procedure was used for measuring NAD-ME, except that the reaction components were 50mM Hepes-KOH (pH7.2), 5mM malate, 5mM NAD, 0.1mM EDTA,

and 0.6mM $MnCl_2$.

NADP-MDH and NAD-MDH

The assay mixture contained 25mM Tris-HCl (pH8.0), 1mM EDTA, 0.5mM oxaloacetate, 0.2mM NADH or NADPH, plus an aliquot of the enzyme. The change in absorbance at 340nm was measured (Hatch and Slack 1969). One unit of enzyme is defined as the amount of enzyme which catalyses 1 μmol of substrate/min under standard assay conditions.

Inhibition of maize enzyme activity by antibody

70μl of each leaf or callus extract containing 200μg of soluble protein was incubated for 30 min or longer period of time with specified amounts of purified PPDK antibody in 0.2ml of 50mM Tris buffer(pH7.5) containing, 5mM $MgCl_2$, 0.1mM EDTA, and 1mM DTT. After incubation for 30 min, the mixture was centrifuged at 12,000xg for 10 min and an aliquot of the supernatant was assayed for PPDK activity.

Isolation of total RNA

Total RNA was isolated essentially as described by Greene (1983) from wheat heads tagged at anthesis and harvested at defined intervals afterwards. 1.2g of wheat seeds were homogenized in a tissue grinder in 10ml of extraction buffer containing 0.1M Tris-HCl(pH8.0), 5mM EDTA, 1.25mg proteinase K and 1% SDS. After centrifugaion of the homegenate at 13,000xg, total RNA was isolated by phenol-chloroform extraction, ethanol precipitation, and pelleting by ultracentrifugation through 5.7M CsCl (Glisin et al 1974). Total RNA content of seeds was determined by spectrophotometric analysis of ethanol precipitates assuming $\varepsilon^{0.1\%}$ (ethanol) at 260nm= 20.

Preparation of mRNA

Total RNA was extracted according to the method of Nelson et

al (1984). In a coffee grinder, 8g of frozen tissue was ground into powder in dry ice and then thawed to room temperature in 4mM guanidium thiocyanate (1ml per g tissue) (Chirgwin et al 1979). Polyadenylated RNA was prepared by fractionation of total RNA on a column of oligo-dT cellulose (Collaborative Research), followed by ethanol precipitation (Aviv and Leader 1972).

In vitro protein synthesis

From 0.2 to 0.5 μg of polyadenylated RNA or 4μg of total RNA was translated in micrococcal nuclease-treated reticulocyte lysate (Amersham) in the presence of ^{35}S-methionine (New England Nuclear) at 30°C for 70 min (Pelham and Jackson 1976) or wheat germ extract in the presence of ^{3}H-leuicine (Marcu and Dudock 1974). Total *in vitro* protein syntheses was determined by measurement of the amount of ^{3}H or ^{35}S in TCA precipitate by scintillation counting.

Immunoprecipitation of in vitro translation products and fluorography

Antibody to PPDK was added to the *in vitro* translation products and mixture was incubated over night at 4°C. Prewashed Staphylococci A cells (Bethesda Research Laboratories) were added to the immunoprecipitated protein according to the method of Kessler(1981). After the precipitate was washed, PPDK was released from the mixture by heating at 80°C for 3min. Following SDS-PAGE of a sample of the supernatant solution, the gel was fixed in protein fixation solution containing 10% trichloroacetic acid(w/v), 10% glacial acetic acid(v/v), and 30% ethanol(v/v) for 1h. The gel was then immersed in Enhance (New England Nuclear) for 1h and then H_2O for 45 min. After the gel was vacuum-dried, it was placed in contact with Kodak X-AR

film with an intensifying screen at -80°C for autoradiography. For estimating the size of the polypeptides, ^{14}C-molecular weight standards (Bethesda Research Laboratories) were run on the same gel adjacent to the samples.

Isolation of bundle sheath strands and mesophyll cell protoplasts

Isolation was carried according to the method of Kanai and Edwards (1973). 7g of maize leaves (2-3 weeks old) were harvested and cut into 0.5mm segments or less with a sharp razor blade. The segments were placed in 50ml of digestion medium consisting of 4% Onozuka R-10 cellulase, 0.2% Macerozyme R-10 (Yakult Biochemical), 0.1% BSA, 1mM $CaCl_2$, 0.5M sorbitol and 10mM MES-KOH buffer (pH5.5). Incubation was carried at 27°C with light density of 600 $\mu Em^{-2}s^{-1}$ for 2.5 h. After the completion of digestion was confirmed by light microscopy, digestion medium was removed by Pasteur pipet. The tissues were washed four times in 5ml of solution A containing 0.5M sucrose, 1mM $CaCl_2$ and 5mM Hepes-KOH (pH7.0). The solution was filtered through 500 µm Nylon net and then 80 µm net. Bundle sheath strands remained on 80 µm net. They were washed 4 times with 7 ml of solution A. At this stage light microscopic observation showed the bundle sheath preparation to be free of mesophyll cells.

Mesophyll protoplasts which were in the filtrate were overlaid with solution B consisting of 0.5M sorbitol, 1mM $CaCl_2$ and 5mM Hepes-KOH (pH7.0) and centrifuged at 100xg for 5min. Purified mesophyll protoplasts were diluted with solution B and centrifuged at 100xg for 2 min. The protoplast pellet was resuspended in 0.3 ml of solution B and kept on ice. The light microscopic observation showed intact protoplasts free of bundle sheath cells.

Light microscopy The mesophyll protoplasts and bundle sheath strands, suspended in solution B and A respectively were examined with Nikon phase contrast microscope with Nikon polaroid camera. Both mesophyll protoplasts and bundle sheath strands remained intact at 4°C for several days.

Protein blot analysis of enzymes in bundle sheath strands and mesophyll protoplast

Bundle sheath strands were homogenized in 1 ml of protein blot extraction buffer using motar and pestle. Mesophyll cell protoplasts suspended in 0.3 ml of solution B were homogenized in a glass homogenizer with 0.6 ml of the above buffer. The homogenate was filtered through Miracloth and then centrifuged at 12,000xg for 10 min. The total soluble protein was determined by the method of Bradford (1976). Chl a/b ratio was determined by the method of Arnon (1949).

Varying quantities of soluble protein (20 to 60 µg) from bundle sheath strands, mesophyll protoplast and whole leaves were loaded for a comparison on gradient gels (6.4-12.8%). After SDS-PAGE, the protein was transferred to cyanogen bromide paper at 0.5 A for 9 h. After transfer, the gel was stained with Coomassie Brilliant Blue and destained to check the transfer efficiency. The transfer paper was probed with anti-PPDK serum or anti-PEPC serum, then 0.5µCi of I^{125} protein A. Autoradiograph was prepared as described previously. After the film was developed, the gel was again probed with either anti-RuBPC or anti-NADP dependent ME sera.

Isolation of intact spinach chloroplasts

35g of young spinach leaves were cut into small pieces into 100 ml of ice cold extraction buffer (0.33M sorbitol, 0.2mM $MgCl_2$ and 5mM

Tricine-NaOH (pH 7.8) and homogenized 3 times for 3s each with a Waring Blender. The brei was filtered through 6 layers of cheese cloth and centrifuged at 2,2ooxg for 30s. The pellet was resuspended in 40 ml of the above buffer and centrifuged again at the same speed for 30s.

The next step in preparation of intact chloroplasts required the preparation of a Percoll gradient as described by Mourioux and Douce (1983). Thirty ml of medium (50% Percoll, 330 mM sorbitol, 50mM Tricine-NaOH, pH 7.8, 2mM EDTA and 0.15% BSA) was pipetted into a centrifuge tube. The tube was placed in a pre-cooled Sorvall SS 90 vertical rotor and centrifuged at 3°C at 10000xg for 100 min. The crude chloroplast pellet was resuspended in a small volume (1-2 ml) of the sorbitol/Tricine-NaOH buffer used in the first step pf the chloroplast isolation and layered on the 30 ml pre-formed Percoll gradient. After centrifugation of the chloroplasts in the gradient for 10 min at 10,000xg at 4°C in the Sorvall SS90 rotor, an intact layerof chloroplasts was obtained as a broad band near the bottom, whereas the stripped chloroplasts and extrachloroplastic membrane system formed a band at the sample-gradient interphase.

Precursor uptake by spinach chloroplasts

Precursor uptake was carried out acoording to the method of Grossman et al (1980) and Bartlet et al (1982) with some modifications. Maize poly(A) RNA was translated in a rabbit reticulocyte cell-free system (Amersham) using 200 µCi ^{35}S-methionine (1126mCi $mmol^{-1}$) (New England Nuclear) at 27°C for 1.5 h, 200µl of the translation product was incubated with intact spinach chloroplasts

which had been prepared by Percoll gradient centrifugation as described above. The incubation mixture (600µl) contained 400µg chlorophyll, 50mM Tricine-NaOH (pH8.0), 8.3 mM methionine, 0.33M sorbitol and 10mM ATP. The chloroplasts were incubated in 5 ml test tubes at 27°C for one h with illumination (8,000 lux) and gentle shaking. For dark controls, test tubes were wrapped with aluminum foil.

After incubation, the chloroplast suspension was diluted with 5 ml of buffer containing 50mM Tricine-NaOH (pH8.0) and 0.33M sorbitol and then was centrifuged at 4,000xg for 3 min. The pellet was resuspended in 0.5 ml of the dilution buffer and treated with 300 µg ml^{-1} of trypsin for 30 min at 0°C. After proteolysis, the chloroplast suspension was diluted with 3 ml of the same buffer containing 1mM PMSF, 1mM benzamidine-HCl and 5mM ε-amino-n-caproic acid, pelleted and resuspended in 1 ml of dilution buffer by gentle mixing. The intact chloroplasts were reisolated by centrifugation at 5,000xg for 5 min through a layer of 5 ml of 50% Percoll gradient prepared by the method described above. To the reisolated chloroplasts, sterile water containing 1mM PMSF, 1mM benzamidine-HCl and 5mM ε-amino-n-caproic acid were added to lyse the chloroplasts. NaCl was added to the solution to the final concentration of 100mM and centrifuged at 12,000xg for 10 min to separate supernatant and membrane fraction. Any PPDK in both fractions were immunoprecipitated overnight at 4°C with 10µl of PPDK antiserum. Prewashed Staphylococci A cells were added and incubated for 2 h then pelleted. The pellet was washed once with 200µl of buffer containing 25mM Tris-HCl (pH 7.5), 0.7M NaCl, 10mM EDTA, 0.15% Triton X-100 and once with the 10 mM Tris-HCl(pH 7.5)

buffer. Staphylococci A cells were removed from PPDK by eluting in the buffer containing 50mM Tris-HCl(pH 7.5), 3% β-mercaptoethanol, 3% SDS, 40% glycerol for 1h followed by heating at 85°C for 2 min and centrifugation at 12,000xg for 5 min. The samples were analyzed by fluorography as described already.

Preparation of chloroplast stroma for protein analysis

The intact spinach chloroplast layer was pipetted out after centrifugation in 50% Percoll gradient and buffer was added to give a chlorophyll concentration of 2mg. ml^{-1}. The chlorophyll content was measured as described already. Sterile water was added to the chloroplast suspension and frozen in liquid N_2. It was thawed to room temperature, vortexed vigorously to lyse chloroplasts, and then centrifuged at 14,000xg for 20 min. The supernatant was brought to 2% SDS, 12% glycerol, 20mM DTT and 2% bromophenol blue before electrophoresis.

Maize and wheat chloroplast stroma protein preparation were made by the method of Sugiyama and Hatch (1981) with minor modifications. About 8 g of each leaf was harvested from young seedling (seven day) and sliced into 1-2 mm sections and blended in 50 ml of extraction buffer consisting of 20 mM Tris-HCl (pH 8.3), 0.4M Sorbitol, 5mM $MgSO_4$, 2mM DTT and 0.2mM EDTA using Polytron (Brinkmann Instruments) equipted with PT 20 ST probe generator for 10 s. The homogenate was filtered through Miracloth and centrifuged at 1,000xg for 3 min. The pellet was resuspended in 2 ml of sterile distilled water and was frozen in liquid N_2; then thawed. After vigorous vortexing to lyse the chloroplasts, the content was centrifuged using a microconcectrator, Centicon (Amicon) until the volume was reduced to 1/40 of the starting

volume, since the protein content assay of the original supernatant of the chloroplast extract showed the protein concentration to be too low (between 0.3-0.7 $mg.ml^{-1}$) to load on the gel directly. The concentrated extract was prepared in the same manner as spinach chloroplast protein before electrophoresis.

Incorporation of labeled carbon dioxide

Plant tissues (70-200 mg) were dissected into small pieces(about 2x2x2 mm). Each sample was placed in 0.5 ml buffer (3mM $NaHCO_3$, 5mM Hepes-OH, pH7.8) in a glass homogenizing tube and pre-illuminated at 650 $\mu Em^{-2}s^{-1}$ for 2 min. The 0.5 ml $NaH^{14}CO_3$ solution was added to give a final concentration of 8.4 mM (25mCi.$mmol^{-1}$), and illumination was continued for 5,10,15,30,60, and 120 s (or specified length of time). Liquid N_2 was poured into each tube at the end of this time to stop the reactions. Methanol was added to give 50% methanol and the mixture was ground as it thawed. Aliquot portion of this mixture was analyzed by two-dimentional paper chromatography and radioautography, and incorporation of ^{14}C into individual compounds was measured by liquid scintillation counting (Pederson et al 1966). For identification of compounds, co-chromatograghy with known labeled compounds or unlabeled standards which were subsequently located using various indicator sprays.

Light microscopy of A188 tissue section

Callus was divided into chlorophyllous and non-chlorophyllous samples which were separately processed. Regenerated shoots of various stages were removed from the plant and cut into small pieces(c.a 5x5 mm). The oldest regenerated leaf was obtained from a regenerated plant which had 5 leaves. The section which is 3 cm from the tip of the 4th

leaf was used. It should be noted that the 4th leaf from this regenerated plant was much smaller in the blade size (c.a. 8 cm) as compared to that of a seedling (20 cm, 3 weeks after planting). As a control, a section which is 3 cm from the tip of the third leaf (the blade length was 10 cm) of a 20 d old maize plant was used.

Callus tissues were fixed in 4% gluteraldehyde in 0.05% phosphate buffer (pH 6.8), dehydrated in an ethanol series and embedded in glycol methacrylate according to the method of Feder and O'Brien (1968). Material was sectioned at 2-5 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 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 parafin. 5 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.

CHAPTER III

RESULTS AND DISCUSSIONS

Part One

PPDK in C_3 plants and maize cells

RESULTS

Occurrence

PPDK was found in various immature seeds of C_3 plants such as wheat, rice, plum and spinach and in some C_3 leaves in wheat, tobacco, spinach (Fig.1), and sunflower, rice and pampusgrass (data not shown). In wheat, PPDK was present in awn and glume (Fig.2A).

The difference in the amounts of PPDK per mg soluble protein depending on the variety of wheat cultivar was compared, however no significant difference could be observed (Fig.2B). The multiple bands which are lower in molecular weight than 94KD PPDK band are probably due to degradation products (see DISCUSSIONS). The percentage of PPDK in soluble protein appears to be less than 0.05% in wheat leaves and even less in spinach and tobacco leaves while in contrast, PPDK can constitute 3 to 10 % of the soluble protein in maize leaves. These estimates are based on densitometric measurements of the darkened areas of the autoradiogram of protein blots and the assumption that PPDK polypeptide from wheat reacts with maize PPDK antibody to the same extent as maize PPDK. The validity of this assumption is suggested by the fact that many Ouchterlony plates prepared with the maize PPDK antibody against crude protein extracts of maize and wheat leaf tissues showed no detectable 'spurs' such as were seen with immature plum fruit protein (Fig.3B), where only partial identity is indicated. These estimates of protein amount must be considered as lower limits.

PPDK was also found in kernels of maize (Fig.4). The amount of PPDK per mg soluble protein is high in later stages of seed development and it is about 20% of that found in maize leaves, amounting to about 2% of the total soluble protein. A similar amount of PPDK was found in

maize husk, but none was found in the silk or root (data not shown).

PPDK has been considered a unique enzyme localized in C_4 leaf mesophyll cells (Edwards and Walker 1973). However small amounts of PPDK protein which is about 1/10 as much in concentration in soluble protein as in the mesophyll cells were also found in bundle sheath cells according to the protein blot analysis (Aoyagi and Nakamoto 1985). The isolated mesophyll cell protoplasts and the bundle sheath strands were free from cross contamination as determined by light microscopy (Fig.5). The chl a/b ratio of the isolated bundle sheath cell was 9-10, whereas that of the mesophyll cell protoplast was 3.2. This difference in chl a/b ratio of the two preparations is consistent with the expected values for the purified cell types (Kanai and Edwards 1973, Girardi and Melis 1983). The amount of PPDK found in bundle sheath cells is higher than that found in wheat leaf so far (Fig. 6A). PEPC was barely detectable in bundle sheath cells (Fig. 6B). NADP dependent ME and RuBPC were exlusively present in bundle sheath cells consistent with the previous reports (Fig. 6B,C) (Hattersley et al 1977, Matsumoto et al 1977).

Properties

Based on the protein blot analysis, the subunit mol wt are similar at about 94 kD except in spinach leaves where the apparent mol wt was about 90 kD (Fig. 1). Maize kernel PPDK seems to be similar as leaf PPDK based on protein blot analysis of two-dimensional gel electrophoresis (Fig.7). The isoelectric focusing point is about pH 6.2, similar to that of leaf PPDK reported by Ashton and Hatch (1983). The protein pattern of leaf PPDK that I have obtained shows a streak which runs vertically. This seems to be attributable to degradation

and needs further investigation.

According to enzyme activity assays, the specific activity of the wheat enzyme is comparable to that of PPDK from maize leaves, but the enzyme level is much lower (Table 1). As in the case with PPDK from C_4 leaves (Hatch et al 1969), PPDK activity from wheat leaves increases during several hours of leaf illumination and decreases in darkness (Table 2). In contrast, PPDK activity in wheat seeds does not change with illumination (data not shown).

The activity of PPDK in a crude extract of wheat seed or leaf is stable for 30 min at room temperature, but decreases rapidly when the enzyme is stored at 0°C (Fig.8). This cold inactivation is reversible, as the activity of the enzyme recovers after 30 min to 90% of the initial value when the temperature is raised to 22°C. This cold lability is similar to that of the enzyme from C_4 plants (Shirahashi et al 1978). In that case, it was reported that the purified enzyme from maize leaves is a tetramer in the active form and reversibly dissociates to give a dimer at lowered temperatures. The pH optimum of the enzyme from wheat tissues is about 7.9 which is similar to the value reported for the maize leaf (Sugiyama 1973).

Location of PPDK in C_3 plants

(A) Leaves

Examination of chloroplast stromal enzyme from maize, spinach and wheat leaves indicates that in both maize and C_3 leaves, PPDK is present mostly in the chloroplast (Fig. 9). Since it is difficult, if not impossible to obtain cytosol uncontaminated with stroma from broken chloroplasts, the upper limit of PPDK amounts that might be in cytosol cannot be accurately determined. We can account for most of the total

leaf PPDK as stromal PPDK, based on amounts per mg soluble protein in leaves and chloroplasts stroma respectively. The amount of PPDK polypeptide was approximately three fold greater in stroma extract as compared to that in the whole cell extract in all three leaves studied. This result indicates that in spinach and wheat leaves, PPDK is probably localized in the chloroplast as in the maize leaves.

(B) Seeds

Green pericarp tissue and endosperm tissue from seeds 12 to 15 and 30 to 33 DAF contained PPDK (Fig. 10). Precise quantitation was not possible in this experiment, but we estimate from densitometry that 90% or more of the PPDK of the 12 to 15 DAF seeds was in the endosperm. The apparent amount of PPDK in the pericarp from the 30 to 33 DAF seeds may be exaggerated due to the fact the precise dissection of these seeds is more difficult than with the 12 to 15 DAF seeds. The aleurone layer tends to stay attached to the pericarp tissue in the older seeds whereas it remains attached to the endosperm in the younger seeds.

The patterns of bands of PPDK subunits obtained by protein blot are quite different for the two tissues (Fig.10). The pericarp tissue gives a number of bands of low mol wt in addition to the main 94kD band seen with endosperm. The multiple bands are probably due to degradation by protease (see Fig. 5 of Part Two). This will be discussed in Part Two. If this pattern of several bands seen with pericarp tissue represents rapid degradation of PPDK, either <u>in vivo</u> or during extraction, it could explain the inconsistency between our finding of PPDK protein in pericarp tissue and the report that PPDK activity is absent from such tissue (Meyer et al 1982). Since the present result is based on immunochemical recognition of the protein rather than

enzymic activity, it eliminates the possibility of a false negative result that might occur due to enzyme inactivation prior to or during tissue preparation. In contrast to PPDK, RuBPC large subunit is seen only in the pericarp tissue which is chlorophyllous (Fig 10B).

DISCUSSION

Although a limited number of C_3 species so far have been examined, PPDK appears to be more widely distributed among C_3 plants than had been generally realized. Negative results from past efforts to detect PPDK enzyme activity in wheat leaves (Meyer et al 1982, Wirth et al 1977) and maize kernel (Meyer et al 1982) may be due to a variety of causes including variability in amount due to age of the tissue and other physiological conditions, inactivation during extraction, and insufficient purification (since there is so little activity, it often cannot be detected in crude extracts). The activity of PPDK per mg soluble protein is in fact much less in C_3 tissues as compared with maize tissues, approximately 1/70th of the enzyme activity. Conditions used in the assay which may be important for obtaining the maximum activity include N_2 bubbling of the extraction buffer as well as inclusion of ascorbate, extraction under full sunlight, and rapid desalting following ammonium sulfate fractionation. Moreover,the PPDK level varies greatly depending on the stage of development (see Part Three). This limited occurrence, together with the difficulty in obtaining the active form, may explain the scarcity of previous reports (Duffus and Rosie 1973, Meyer et al 1982, Schnabl 1981) of its presence in such tissues.

There are many similarities between the C_3 PPDK (in wheat seeds and leaves) and the enzyme from maize. For example, the mol wt (94kD) of the subunit is the same. Based on the Ouchterlony double diffusion method, wheat seed or leaf PPDK appeared identical with maize leaf PPDK, when tested with maize leaf PPDK antibody.

Two dimensional gel electrophoresis and protein blot analysis of maize leaf and kernel PPDK showed a similar pattern. The presence of lower mol wt bands was specific to the chloroplastic PPDK and is probably due to degradation by protease . A similar pattern was observed with pericarp tissue and trypsin treated samples (Fig. 5 Part Two). Purified PPDK separated by SDS-PAGE showed the same pattern (Ashton and Hatch 1983). Further study is necessary to determine the cause for the multiple bands.

Enzyme properties such as in vivo light activation (in leaves only), cold instability, and pH profile of the wheat enzyme are all similar to those found in C_4 maize leaf. Meyer et al (1978,1982) reported that Km values for ATP and Pi of the wheat seeds and leaves are also similar to those from C_4 maize leaf. Km (pyruvate) of wheat seed PPDK was 24 μM whereas that of maize leaves was 140 μM (Sugiyama 1973). Therefore the wheat seed PPDK shows greater affinity to pyruvate than maize leaf PPDK. Burnell (1985) reported that PPDK from both wheat leaf and maize kernel served as substrates for PPDK regulatory protein which mediates activation of maize leaf PPDK, so that a similar mechanism of enzyme activativation/inactivation may exist for PPDK from both tissues.

The occurrence of PPDK in bundle sheath cells at appreciable quantities indicates that PPDK is more widely present in maize leaves than previously considered. In contrast, NADP-ME and RuBPC are exclusively localized in bundle sheath cells and PEPC is exclusively localized in mesophyll cells (Fig.6) The possibility of cross contamination may be estimated not only by light-micrography, but also by the result of protein blot analysis of bundle sheath strands

fraction probed by PEPC antibody. Perrot-Rechenmann et al (1982) has demonstrated exclusive localization of PEPC in the cytoplasm of maize mesophyll cells. I have detected minute amounts of PEPC only once (Fig. 6B) and not in the other repeated experiments, making it likely that the very small amount of PEPC in bundle sheath chloroplast preparation represents a good estimate of mesophyll contamination. Therefore this result may be used to judge the purity of the bundle sheath strands preparation. PPDK gave much stronger band than PEPC so that it is difficult to attribute the PPDK band to contamination by mesophyll cells.

In order for C_4 photosynthesis to function most efficiently, it is reasonable that certain enzymes, notably PEPC, RuBPC and NADP-ME, have complete differential localization between the two photosynthetic cell types. However, some other photosynthetic enzymes, including those for reduction of PGA to glyceraldehyde 3-phosphate, function in both mesophyll and bundle sheath cells. In maize, some of the pyruvate generated from malate decarboxylation through NADP-ME may be converted to PEP through PPDK in bundle sheath cells. Thus, both cell types may share in the regeneration of the substrate for PEPC. In addition if some PGA is converted to PEP in mesophyll cells (Huber and Edwards 1975) it would not be necessary that all of the pyruvate from malate decarboxylation return to the mesophyll cell. These possibilities add flexibility in how the level of carbon intermediates of the C_4 cycle may be maintained.

The occurrence of PPDK in maize organs, such as husk and kernels, suggests that the role of PPDK in maize is not restricted to C_4 photosynthesis which takes place in a leaf blade. Soldatini et

al (1982) reported that all aerial organs metabolically related to the ear in maize are able to fix $^{14}CO_2$ both in the light and in the dark mostly into aspartate plus malate. Leaf sheath and hypsophylls did not photorespire, even though they do not possess Kranz anatomy. This wide distribution of PPDK both in C_3 and maize adds additional information to the previous reports (Meyer et al 1982) and indicates that PPDK is present in tissues without Kranz anatomy.

It is interesting that the concentration of PPDK in the soluble protein found in maize kernels and bundle sheath cells is still greater than that found in wheat leaves and seeds. Thus the greater expression of this enzyme seen in maize leaves seems to be carried over into the seed, at least when maize and wheat are compared.

The location of PPDK within the wheat and spinach leaves is in the chloroplast as in the maize leaves. In maize mesophyll cells, the role of PPDK in the CO_2 fixation and transport pathway is well known (Hatch and Slack 1968). Such an intercellular C_4 transport does not apparently occur in C_3 leaves, but the possibility for intracellular transport remains. This aspect will be discussed in detail in Part Three.

The location of PPDK in seed tissue is not primarily in photosynthetic CO_2 incorporation as it is in C_4 leaf tissue. This will be discussed also in Part Three.

Table 1. PPDK activity and amount in wheat leaves and seeds, and in maize leaves

	Amount μg/mg soluble protein	Enzyme Activity U/mg Chl	U/mg soluble proteinx10^{-3}	U/g fresh wt
Maize leaf	30	3.2	110	1.1
Wheat leaf	0.4	0.44	9.7	0.05-0.61[a]
Wheat seed	0.5	3.3	5.9	0.05-0.39[b]

a/b Values may vary depending on the condition of extraction and the material.

Table 2. Light activation and dark inactivation of PPDK activity in wheat leaves

Treatment	PPDK Activity U/g fresh wt
Control[a]	0.22
Dark[b]	0.022
30 min dark[c]	0.043
Light[d]	0.62

[a] Flag leaves were harvested at 9 AM (sunrise was at about 6 AM).

[b] The plant was kept in darkness overnight.

[c] The plant was placed in a dark room for 30 min at 9 AM before harvesting.

[d] The plant was left in sunlight until harvested at 3 PM.

Fig. 1. Occurrence of PPDK in C_3 plant tissues. The total soluble protein was separated on SDS gradient gel (6.4-12.8%). The transfer paper was probed with antimaize-PPDK. A. Lanes 1 and 8, maize leaf(o.13 mg); lanes 2 and 7, purified maize leaf PPDK (0.5 µg); lane 3, wheat leaf (0.22mg); lane 4, spinach leaf (cv 5286 America, 0.44 mg); lane 5, tobacco leaf (0.17 mg); lane 6. tobacco callus (0.02 mg). B. Lane 9, spinach leaf (cv Highpack, 0.38 mg); lane 10, purified maize leaf PPDK (0.25µg). Although PPDK was not detected in the blot test in lane 4, other test such as shown in lane 9 gave a positive result. The apparent mol wt of the spinach PPDK subunit seems smaller (estimated 90 kD) than that of maize leaf PPDK.

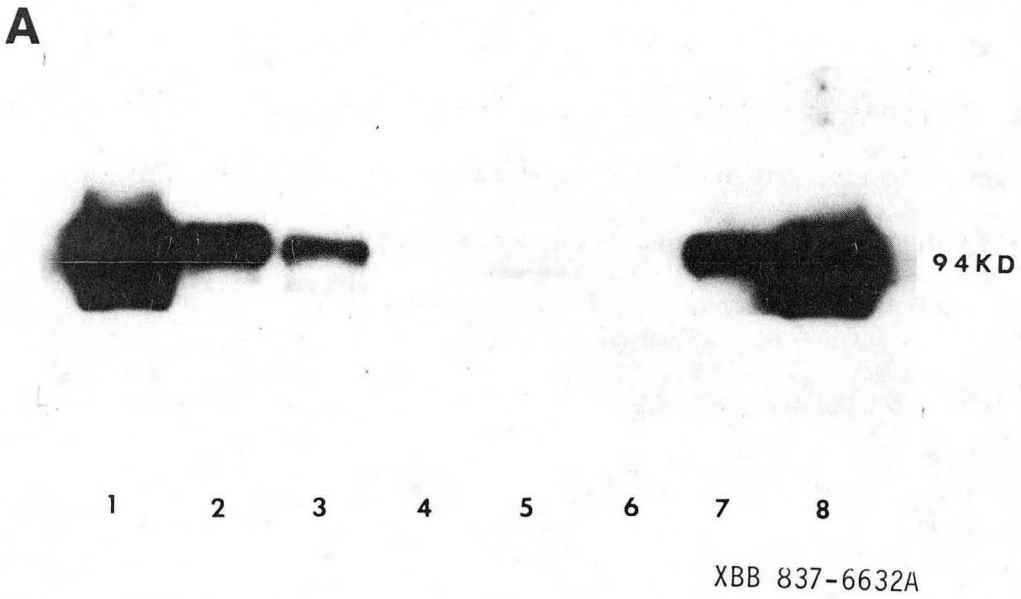


Fig. 1

Fig.2A. Comparison of PPDK from leaves and seeds of wheat and maize leaves, by protein blot method. Lane 1, 0.25 μg purified maize PPDK; lane 2, 0.5μg PPDK; lane 3, 1.0 μg PPDK; lane 4 to 8, each 220 μg total soluble protein from wheat tissues as follows: lane 4, leaf; lane 5, endosperm; lane 6, pericarp; lane 7, awn plus glume; lane 8, whole grain; lane 9, 0.5 μg purified maize PPDK; lane 10, 14 μg of crude maize leaf extract. Lnae 6 showed multiple bands due to degradation of the enzyme during extraction. The quantity of polypeptide was estimated from x-ray film darkening standardized by exposure to known amounts of labeled PPDK.

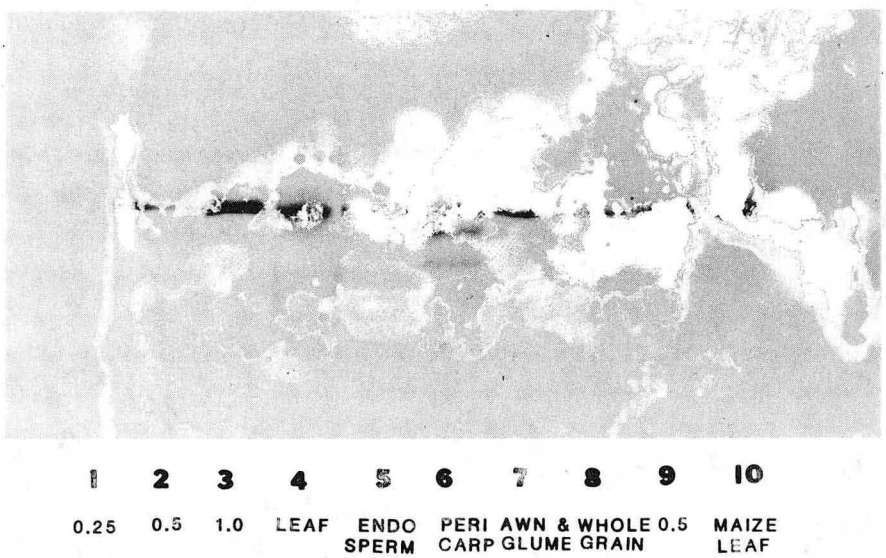
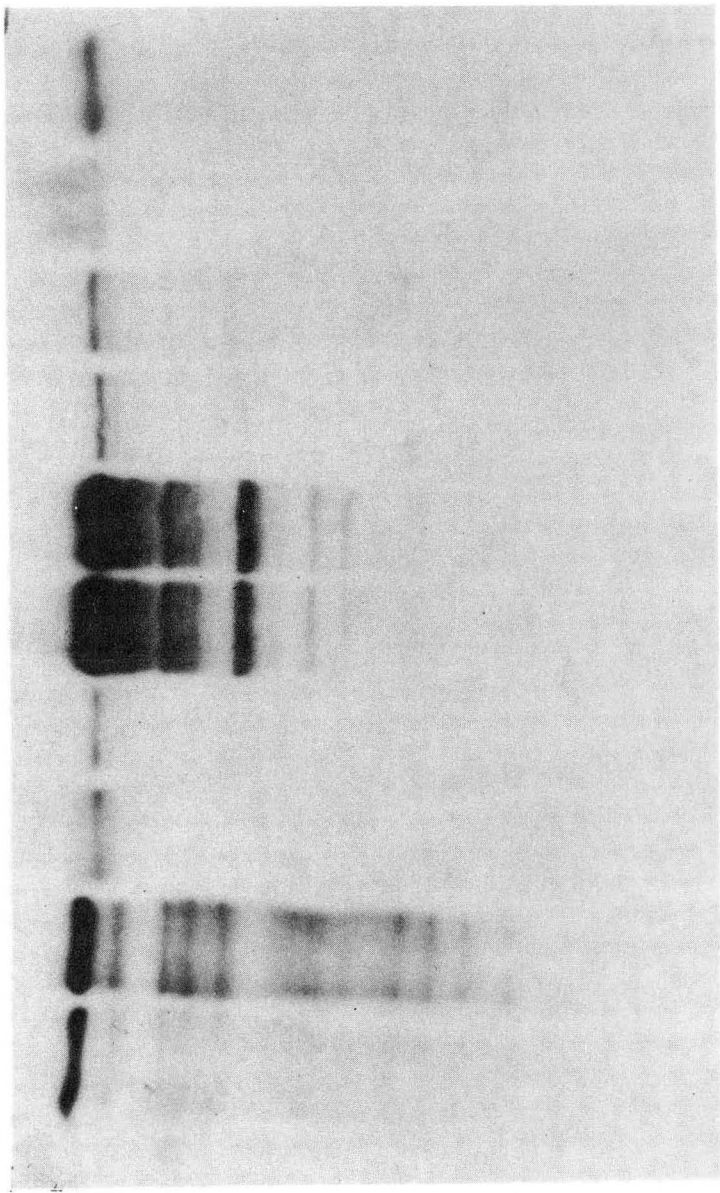


Fig. 2A

BB 837-6010

Fig. 2B Comparison of PPDK content in various wheat cultivars Lane 1 and 2, purified PPDK standard (0.125μg and 0.25μg respectively); Lane 3 to 10, extract (300μg) from lane 3, Anza leaf(upper); lane 4,Anza leaf (lower); lane 5, Anza seed lane 6, Cheyenne seed; lane 7, Cheyenne leaf (lower); lane 8, Cheyenne leaf (upper), lane 9, Rojo leaf (upper); lane 10, Rojo leaf (lower)

* () indicates the relative position of the leaf on the stalk.



XBB 848-6271A

Fig. 2B

Fig. 3 Ouchterlony double diffusion of maize leaf and plum immature fruit PPDK. (A) Well A, Anti-PPDK serum; well B, purified PPDK from maize leaf; wells C and D, crude enzyme extract from maize leaf. (B) Well A, Anti-PPDK serum; Well B, purified PPDK from maize leaf; wells C and D, the crude enzyme extract from immature plum fruit. The spur (arrow) indicates partial identity between plum PPDK and maize leaf PPDK.

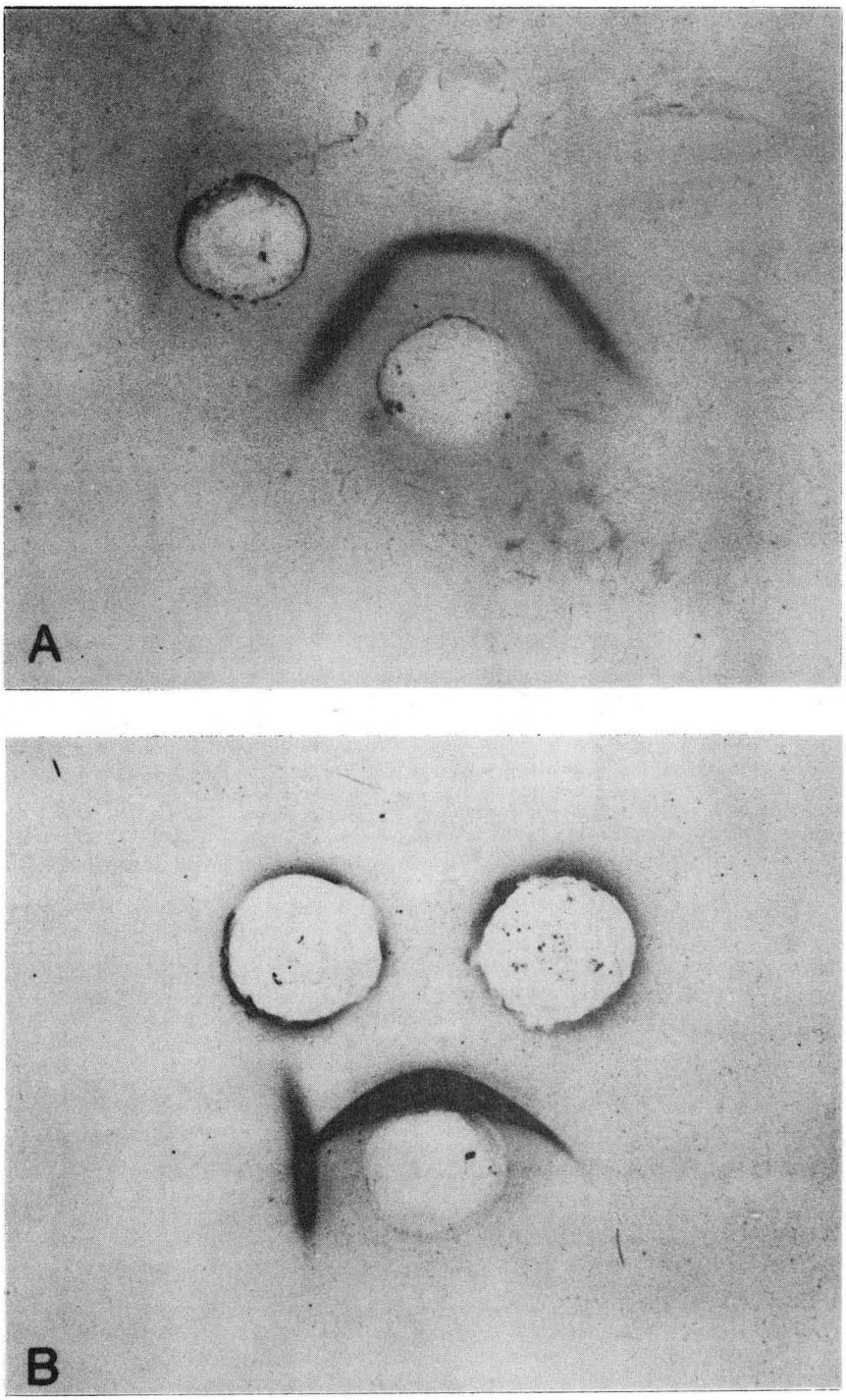


Fig. 3

XBB 837-6555A

Fig. 4 Occurrence of PPDK in maize kernels according to the protein blot method. Lane 1, maize leaf obtained from mature plant about 70 d old (0.05 mg soluble protein); lane 2, purified maize leaf PPDK (0.25μg); lane 3, immature maize kernel at early stage (0.36 mg soluble protein) when the kernel is light green, average diameter of a kernel is 3 mm, and the ear is 5 cm long; lane 4, immature maize kernel at a later stage (0.5 mg soluble protein), when the kernel is yellow, average diameter of a kernel is 1 cm, and the ear is 7 cm long; lane 5,the ear is at later stage; lane 6, purified maize leaf PPDK (0.5μg).

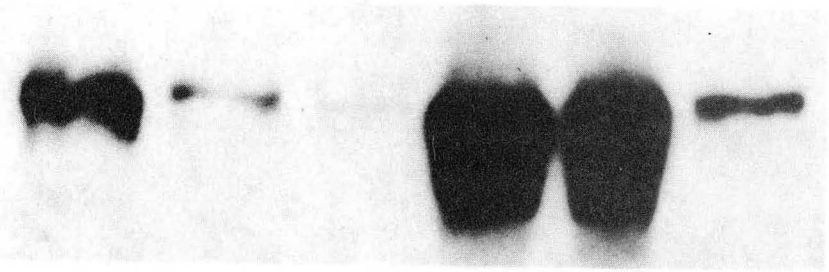


Fig. 4 XBB 839-7793

Fig. 5 Light microscopy

A. Mesophyll cell protoplasts observed under a light microscope, x160.

B. Bundle sheath strands, x80.

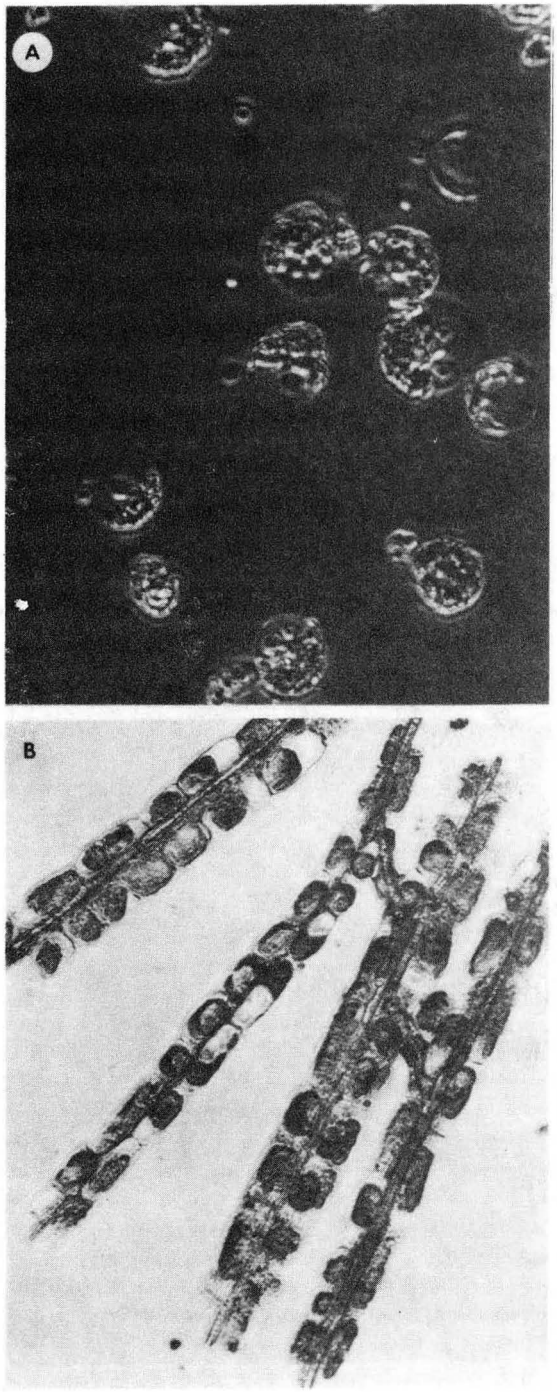


Fig. 5 XBB 851-626A

Fig. 6 Protein blot analysis for PPDK of various samples.

A. PPDK

lane 1, Wheat leaf tip (400μg)

lane 2, Maize bundle sheath cells (20μg)

lane 3, Maize mesophyll cells (20μ g)

lane 4, Maize whole leaf (20 μg)

* ( ) indicates the amount of soluble protein loaded per lane.

B. PEPC and NADP dependent ME in maize leaf

lane 1, Bundle sheath cells

lane 2, Mesophyll cells

lane 3, Whole leaf

* In all cases, 60 μg of soluble protein was loaded per lane.

C. PPDK and RuBPC in maize leaf

lane 1, Bundle sheath cells

lane 2, Mesophyll cells

lane 3, Whole leaf

* In all cases, 40 μg of soluble protein was loaded per lane.

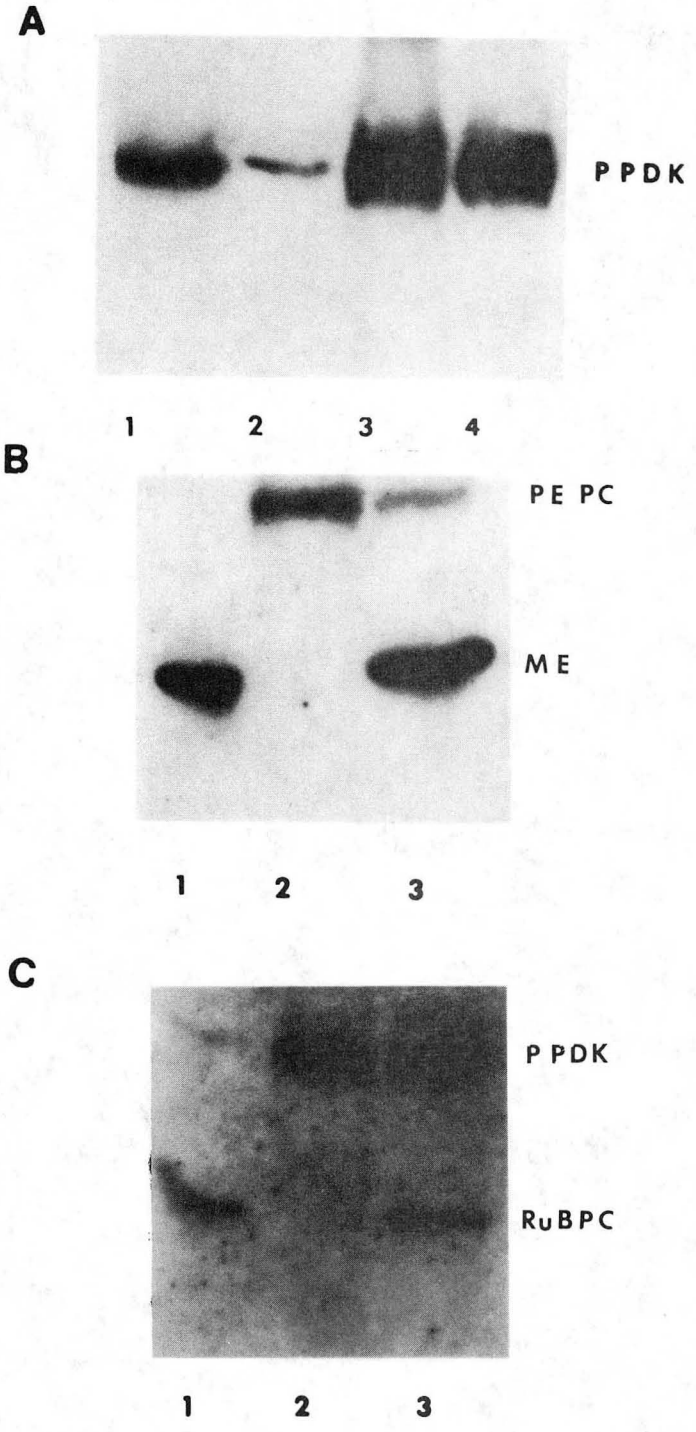


Fig. 6

XBB 851-636B

Fig.7

Two-dimenional gel electrophoresis of maize kernel PPDK analyzed by protein blot.

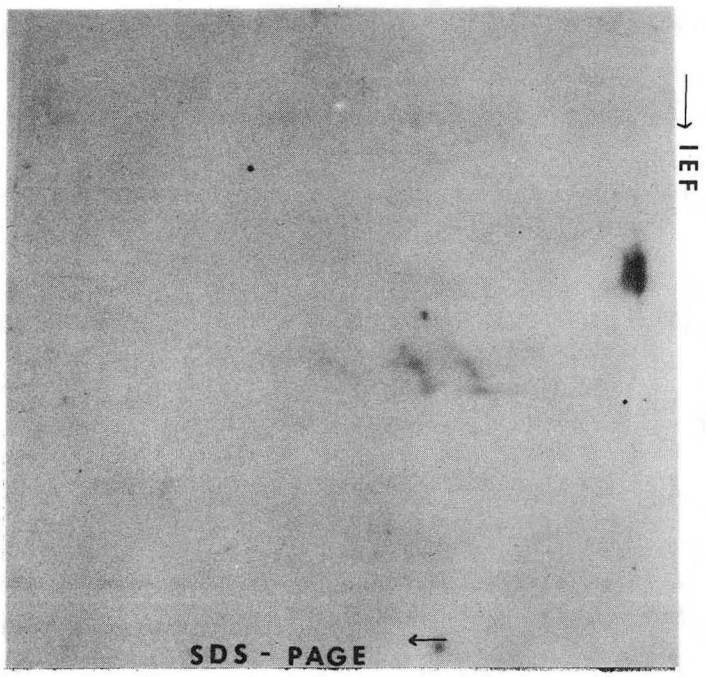
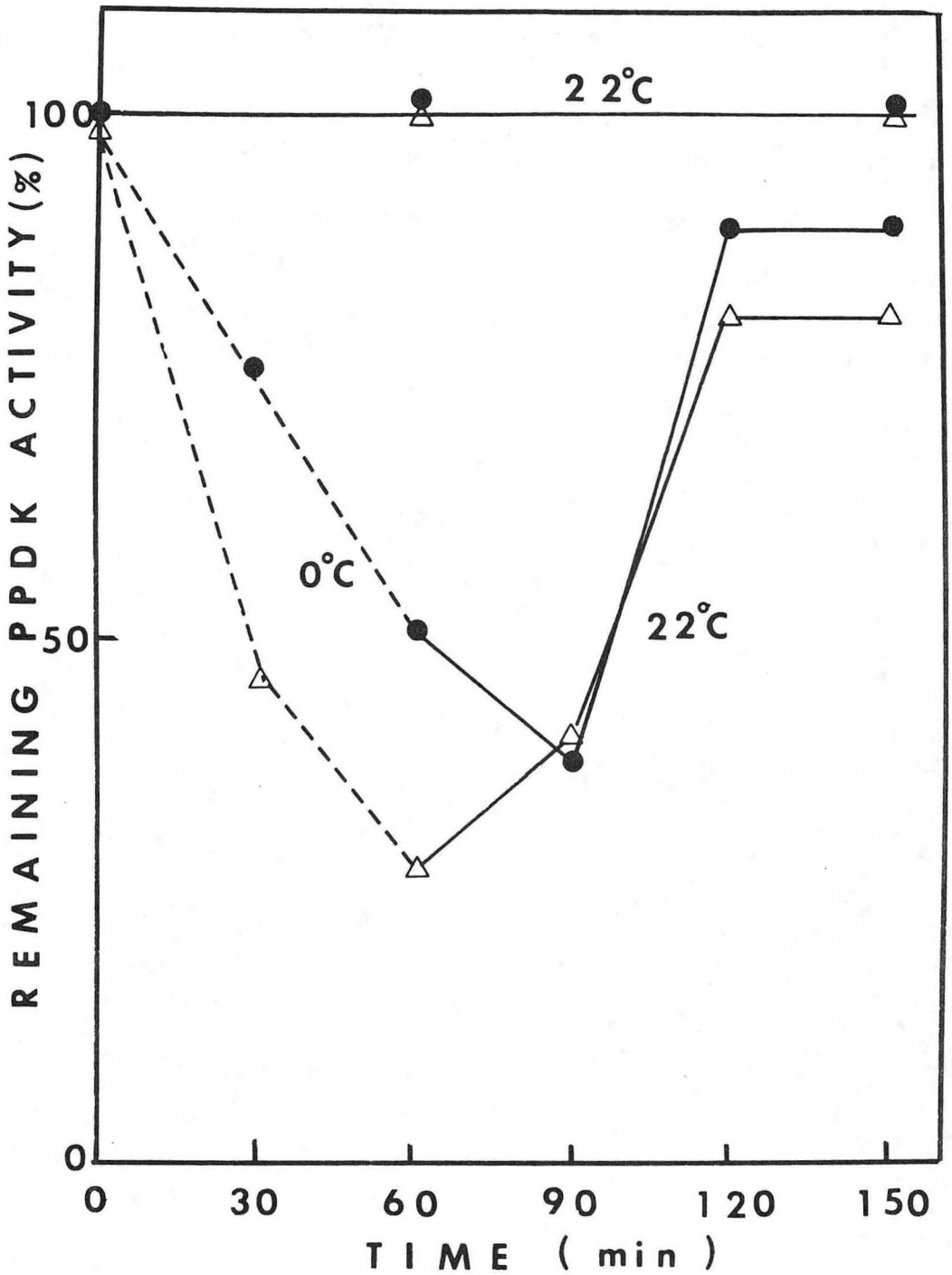


Fig. 7

XBB 853-2347

Fig. 8

Reversible cold lability of PPDK from wheat. Leaf enzyme (Δ); seed enzyme (●). (.....), 0°C; (_____), room temperature (22°C). The crude extract was prepared under sunlight as described previously. The enzyme was then stored 30 and 60 min at 0°C, then transferred back to room temperature and assayed after 30, 60, and 90 min.



XBL 838-10877

Fig. 8

Fig. 9

Protein blot analysis of C_3 leaf chloroplast stroma

lane1, Purified maize leaf PPDK standard (0.375 µg)

lane 2, Maize chloroplast stroma protein (30 µg)

lane 3, Wheat chloroplast stroma protein (400µg)

lane 4, Spinach chloroplast stroma protein (300µg)

Fig. 9

XBB 847-5303

Fig. 10

Presence of PPDK and of RuBPC in endosperm and pericarp tissue. A.PPDK. B.RuBPC (large subunit). RuBPC is located only in the pericarp tissue. The seed extract were analyzed by protein blot. Lane 1, 0.5 µg of purified maize PPDK standard. Lanes 2 through 7 each receivedd 0.5 mg of soluble protein from wheat seed tissues, but the number of seeds from which this amount of protein was derived varied from about 0.2 to 1.1. In lanes 2 and 4 (pericarp, 12-15 DAF) the protein was derived from about twice as many seeds as in lanes 3 and 5 (endosperm, 12-15 DAF), but in lanes 6 (pericarp, 30-31 DAF) and 7 (endosperm, 12-15 DAF) the protein was from about the same number of seeds.

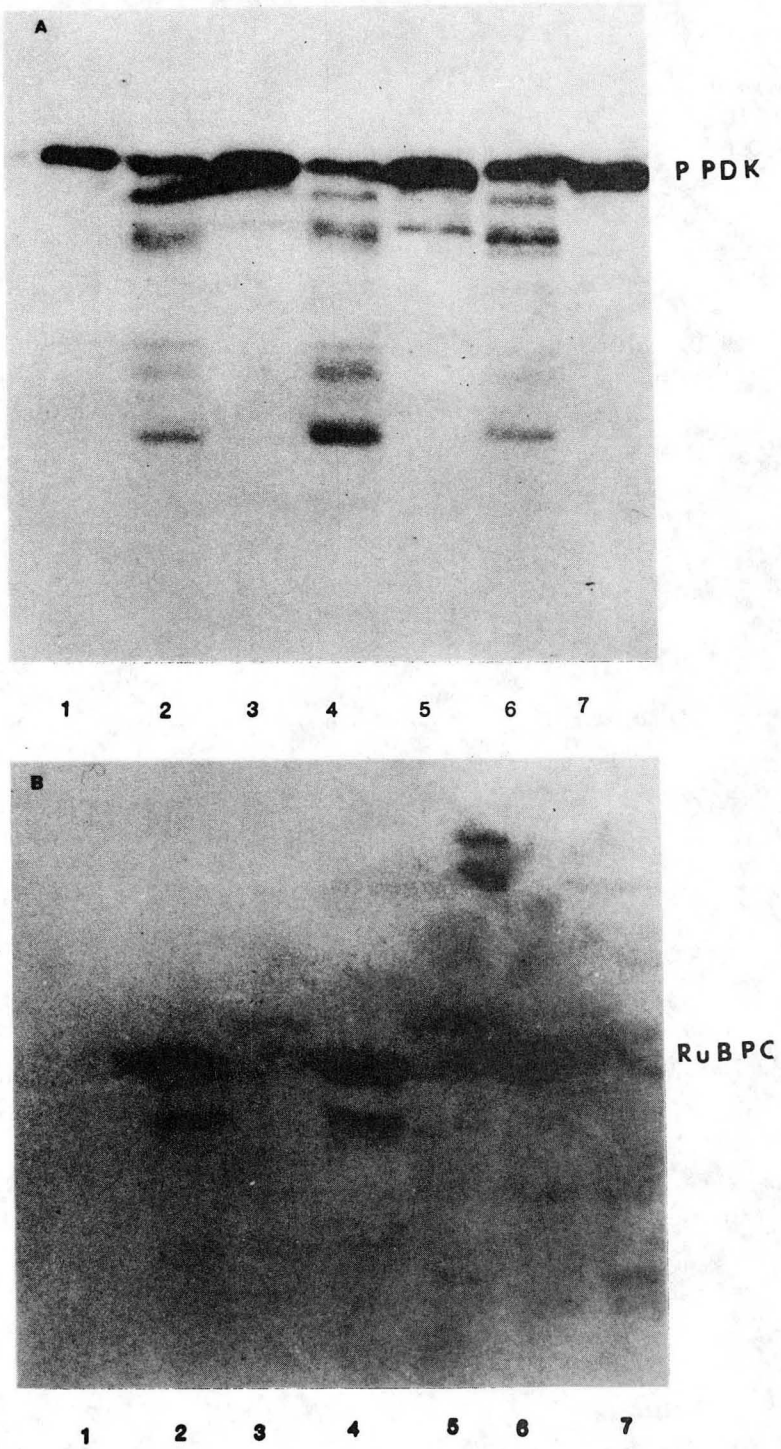


Fig. 10

XBB 830-8731B

Part Two

Organ Specific Gene Expression

RESULTS

Presence of Organ Specific Polypeptide

With antibody to maize leaf PPDk as a probe against the polypeptides in the *in vitro* translation products of the poly(A)RNA extracted from the several sources (Fig.1) only approximately 110 kD polypeptides were detected when the poly(A)RNA was obtained from either maize leaves (lane 2) or wheat leaves (lane 4). In contrast, only approximately 94 kD polypeptides were immunoprecipitated among the translation products of mRNA from either maize kernels (lane 3) or wheat seed (lane 5). In the case of maize kernel translation products, there was an indication of a doublet of polypeptides differing from each other only very slightly in mol wt (lane 3), and several smaller mol wt bands, each result reported here was reproducible from at least six experiments.

Chlorophyllous A188 callus showed two bands, one corresponding to 110 kD precursor polypeptide and the other corresponding to 94 kD polypeptide. The similar two band-pattern was observed in maize leaf when poly(A)RNA was isolated from the first leaves of young seedlings (8d after planting) (Fig.2, lane 6), but never observed when expanded second or later leaves were used (Fig.1, lane 2 and Fig.2, lane 5). Non-chlorophyllous callus only showed one band of mol wt 94 kD (data not shown).

Precursor uptake by spinach chloroplasts

Maize leaf poly(A)RNA was translated *in vitro* and the products were incubated with intact spinach chloroplasts. After uptake of *in vitro* synthesized protein, PPDK in spinach chloroplast stromal extract was immunoprecipitated with PPDK antibody prepared against maize leaf PPDK. The largest polypeptide band of an apparent molecular weight of 94 kD was observed (Fig. 3). This is in contrast with the 110 kD

polypeptide obtained by both Hague et al (1983) and the previous studies on immunoprecipitation of *in vitro* translation product of poly(A) RNA isolated from maize leaf (Fig. 1, lane 2). The results suggest the processing of a 110 kD precursor polypeptide into a mature-sized one in spinach chloroplasts.

It appears that the uptake of the precursor polypeptide is light-dependent (Fig. 4); however, uptake dependence on added ATP could not be demonstrated unequivocally so far. Spinach chloroplasts are reportedly less favorable than pea chloroplasts for demonstrating ATP-dependence of precursor polypeptide (at least in the case of RuBPC-SS) (Grossman et al 1980), possibly because of higher endogenous levels of chloroplast ATP even in the dark (Inoue et al 1978, Santarius and Heber 1965). We chose spinach for these studies, however, since we have detected PPDK in spinach leaves but so far did not see PPDK in pea chloroplasts. When light-dependent RuBPC-SS precursor uptake into isolated chloroplasts has been previously observed it was considered evidence for an ATP requirement; thus, my failure to observe ATP-dependent PPDK precursor uptake in spinach chloroplasts may be due to too slow uptake of ATP by these chloroplasts, perhaps because of lack of optimal medium pH, Pi, or other factors (Grossman et al 1982).

Heterotrophic PPDK in A188 callus

Based on protein blot, the mol wt of PPDK subunit in A188 callus is 94 kD, the same as the maize leaf PPDK. However, A188 callus forming roots shows a major band of mol wt 60 kD and the 94 kD band is very faint. Trypsin digestion of PPDK causes a shift of PPDK band from 94 kD to lower mol wt bands including 60 kD (Fig. 5).

A188 callus PPDK polypeptide level is not affected by dark treatment.

Non-chlorophyllous callus grown in dark for up to 5 weeks showed no change in PPDK content (Fig. 6). Non-chlorophyllous callus PPDK is active and the enzyme activity is inhibited in the similar manner as the enzyme from leaf when incubated with antibody raised against maize leaf PPDK (Fig.7).

DISCUSSION

Hague et al (1983) suggested that PPDK subunits are synthesized in the cytoplasm of maize leaf cells as 110 kD polypeptides with a transit sequence that facilitates transport into the chloroplasts and processing to the 94 kD PPDK polypeptide of the native protein. My results suggest a similar mechanism may be present in leaves of wheat, a C_3 plant.

The different SDS-PAGE mobility of PPDK polypeptides could be due to other reasons than mol wt difference. It seems unlikely that different migration is due to conformational difference, however, since it persists through denaturing conditions. Post-translational modification such as glycosylation could cause different electrophoretic migration, but for this to happen with in vitro translation by the cell-free system appears unlikely. All the results presented were seen in repeated experiments (6 or more), so experimental artifacts are improbable. The most probable explanation for differential mobility is difference in size, as Hague et al (1983) proposed for the maize leaf case.

The origin of several fast-migrating bands ranging in mol wt between 14 and 23 kD in the case of the maize kernel (Fig. 1, lane 3) is not known. Neither the intensity of the bands nor their apparent mol wt support the possibility that they are cleaved transit sequences.

The presence in wheat leaves of mRNA which when translated gives a 110 kD polypeptide but not 94 kD suggests that in wheat leaf cells as well as in maize leaf cells, PPDK might be a chloroplast enzyme. The location supports this hypothesis, even though there is a possibility that a low level of PPDK is present in the cytosol in C_3 leaf cells.

I propose that the mRNA from seed tissue is translated to give

polypeptides without the transit sequence since they need not enter the plastids but instead function in the cytoplasm. There is no necessity for a transit sequence which would permit entry into the plastids.

Other examples of the plant isoenzymes synthesized with or without transit sequences for function in chloroplasts or cytoplasm, respectively, have been reported, including glyceraldehyde-3-P dehydrogenases (Cerff and Kloppstech 1982) and triose-P isomerases (Pichersky et al 1984). Differences were noted in the regulatory properties of the cytoplasmic and chloroplastic enzymes. The physicochemical properties of seed and leaf PPDK from wheat and maize appear to be remarkably similar (Aoyagi and Bassham 1984, Burnell 1985) but differences may be found with further study.

The organ specificity of type the of mRNA for PPDK observed in this study with maize and with wheat suggests some mechanism of organ regulation of type of mRNA formed. One possibilty is that there are different PPDK genes which are differentially expressed in leaf and seed tissue.

The light-dependent uptake of maize PPDK precursor polypeptide by spinach chloroplasts provides further evidence for the location of PPDK in the chloroplasts of wheat and spinach and a mechanism of synthesis, transport, and processing similar to that already known for RuBPC-SS and some other chloroplast proteins. The nature of the apparant light-dependent uptake needs further investigation.

The great majority of chloroplast proteins are synthesized on cytoplasmic ribosomes (Gilham et al 1978). There remains much to be learned about the size and specificity of "transit sequences" of the polypeptides of these proteins with respect to both species and protein.

Recognition of the polypeptide precursor of RuBPC-SS seems not to be species specific since the Chlamydomonas precursor polypeptide is taken up by spinach and pea choloroplasts (Mishkind et al 1985). The entry of maize PPDK polypeptide into isolated spinach chloroplasts suggests nonspecific recognition with respect to species in this case also. It was noted earlier that in terms of immunochemistry, the PPDK polypeptides from maize and from several C_3 species appear to be rather similar (Aoyagi and Bassham 1984).

The molecular weights of mature PPDK polypeptide subunits are about 94 while that of the precursor is about 110 kD, so that the "transit" sequence may be about 16 kD. As noted previously (Hague et al 1983), this size is comparable to the 12 kD size reported for a subunit of another enzyme of C_4 carbon metabolism, NADP-dependent malic enzyme, found in bundle sheath chloroplasts (Collins and Hague 1983). These are the largest "transit" sequences reported thus far for chloroplast stroma polypeptides, but sequences of similar size have been reported to proteins of chloroplast membranes synthesized in the cytoplasm (Grossman et al 1982). Whether or not there is any specificity of recognition sites with respect to "leader" size is unknown.

The presence of the leaf-type polypeptide in the in vitro protein synthesis product of chlorophyllous callus mRNA and the absence of that in non-chlorophyllous callus indicates that the presence of chloroplasts is correlated with the expression of leaf-type PPDK. However the presence of Kranz anatomy does not seem necessary, because the chlorophyllous callus does not have developed leaf morphology at this stage. (See Fig 11, 12a, Part Four). This is consistent with the results of finding PPDK protein in tissues without Kranz anatomy as discussed earlier in Part

One. The presence of both leaf-type polypeptide and heterotrophic type polypeptide observed in young maize seedlings, but never in older seedlings suggests that upon acquiring autotrophy the leaf type polypeptide becomes the major one. The fate of the heterotrophic type polypeptide is not known. If it is present in older leaves, it must be too insignificant in the quantity to be detected. It should be remenbered that the importance of Kranz anatomy for the accumulation of PPDK and other carbon metabolism enzymes in maize leaves cannot be compared directly with the A188 callus case since callus does not carry out C_4 carbon transport (see Part Four).

In sharp contrast to the leaf-type PPDK (Hatch and Slack 1969), the activity of the heterotrophic type PPDK in maize callus is not regulated by light with respect to biosynthesis and enzyme activity.

Hague et al (1983) found light independent mRNA of PPDK in etiolated maize leaf. This form of PPDK in dark-grown seedling is unknown. Enzyme activity inhibition assay suggests that callus enzyme is active and is closely related to the leaf enzyme in protein structure. The role of PPDK in callus tissue will be discussed in Part Four.

Fig. 1. *In vitro* translation of PPDK mRNA from leaves and seeds from maize and wheat. Analysis was by SDS-PAGE followed by fluorography. Lane 1. ^{14}C-labeled mol wt standards.

Lanes 2-5. Immunoprecipitated polypeptides synthesized in rabbit reticulocyte lysate system in the presence of 0.5μg of poly(A)RNA from: maize leaf (lane 2), maize kernel (lane 3), wheat leaf (lane 4), and wheat seed (lane 5).

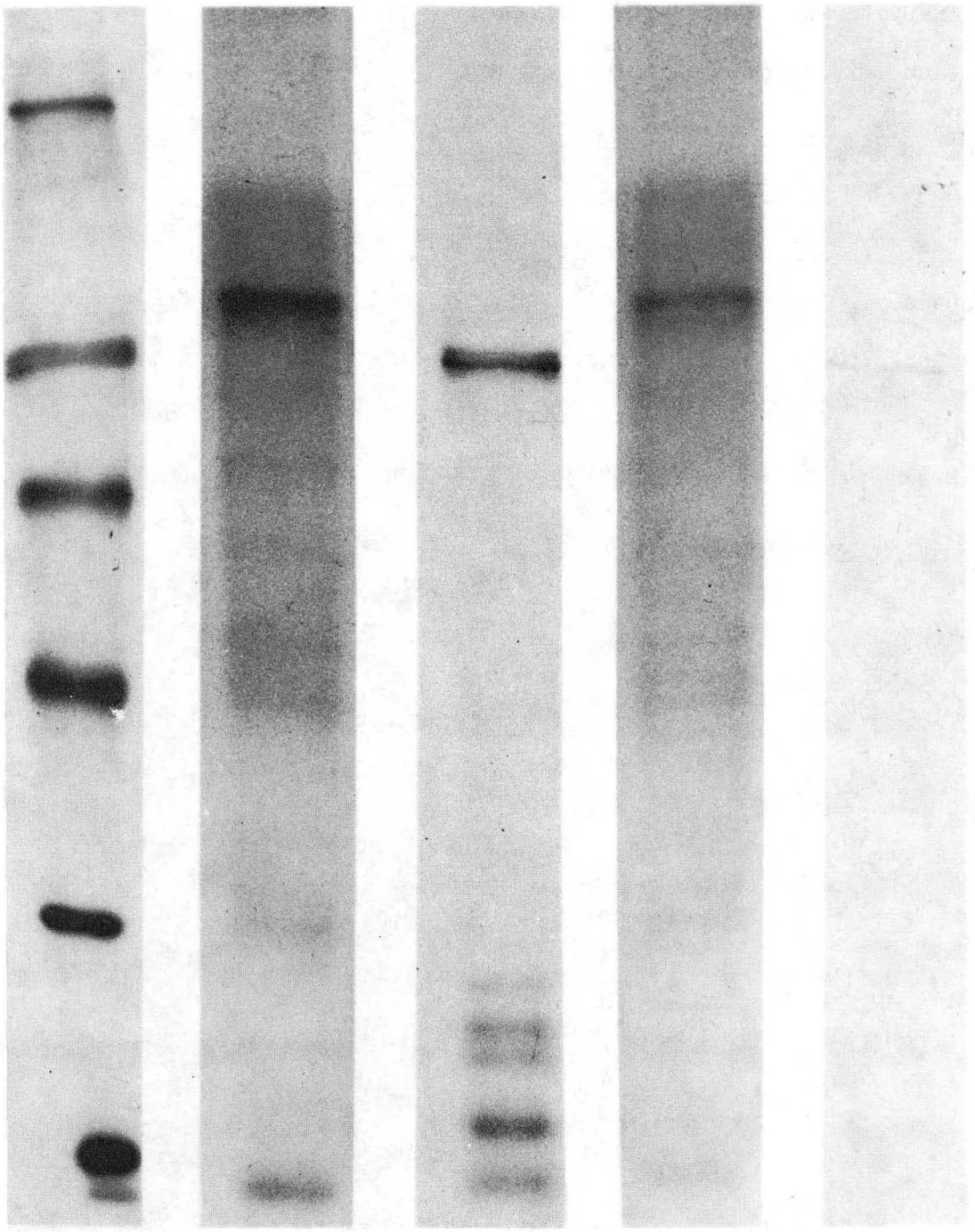


Fig. 1

XBB 845-4124A'

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}C-labelled mol wt standard

Lane 2. *In vitro* translation in the absence of mRNA

Lane 3. *In vitro* translation of leaf poly(A)RNA

Lane 4. *In vitro* translation of callus poly(A)RNA

Lane 5. Immunoprecipitation of translation product of lane 3.

Lane 6. Immunoprecipitation of translation product of leaf poly(A)RNA isolated from the first leaves.

Lane 7: Immunoprecipitation of translation product of lane 4.

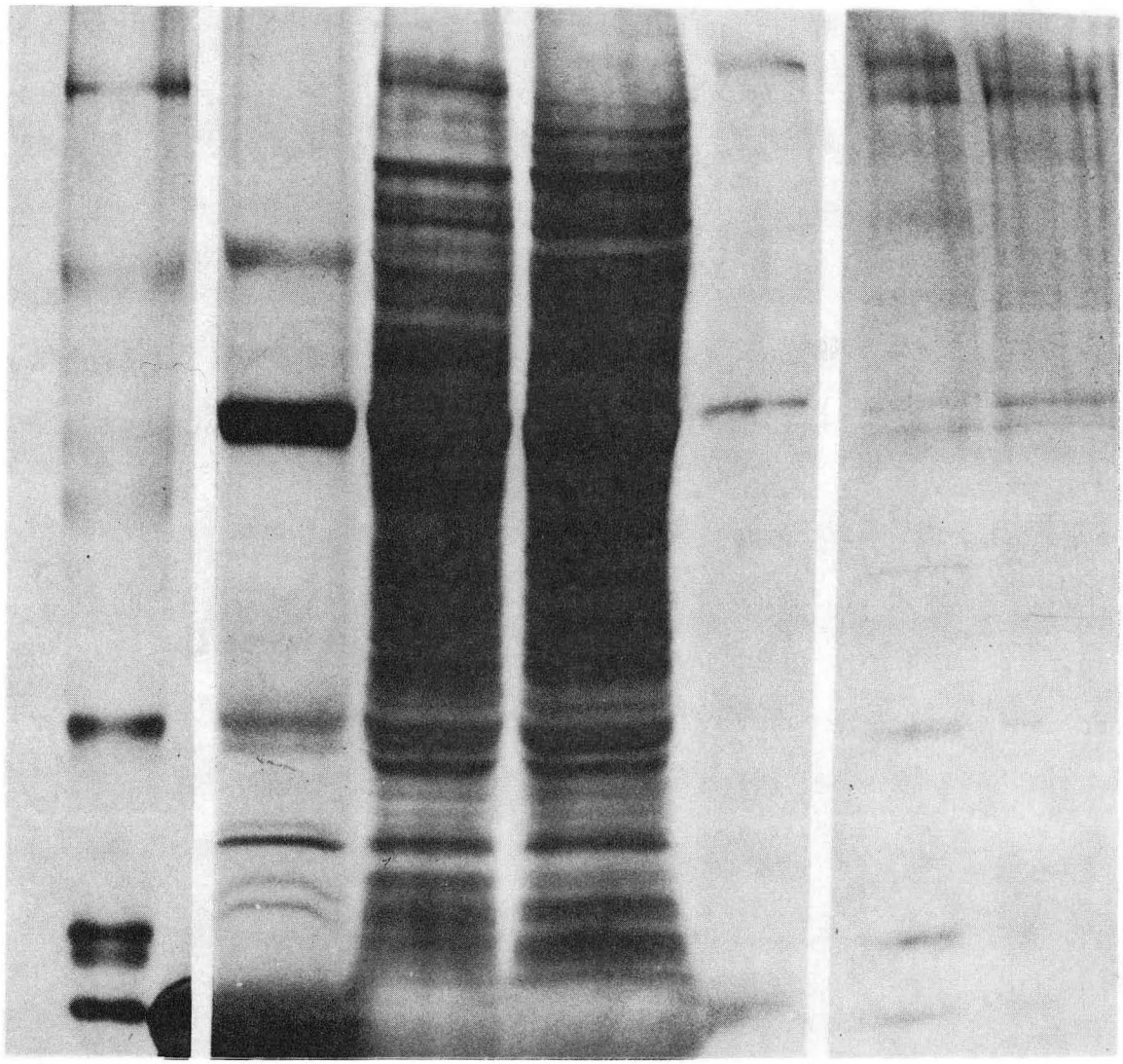


Fig. 2

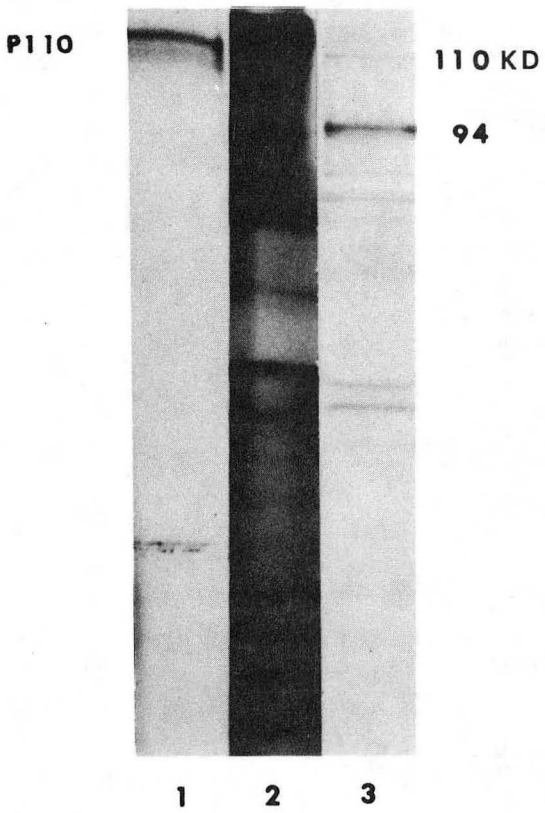
XBB 848-6270A

Fig. 3. Maize PPDK precursor uptake by spinach chloroplasts. The *in vitro* translation products of maize leaf poly(A)RNA were incubated with intact spinach chloroplasts. Subsequently, the chloroplasts were lysed and the stroma extract was reacted with maize PPDK antiserum (see Materials and Methods for details).

Lane 1: PPDK precursor of 110 kD (P110). 1/20 aliquot of total translation product precipitated by PPDK antiserum.

Lane 2: Total *in vitro* translation product, 1/20 aliquot.

Lane 3: Chloroplast-processed PPDK after uptake. The suspended chloroplasts were exposed to 9/10 of the total translation product; after lysing, the entire stromal extract was loaded on this lane. The faint band above the 94 kdalton band is at about 110 kdaltons and may be the unprocessed precursor.



XBB 8412-9574A

Fig. 3

Fig. 4. Light-dependency of chloroplast uptake of PPDK precursor. The cell-free protein synthesis products which were transported into chloroplasts in light or dark and with addition of ATP or without addition of ATP were reacted with PPDK antiserum (lane 3 to 6).

Lane 1: ^{14}C-labelled polypeptide molecular weight standards. Mol wt of the bands are 92.5, 69, 46, 30, and 14 (kD) from the top to bottom.

Lane 2: Precursor PPDK polypdptide.

Lane 3: Incubation in light + ATP

Lane 4: Incubation in light - ATP

Lane 5: Incubation in dark + ATP

Lane 6: Incubation in dark - ATP

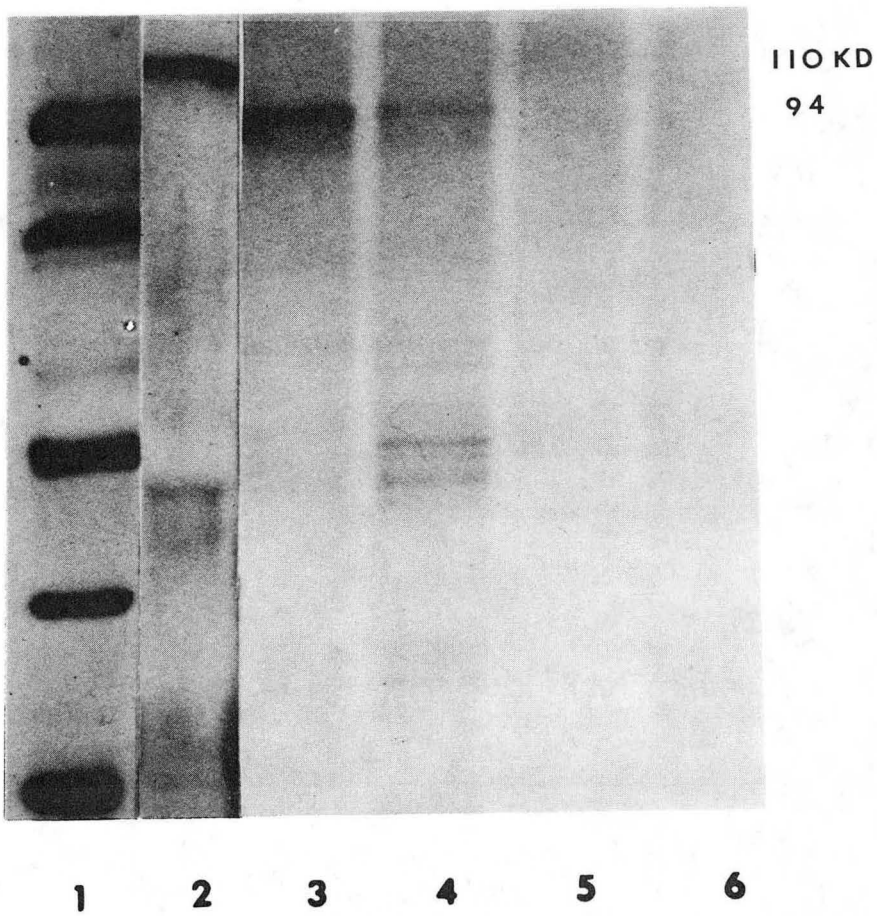


Fig. 4

XBB 842-9379A

Fig. 5A. Different mol wt of root forming maize callus

Lane 1 to 3: Purified maize leaf PPDK (0.125, 0.25, 0.5 μg respectively)

Lane 4: White callus extract (200μg soluble protein)

Lane 5: Root-forming callus extract (the same amount as above)

Lane 6: Maize leaf extract (20μg of soluble protein)

B.The effect of trypsin digestion on PPDK molecular weight

Lane 1: Trypsin digested maize leaf extract

Lane 2: Maize leaf extract prepared in the presence of PMSF

Lane 3: Maize leaf extract kept at 0°C.

* 20 μg of soluble protein was loaded in each lane.

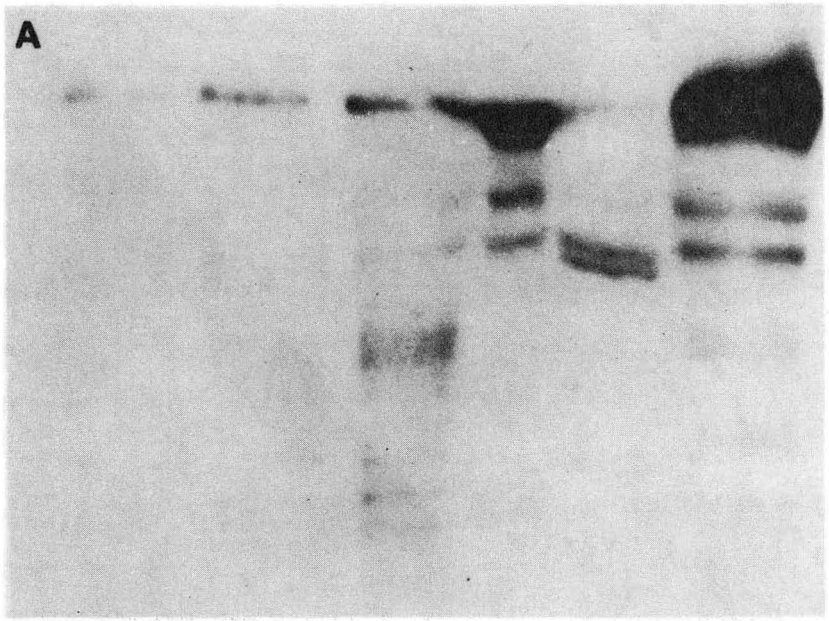


Fig. 5A

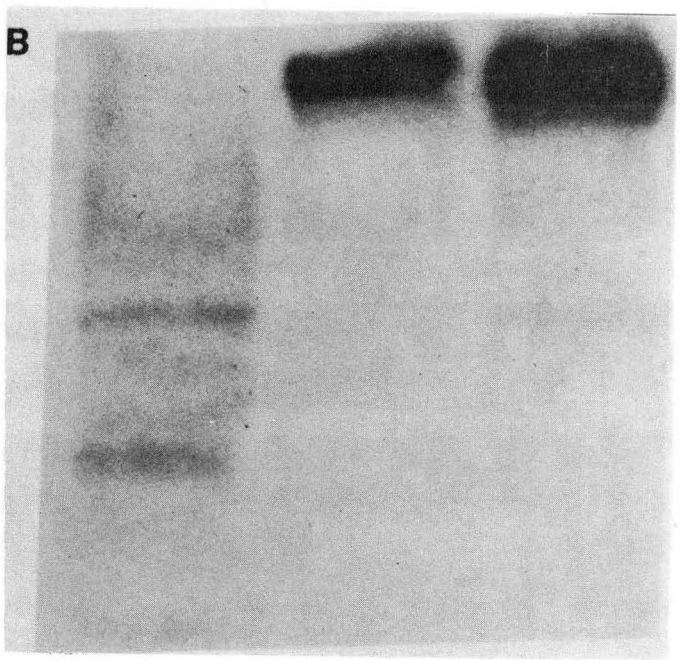


Fig. 5B

XBB 853-2092B

Fig. 6. Effect of dark treatment of maize callus on PPDK content by protein blot.

Extract (0.2 mg soluble protein) from green callus (lane 1) and non-chlorophyllous callus which was grown in darkness for the various length of time were loaded for comparison. Lane 2 (0 d), lane 3 (1d), lane 4 (4 weeks),and lane 5 (3 weeks in darkness and 1 week in light).

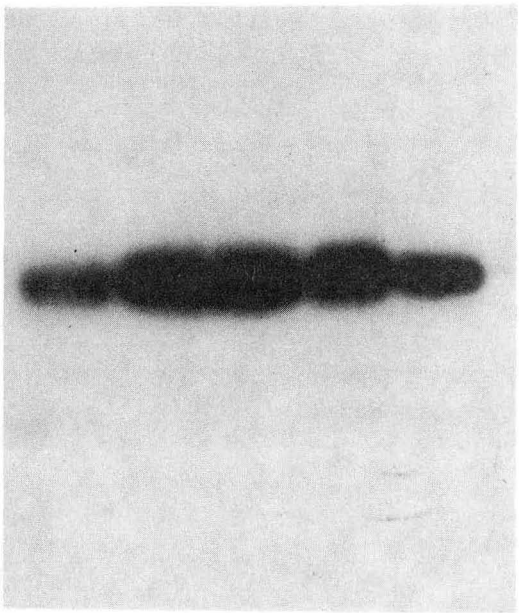
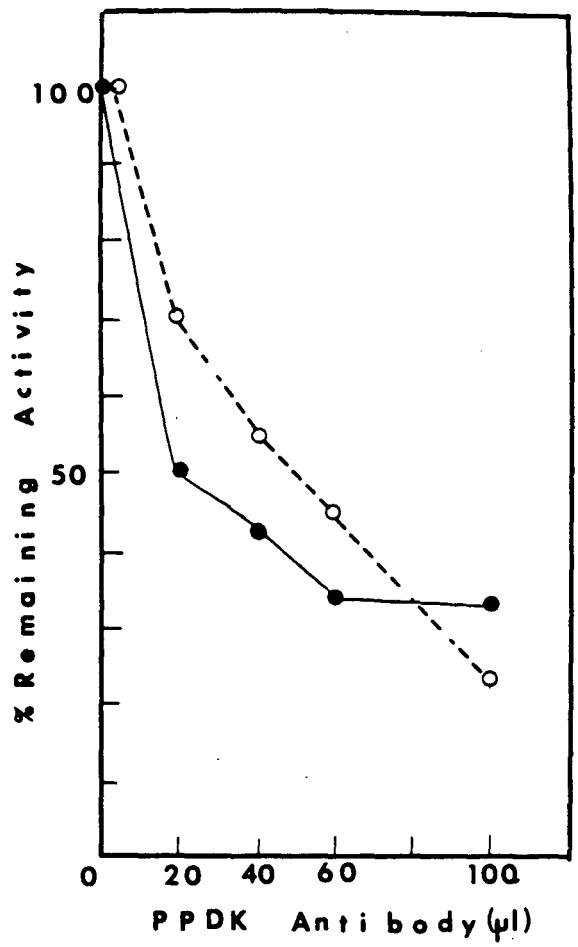


Fig. 6 XBB 848-6272

Fig. 7. Inhibition of enzyme activity by PPDK antibody maize leaf extract (o- - - -o) and callus extract (●——●) were incubated with purified PPDK antibody. The loss in enzyme activity increased with increased amount of antibody added.



XBL 847-2894

Fig. 7

Part Three

Developmental Regulation of Gene Expression in Wheat

RESULTS

(A) PPDK gene expression in developing wheat seeds

Developmental profile of wheat seed PPDK content

Under the growth conditions used, Cheyenne wheat seeds reach maximun fresh and dry weight approximately 35 days after anthesis (DAF). The amount of PPDK protein in seeds began to increase at about 6 d after anthesis, reached a maximum value between 20 and 25 d, and declined to the initial value between 35 and 40 d past anthesis (Fig.1). This result is complementary to that of Meyer et al (1982), who reported that the activity of the enzyme increased from being barely detectable at 7d after anthesis to a maximum about 25 d after anthesis. The agreement between my results and those of Meyer et al (1982) suggests that the activity of PPDK in developing seeds is in direct relation to the amount of enzyme present, and not defined by enzyme activation.

In agreement with Meyer et al (1982), I have observed that the increase and subsequent decline in PPDK content occur later during seed development than does that of seed Chl (Fig.1). The peak in PPDK content also occurs later in seed development than that of RuBPC. This is seen when autoradiographs of protein blots for the two enzymes are compared as a function of seed development (Fig.1). The wheat PPDK subunit migrates as a single band in SDS-gels with an apparent subunit mol wt of about 94kD, while the RuBPC large subunit migrates at about 55kD (Akazawa and Osmond 1976). The profile of RuBPC is similar to that of Chl.

Developmental profile of PPDK mRNA activity

In order to compare seed content of PPDK with the seed's potential

for synthesis of this enzyme, I isolated total RNA from seeds at different times after flowering, and followed PPDK mRNA activity by immunochemical analysis of *in vitro* translation products. SDS-gel analysis of anti-PPDK immunoprecipitated *in vitro* translation products shows a prominent band at 94kD (Fig.1, lane5 in Part Two), similar to that of authentic PPDK subunit.

The level of PPDK mRNA activity, per μg RNA, begins to rise at about 9 DAF and reaches a maximum about 24 DAF (Fig.2). This profile is similar to that of total seed mRNA activity per μg total RNA and to that of total seed mRNA activity per seed. The change in *in vitro* PPDK mRNA activity with seed development, including the decline after 25 DAF, is also similar to the change in seed PPDK content (Fig.1). This suggests that the enzyme undergoes a relatively rapid rate of metabolic turnover, and that its content in the seed is determined mainly by its rate of synthesis.

^{14}C incorporation analysis

Analysis of photosynthetic fixation of ^{14}C-labeled bicarbonate by wheat seed green tissue (25 d after flowering) showed that the fixation at the shortest times is predominantly into compounds derived from oxaloacetate(malate, aspartate, fumarate, and citrate)(Fig.3). After 5 s labelling, malate accounted for 42% of radiocarbon found in compounds on the paper chromatogram. After longer periods of photosynthesis with labeled carbon, the normal pattern of C_3 assimilation (sugar phosphates, phosphoglycerate) becomes more prominent. Experiments with green seed tissue 18 d after flowering gave similar results (not shown), although labeling of C4 compouns was slightly less dominant. Fixation of labeled carbon was not linear, being at a somewhat greater rate during the

first few seconds.

(B) PPDK Gene Expression in Developing Wheat Leaves

Appearance and accumulation of enzymes

The profiles of appearance and accumulation of RuBPC, PEPC, PPDK and NADP-dependent ME in wheat leaves are very different from the profiles in maize leaves (see Part Four, Mayfield and Taylor 1984). Based on Protein blot analysis, in wheat primary leaf sections, RuBPC is already present in the first 0-1 cm (Fig.5) at about 12% of the final level of accumulation. RuBPC increased rapidly from the base to tip where the amount of RuBPC is about 56% of the total protein (Dean and Leech 1982). PPDK appears in the 4-6 cm section and increases ten-fold with development (Fig.4).

PEPC is present in the basal section; however the level is low, and it never increases much. NADP-dependent ME appears in the 4-6 cm section; however, its level also remains relatively low compared to that of a C_4 leaf (See Fig.7 in Part Four).

The increase of total amount of PPDK and RuBPC-LS is due both to the increase in their proportion of total soluble protein with development and also to an increase in the amount of soluble protein. The increase can be seen either on a soluble protein basis or fresh weight basis. The pattern of enzyme accumulation is closely related to that of soluble protein (Table 1).

Effect of leaf position on the profile

When upper leaves of wheat were used, the pattern of appearance and accumulation was basically the same. Only the level of enzymes was higher (Table 2). In mature wheat leaves, the portion of soluble protein allocated to RuBPC increased from base to tip 4-fold whereas

PPDK did not show any increase after the 2nd section (Fig.5)

PEPC activity

The enzyme activities for primary wheat leaf tips and base sections were 0.35 and 0.26 U per g fresh weight respectively, and for mature leaf tips, 0.64U per fresh weight. The Km (PEP) was 0.2 mM and the V_{max} was 0.6 U per g fresh weight in each case.

MDH-NADP activity and its location in wheat leaves

MDH-NADP activity was found in the stroma of the chloroplasts. Surprisingly, the supernatant fraction after centrifugation of the intact chloroplasts did not contain a detectable level of this enzyme activity.

Apparently there was relatively little chloroplast breakage during homogenization, because PPDK polypeptide also was not detected in the supernatant by the protein blot method. The level of MDH-NADP in the stroma was determined to be 0.52U per mg chlorophyll present in the chloroplasts before lysing. Since the leaves contained 0.9 mg chlorophyll per g fresh weight, there was 0.58 U of enzyme activity per g fresh weight of leaf.

$^{14}CO_2$ fixation pattern of wheat leaves

Total ^{14}C fixation was 1.5 to 2 times greater for the tips than the base. The part of total ^{14}C incorporated into C_4 acids after 10 s for the base section was 43%, whereas PGA and sugar phosphates (fructose 1,6-bisphospate, fructose-6-phosphate, RuBP) were less than 10% (Fig.6A). The part of total ^{14}C incoporation into C_4 acids after 10 s for the tip section was 7%, whereas that into PGA and sugar phosphates was 82% (Fig.6B). Besides C_4 acids, ^{14}C was fixed into glutamine, alanine (about 10%) and other compounds (Fig.7).

DISCUSSION

(A) Wheat seed

In the present study I have examined developmental aspects of PPDK accumulation in wheat seeds to gain insight into the nature of control of such expression, and also to seek further clues about the possible metabolic role of this enzyme in seeds. I have observed that in wheat seeds there is a direct correlation between the amount of PPDK protein and the level of PPDK mRNA activity, and that these two parameters vary with seed development in a manner similar to that of PPDK activity as reported by Meyer et al (1982). Therefore, these all are correlated with time of development. Furthermore, these profiles are correlated with toal protein mRNA activity (unlike the case for RuBPC, for example).

These data suggest that PPDK gene expression in the developing wheat seed, as indicated either by enzyme activity or enzyme protein, is controlled by the level of its mRNA.

In maize mesophyll leaf cells PPDK mediates conversion of pyruvate with ATP and Pi to provide the PEP for carboxylation leading to formation of oxaloacetate (Hatch and Slack 1966). In wheat seeds, absence of Kranz anatomy, the location of PPDK both in the white endosperm tissue and in the green pericarp, and the different developmental profile for PPDK activity as compared with Chl and RuBPC, all suggest that the principal role of PPDK in seeds is not direct participation in photosynthetic carbon fixation leading to sugar formation. The two steps from pyruvate to oxaloacetate may be important in seed development nevertheless. In seeds, PPDK may supply PEP for photosynthetic and dark carbondioxide fixation. Photosynthesis

by developing wheat grains can account for as much as 34% of the weight gain of the grains (Evans and Rawson 1970). Considerable additional photosynthesis by the grains may be involved in the refixation of respiratory CO_2 (Kriedman 1966). Stomata were observed on wheat and barley grains toward the brush end on the flanks of the crease by Cochrane and Duffus (1979) who examined the ultrastructure of immature grains and discussed it with relation to the transport of gasses and metabolites and to the relatively low rate of photosynthetic $^{14}CO_2$ fixation of intact seeds compared to the much larger rate seen upon removal of the outer pericarp (Radley 1976).

The pattern of ^{14}C-labeled compounds formed during short periods of photosynthesis with ^{14}C-labelled bicarbonate in wheat seeds 25 d after flowering indicates a very high PEPC activity (Fig. 4). Seeds at this late developing stage were chosen for the highest level of PPDK is seen in such seeds. Moreover even though the total fixation is higher at earlier stage (18 DAF) when Chl content of the seeds is higher, the fraction of C_4 compounds was minor in the preliminary study. There is a preponderance of labeled products of PEP carboxylation such as aspartate, malate, citrate, and glutamate. The continued high percentage of labeling of these compounds to 1 min seems to suggest a relatively high rate of net synthesis of C_4 and C_5 compounds. The higher rate of fixation at the shortest time may indicate that accumulated PEP was rapidly used up when bicarbonate was administered to the exposed tissue.

Nutbeam and Duffus (1976) reported predominant labeling of malic acid at 1 min of photosynthetic $^{14}CO_2$ fixation by barley pericarp tissue (25-30 DAF), with labeling moving to sucrose and hexose phosphates at longer

times, and they considered this to be evidence for C_4 photosynthesis in that tissue. Similar results were reported to have been obtained for wheat pericarp, but the data were not shown. In any event, the time scale for the kinetics of these results with barley pericarp are quite different from those results of the present study in which the labeling of malate and aspartate, although declining after the first 15 s, continued to be prominent. Wirth et al (1976) reported ^{14}C labeling in wheat pericarp at 10 s only, and found a predominace of labeled products characteristic of C_3 photosynthesis. Such differences in results from different studies may well be related to physiological differences in the seed tissue used, especially in stage of development and variety. In fact, when another variety Anza at 18d after anthesis was used, I have obtained a predominantly C_3 fixation pattern whereas at later stage (25 d after anthesis) C_4 compounds were predominant.

Given the greater activity of PEPC than of PPDK (Duffus and Rosie 1973), some PEP for carboxylation may come from glycolysis of stored carbon. However, conversion of pyruvate to PEP, mediated by PPDK, may be an important source of the PEP, a substrate for carboxylation. In particular, PEP carboxylation and a preceeding conversion of pyruvate to PEP mediated by PPDK may be required for the net conversion of the C_3 amino acid carbon skelton of alanine to C_4 and C_5 amino acids in wheat grains. Amino acids present in major amounts in flag-leaf phloem are glutamate, aspartate, serine, alanine, and glycine (Simpson and Dalling 1981). Conversion of this mixture of amino acids to that required for seed protein synthesis must occur in the seeds (Kirkman and Miflin 1979).

Wheat storage protein such as gliadin is very rich in glutamate and proline (Jennings and Morton 1963) which is made from glutamate. During

the synthesis of such proteins, the utilization of other amino acids including alanine might lag, allowing these amino acids to accumulate in the endosperm during the first 3 weeks of seed development. Alanine is the major soluble amino acid of the endosperm of wheat grains, and when plants are not irrigated, alanine can constitute as much as 47% of total soluble amino acids (Singh 1981). In this case, the peak in quantity of alanine occurs at about 25 d after flowering, at about the same time as the maximum in PPDK protein. Perhaps during the last 2 weeks, conversion of excess accumulated alanine to glutamate is needed for efficient utilization of this stored alanine. Another possibility, the conversion of alanine to aspartate (Meyer et al 1982) seems less important since a much smaller amount of C_4 amino acids than C_5 amino acids are required in the storage protein (Jennings and Morton 1963). For similar reasons, other possibilities such as the conversion of malate to pyruvate followed by reduction to sugars seems less likely.

In maize, alanine is the amino acid occurring in highest amount among the free amino acids in the developing kernel (Arruda and Silva 1979). There may be, therefore a necessity in corn kernels for the conversion of alanine to C_4 and C_5 amino acids during the latter stages of kernel development. Both PPDK protein and activity were found in immature maize kernels. If the role of seed PPDK is as just discussed, it may be placed among those enzymes that contribute to the synthesis of seed reserve material, such as glutamate-pyruvate aminotransferase , glutamate-oxaloacetate aminotransferase, (Rijiven and Cohen 1981), and ADP-glucose pyrophosphorylase (Turner 1969). Developmental activity profiles reported for these enzymes in cereal seed endosperm tissue are similar to that of total seed mRNA activity, a large fraction of which

codes for seed storage proteins (Greene 1983), and to the profile of PPDK mRNA activity observed in this study. It is possible that the expression of the genes coding for these enzymes is coordinated in the same way as their observed metabolic activities are. The inclusion of seed PPDK in such a coordinated expression would be consistent with all of its observed properites.

(B) PPDK gene expression in developing wheat leaves

Terms such as "C_4 metabolism", though widely accepted to denote photosynthetic carbon transport via C_4 acids in C_4 plants such as maize and sugar cane, can become rather ambiguous when applied to possible C_4 functions in C_3 plants. The anaplerotic C_4 function employs β-carboxylation with PEPC to convert C_3 carbon skeletons to C_4 and C_5 amino acids, as well as to 5-aminolevulinic acid (Klein et al 1975), and thence to end products such as chlorophyll, cytochromes, etc. The carbon transport function occurs during photosynthesis in C_4 plants when the CO_2 incorporated by β-carboxylation in mesophyll cells is released later inside chloroplasts of bundle sheath cells to be reincorporated by carboxylation of ribulose 1,5-bisphosphate, mediated by RuBPC.

The anaplerotic C_4 function is employed in both C_4 and C_3 plants, in leaves and other tissues, in light and in dark, and thus can be either non-photosynthetic (heterotrophic) or photosynthetic (autotrophic). When PPDK is present in developing seeds, the substrate, PEP, may be formed from alanine, via pyruvate. During photosynthesis, however, the PEP may be derived from phosphoglyceric acid formed by the photosynthetic carbon reduction cycle, even if the product is, for example, glutamate. The anaplerotic pathway in this

case is photosynthetic.

Much of the search for C_4 characteristics in C_3 species has been focused on plant species with leaf anatomy resembling Kranz anatomy, or in closely related C_4 and C_3 species,and in hybrids of such species (Bjorkman 1976). Wheat is not a species with such anatomy, and intercellular CO_2 transport would hardly be expected.

A carbon transport function could conceivably occur as an intracellular process in C_3 plants, perhaps from cytoplasm to chloroplast, as discussed later. Substantial ^{14}C labelling of C_4 acids in the presence of $^{14}CO_2$ can occur with either anaplerotic or carbon transport function, or both, so it is not a sufficient criterion for carbon transport. An accepted criterion for intercellular C_4 carbon shuttling is movement of ^{14}C-label from C_4 acids to sugar phosphates and phosphoglycerate in pulse-chase experiments (Hatch and Slack 1966). Demonstration of such tracer movement might be difficult, if not impossible, for intracellular carbon transport, however, since metabolite pool sizes might be much smaller and time for transport much shorter. While there are other criteria for reduced photorespiration (such as lowered compensation point), they are not unique to C_4 carbon transport , since other active CO_2 transport mechanisms (as in some algae) can also lower photorespiration. Low $\delta^{13}C$ values do indicate an increased PEPC/RuBPC activity ratio (Smith and Epstein 1971), however, and there are some indications for changes during wheat leaf development (Winkler et al 1978), though the values are in all cases within the C_3 plant range.

The purpose of the present study was to examine the appearance of "C4" enzymes in developing wheat leaves for biochemical clues that

might indicate the presence of some type of C_4 carbon transport role, possibly intracellular, in wheat leaves. The levels in C_3 leaf tissue of PPDK and some other enzymes required for C_4 carbon transport in C_4 plants are low compared to those of a C_4 plant. Nevertheless, if such a pathway occurs in wheat, it would suggest that wheat might have some ability to diminish losses due to photorespiratory conditions. It also could indicate possible evolutionary sequences leading to development of C_4 photosynthesis. Knowledge of such a "C_4in C_3 plant" path might help in planning future plant breeding or even molecular genetic engineering designed to impart C_4 characteristics to C_3 plants.

In figure 8 I present one possibility for intracellular carbon transport involving C_4 acids. Other hypotheses involving C_4 transport are possible, but the scheme shown seems consistent with what is known about enzyme locations and activities, although more quantitative data on oxaloacetate uptake and PEP export from wheat chloroplasts is needed. I find two types of evidence in wheat leaves. One is the location of PPDK in chloroplasts in leaves. The other is an increase with leaf development in the levels of PPDK even as CO_2 fixation by RuBPC becomes quantitatively dominant over β-carboxylation.

PPDK in chloroplasts is probably active only in light; photosynthetic formation of phosphoglyceric acid via the reductive pentose phosphate cycle would likely supply all the PEP needed for biosynthesis. A role for PPDK in making PEP for the purpose of conversion of alanine to aspartate and glutamate thus seems unlikely in illuminated leaves. A role in photosynthetic carbon transport does seem possible, however, considering the developmental profile for PPDK activity as compared to that

of RuBPC, PEPC, and other key enzymes.

The appearance of RuBPC in the first section of wheat primary leaf is as previously reported (Dean and Leech 1982). PPDK appears at about 4 cm from the base section and increased from base to tip by 10-fold, is increasing at a time when PEP generation from the reductive pentose phosphate cycle would be adequate for anaplerotic C_4 formation (e.g. glutamate synthesis). The need for additional PEP formation from pyruvate suggests a role in a carbon transport cycle. The level of PPDK also increased with ascending leaf position on the stalk so that upper leaves contain more PPDK per fresh weight. The maximum level of PPDK reached is small compared to a C_4 plant, but the large increase seems significant.

In wheat leaf tips (where the maximum PPDK is found) the activity of PEPC is still more than ten times that of PPDK. This ratio is similar to that in maize, where the total activities are much greater (Sugiyama and Hirayama 1983). The increase in PPDK activity from wheat leaf base to tip would provide a more rapid formation of PEP, accelerating the PEP carboxylation reaction in direct proportion to the increase in PPDK activity. The wheat leaf tip PEPC Km of 0.2 mM and V_{max} of 0.6 U per g fresh weight that I find are similar to the values reported (Ting and Osmond 1973) for other C_3 plants. The increase in PEPC towards the leaf tip therefore is not due to the appearance of a "C_4" type of PEPC.

Similarly, the activities of another enzyme of a putative intracellular carbon transport pathway, ME is in excess over PPDK activity at each developmental stage investigated. The developmental profile of MDH-NADP was not studied, but I did confirm its intracellular location. Nainawatte et al (1974) did not find this

enzyme in wheat chloroplasts. It might be expected to be present in C_3 chloroplasts, since it would be required for the operation of malate/ OAA shuttle from the chloroplast to cytoplasm (Heber 1974). Oxaloacetate reduction would be expected to occur inside the chloroplasts rather than in the cytoplasm in wheat, given the known hydrogen shuttle mechanism (Heber 1974). The MDH-NADP activity found in the chloroplasts was in excess of the maximum PPDK activity.

Thus, the intracellular location of C_4 enzymes is the same in wheat as in maize: PEPC in the cytoplasm, and PPDK, ME, and MDH-NADP in chloroplasts. In wheat cells, the latter enzymes are together with RuBPC in the same chloroplasts, rather than in different cells. The amount of PPDK present in wheat leaf cell chloroplasts is, however, not even as much as has been found to be present in maize bundle sheath cells . The levels of PEPC and of NADP-ME do not increase but are in excess of the PPDK activity. Provided oxaloacetate is taken up by the chloroplasts and PEP is translocated from the chloroplast to the cytoplasm at rates greater than that of pyruvate to PEP conversion, PPDK activity would catalyze the rate-limiting step, and its increasing level could control the amount of carbon shuttling. If such an intracellular carbon shuttle does operate in fact, it may be of minor quantitative importance.

Wirth et al (1976) reported some capacity for "C_4 metabolism" in wheat primary leaf and tissues surrounding the grain, but the ratio of C_3/C_4 products increased markedly in the mature leaf, probably due to an increased RuBPC/PEPC ratio. The relative level of PEPC was higher in the primary wheat leaf than the mature leaf, and RuBPC level continues to increase with higher leaf position. Since the amount of

PPDK and of PEPC are low relative to RuBPC in wheat leaves, C_4 products are relatively minor. In the wheat leaf tip, however, I found the fraction of total ^{14}C fixation into C_4 acids after 10 s to be 7-12%, somewhat higher than the value (3%) reported in barley leaf (Kestler 1975). In the base section of wheat leaf I found 42% in C_4 acids after 10 s. The large increase in RuBPC with leaf development tends to obscure the effect of the smaller, yet significant increase in PPDK level, when C_4 labelling is observed.

The uptake of oxaloacetate into the chloroplasts would compete with uptake into the mitochondria. The large concentration gradients of C_4 acids required for intercellular transport in C_4 plants probably would not be seen in the case of intracellular transport. The appearance of large amounts of ^{14}C-labeled C_4 acids, noted in photosynthesizing C_4 plants or in C_3 plants when there is significant anaplerotic β-carboxylation, probably would not be expected during intracellular carbon shuttling.

Such a carbon shuttle would seem to offer advantages to wheat under conditions of high light intensity. I would not expect intracellular carbon transport to be as effective as intercellular transport (as in C_4 plants), since O_2 generation near the site of the RuBP carboxylation reaction would not be reduced as it apparently is in C_4 bundle sheath cells (Downton 1971) and also since the effective increase in CO_2 levels in the chloroplasts at that site might not be increased as much. Recovery of photorespiratory CO_2 and its concentration in the chloroplasts could nevertheless be quite effective in increasing the rate of net photosynthesis, since all the green cells would be carrying out the reductive pentose phosphate cycle.

That the putative carbon transport pathway seems to be not more significant in T. aestivum could be that the result of plant breeding of wheat for characteristics other than photosynthetic rate at high light intensity and temperature. It has been suggeted (Evans and Dunstone 1970) that the rate of photosynthesis in wheat may have fallen in the course of evolution, since the highest photosynthentic rate (63 mg CO_2 dm^{-2} h^{-1})among tested wheat species with bright light (7,000 f.c) was found in Triticum boeoticum (Evans and Dunstone 1970), an ancient species grown in 8,000 B.C. in the fertile crescent (Harlan and Zahary 1966). This rate is 70% higher than the rate found under the same conditions in that study for T. aestivum, and in fact compares favorably with rates reported elsewhere for maize in bright light (Downton 1971). T. boeoticum, however, exhibited the same degree of increased rate with lowered O_2 pressure as did other wheat species, so diminished photorespiration might not be the most important factor in its high rate. Perhaps a breeding program to increase the expression of PPDK while retaining desirable features of grain and leaf size, etc. would ultimately prove beneficial to the goal of higher yields.

Table 1. Content of chlorophyll and soluble protein of primary wheat leaf sections.

Distance from base (cm)	Total Chl (μg/g FW)	Chl a/b	Soluble protein (mg/g FW)
0-1	40.5	2.0	22
1-2	70.2	2.2	43
2-4	271	2.9	48
4-6	654	3.2	55
6-8	1080	3.3	60
8-10	1380	3.3	65
10-tip	2200	3.1	72

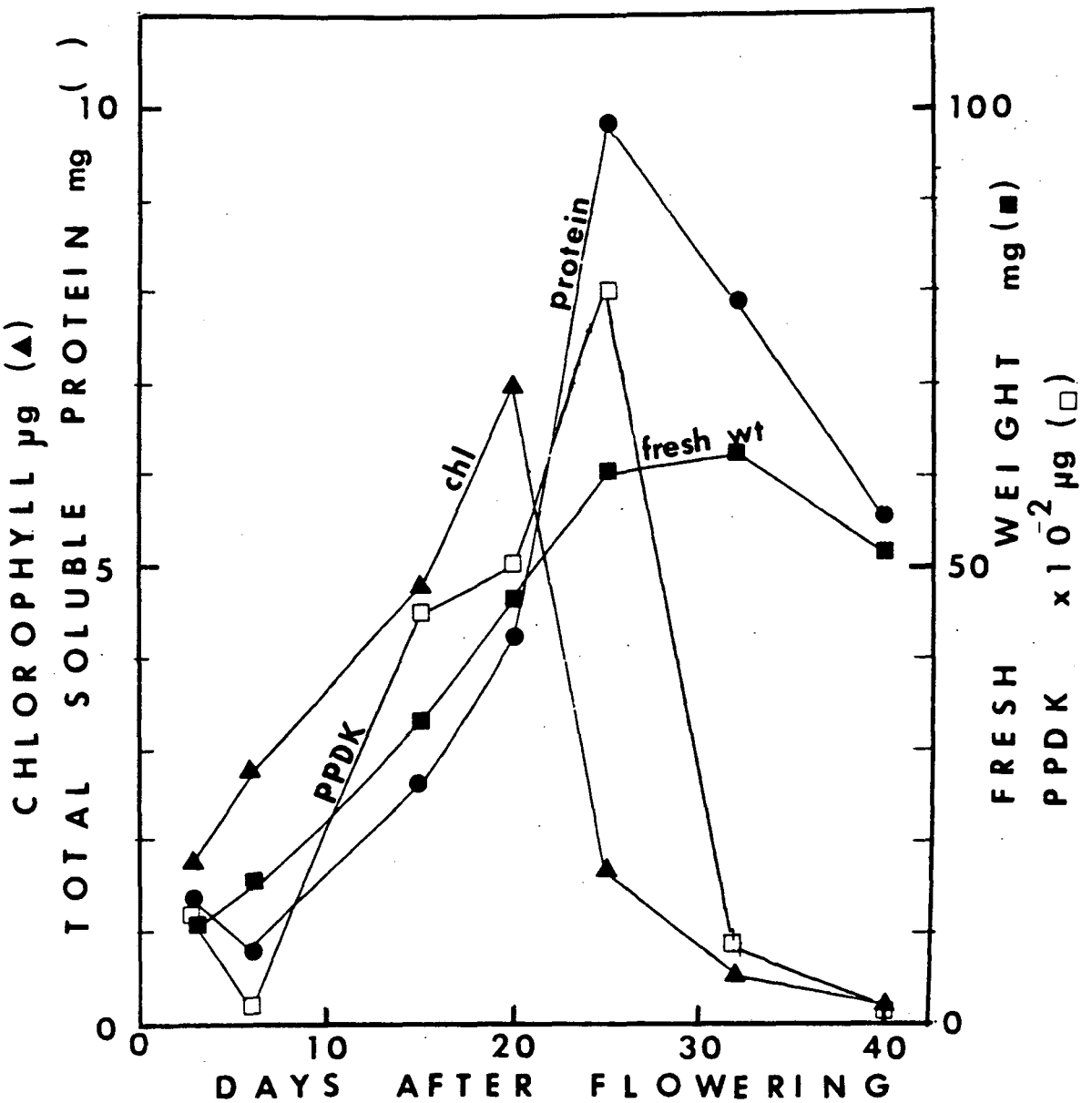
Table 2. Relative levels of enzyme proteins in different leaf positions of wheat plants.

Protein blots of wheat leaf sections obtained from the primary, the third and the mature leaf (4th leaf having the blade length of c.a. 50 cm) were used to estimate the relative levels of enzyme protein. Relative enzyme protein level per g fresh weight (levels in tip of primary leaf= 100 %)

Leaf position	PPDK	PEPC	RuBPC	ME
primary base	0	95	10	0
primary tip	100	100	100	100
3rd (max)*	120	105	120	105
mature (max)	200	110	150	110

*(max), the maximum level of enzyme polypeptides found in the leaf was not in the tip section, but were found in the sections less distal than the tip section.

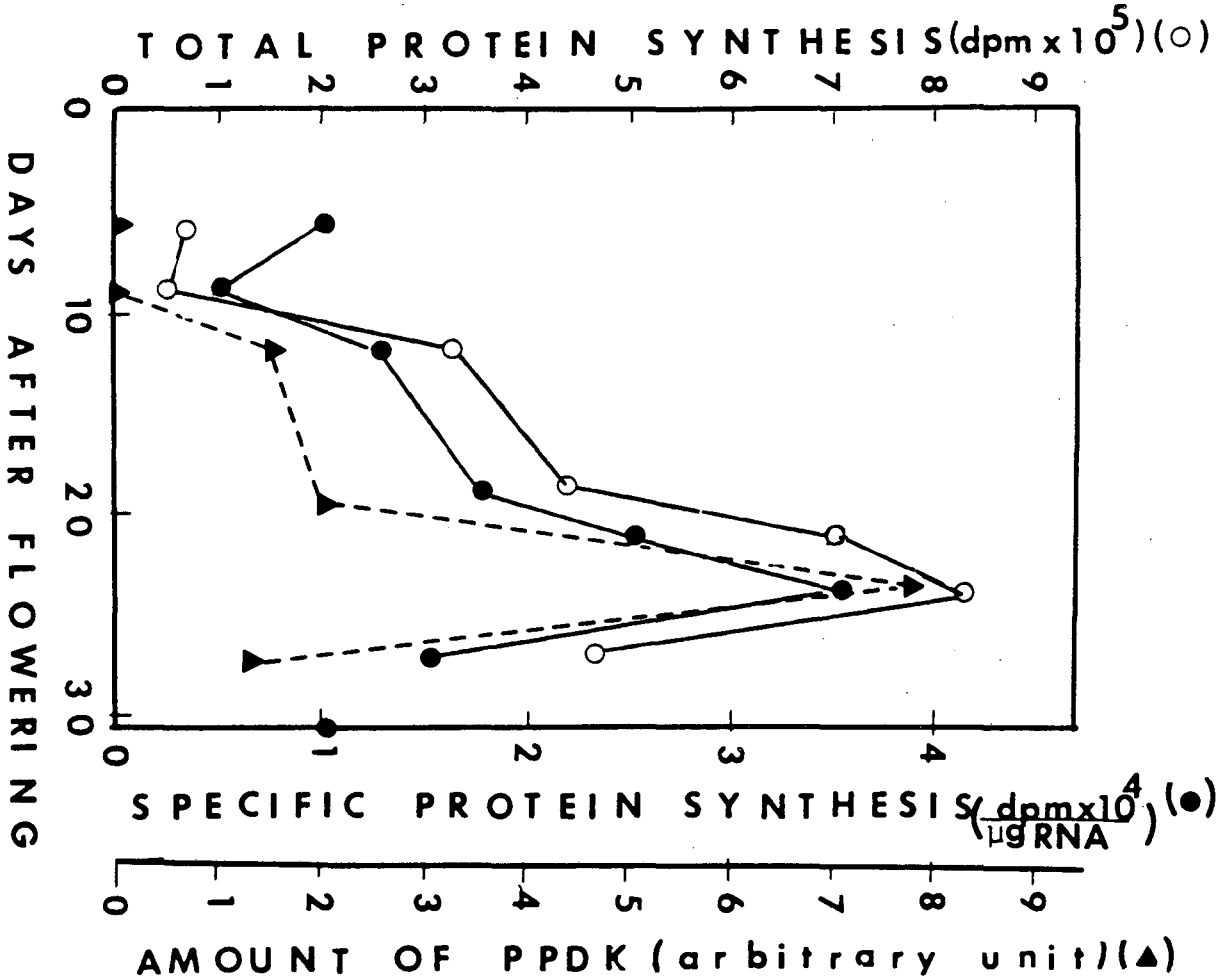
Fig. 1 Developmental profile of wheat seed PPDK protein and of RuBPC large subunit. Fresh weight, total soluble protein, Chl, the amount of PPDK and RuBPC large subunit were estimated from the intensity of the protein blot autoradiogram using densitometry.



XBL 838-10876

Fig. 1

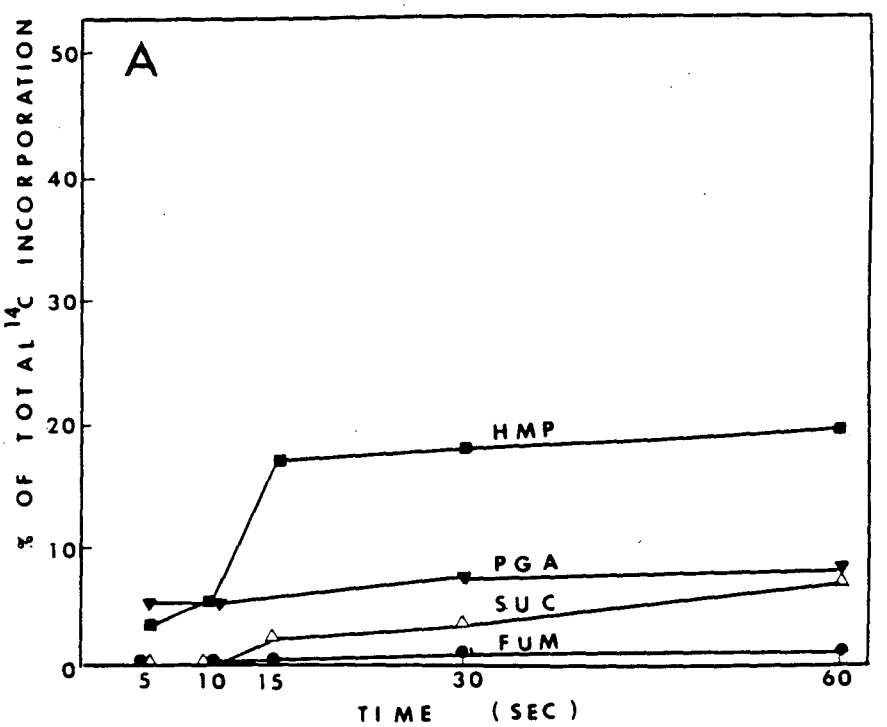
Fig.2 Developmental profiles of PPDK mRNA activity as measured by *in vitro* protein synthesis and detection by immunoprecipitaion. Curve A, PPDK mRNA activity per μg total wheat seed RNA; curve B, total wheat seed protein mRNA activity per μg total seed RNA; curve C, total wheat seed protein mRNA activity per seed.

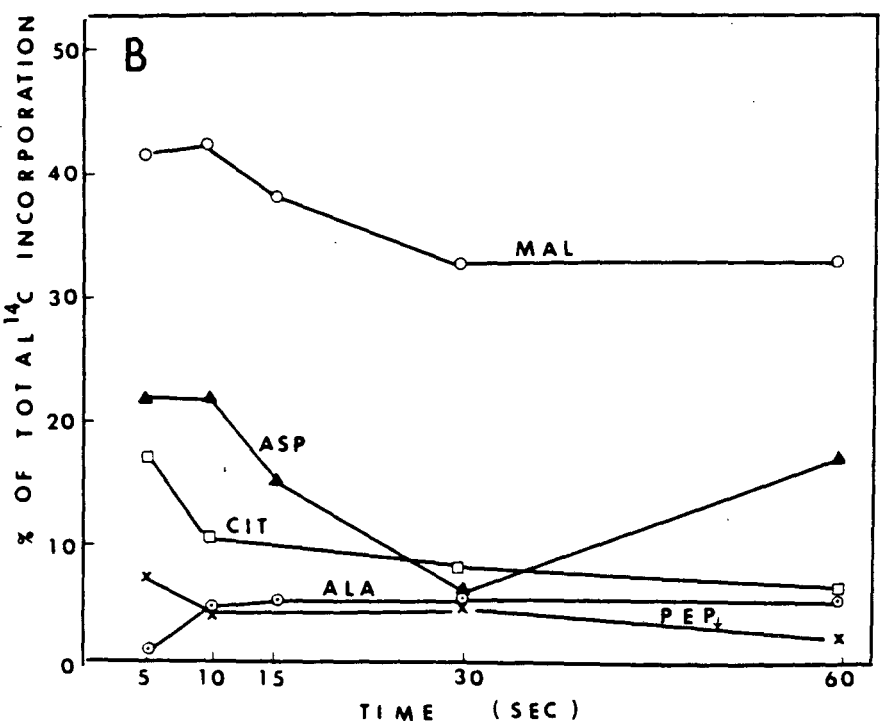


XBL 838-11256

Fig. 2

Fig. 3 Labeling of metabolites during short periods of photosynthesis by green wheat seed tissue in the presence of $^{14}CO_2$. Seeds at 25 d after flowering were dissected and after removal of endosperm, pericarp and adjoining tissue was used. (A) HMP, hexose monophosphates; PGA, 3-phosphoglycerate; SUC, sucrose. (B) MAL, malate; ASP, aspartate; CIT, citrate; ALA, alanine; PEP, phosphoenolpyruvate; FUM, fumarate. Two-dimensional chromatograghy and autoradiograghy analyses were used. The individual compounds were identified by cochromatography with known amounts of either labeled or unlabeled amino acids standard. After the x-ray films were developed, the chromatograms were sprayed with Ninhydrin solution to develop colors.





XBL 8310-12073A

Fig. 3

Fig. 4 Accumulation of enzyme polypeptides of PEPC, RuBPC, PPDK, and NADP-ME in sections of primary wheat leaf. The relative level is expresed as percentage of the final level (in the tip). The levels were determined by densitometry of the radioautographs of the protein blot.

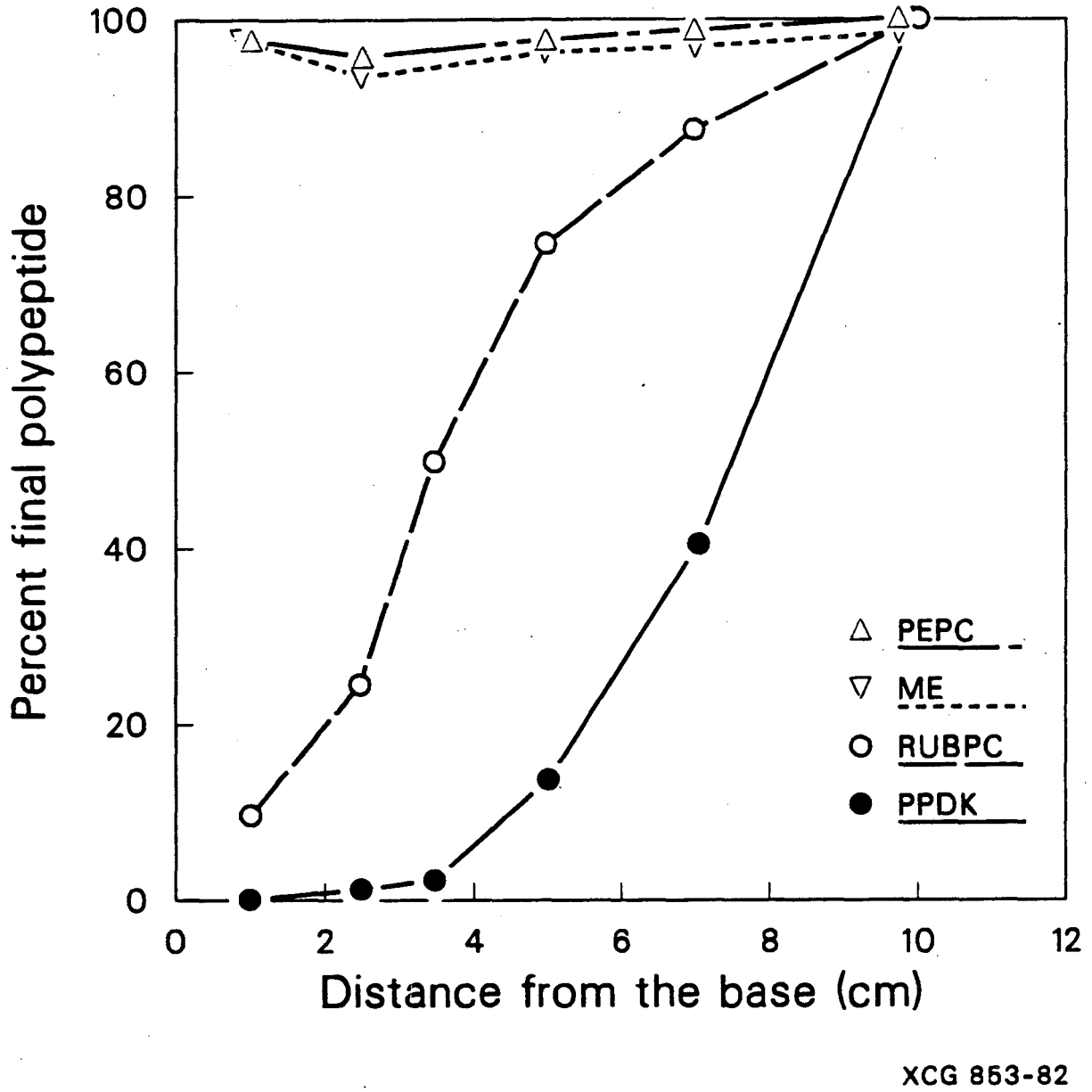

Percent final polypeptide
100
80
60
40
20
0
0
2
4
6
8
10
12
Distance from the base (cm)
PEPC
ME
RUBPC
PPDK
XCG 853-82

Fig. 4

Fig. 5 The profile of accumulation of PPDK in wheat leaf sections.

A. Primary leaf

In each lane extract containing 400μg soluble protein was loaded. Lanes 1-6 are from leaf sections respectively (in cm): base-2, 2-3, 3-4, 4-6, 6-8, and 8-tip.

B. Mature leaf

Lane 1: maize leaf extract (50μg); lanes 2-8 each were loaded with extracts containing 400μg soluble protein from sections respectively (cm); 0-4, 4-8, 8-15, 15-22, 22-29, 29-36, 36-tip. Lane 9 was loaded with the same amount of soluble protein from primary wheat leaf (as in Fig.4) for a comparison.

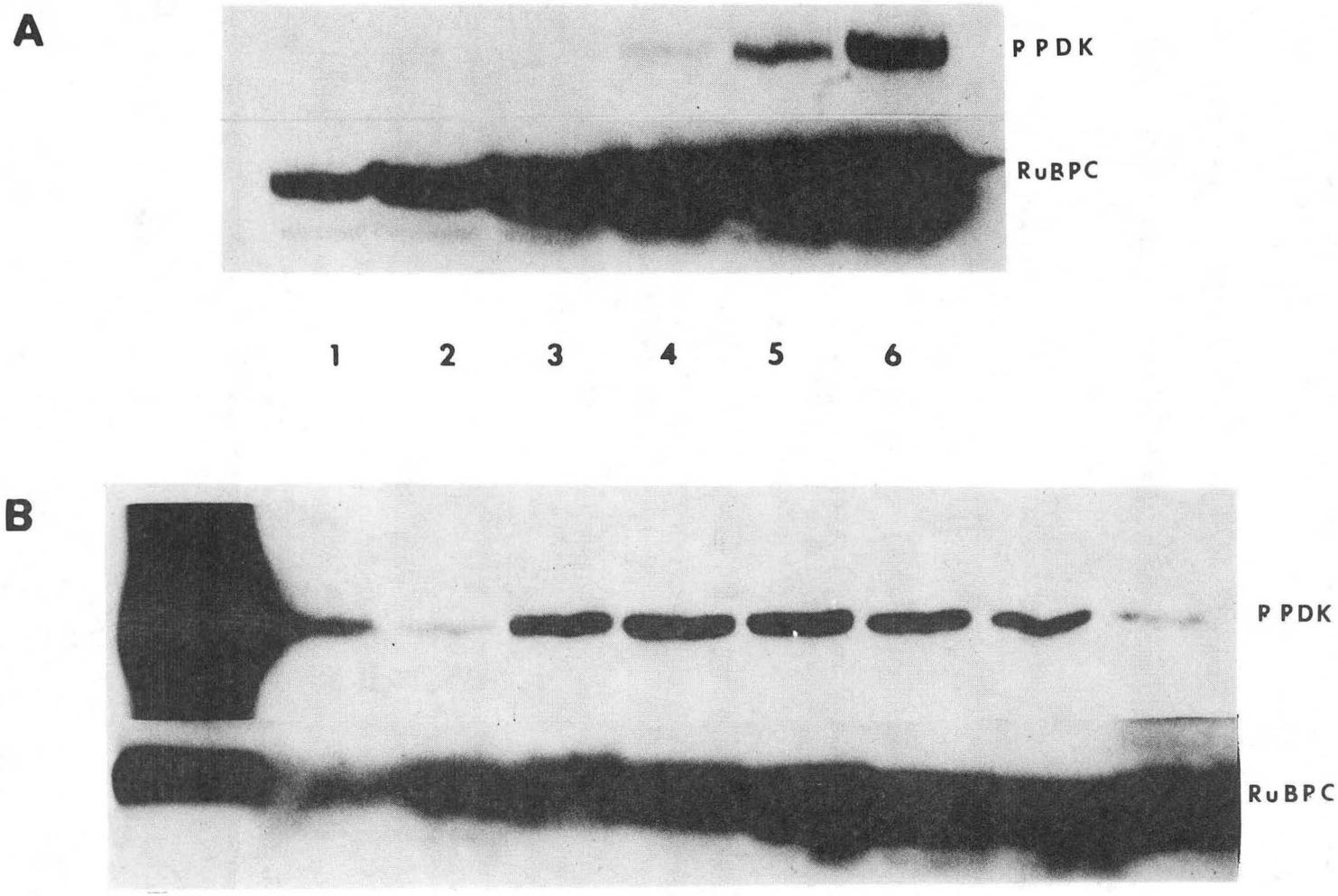


Fig. 5

XBB 852-1743B

Fig. 6 Incorporation of ^{14}C from $^{14}CO_2$ into C_3 and C_4 compounds in wheat leaf sections

A. Base section

B. Tip section

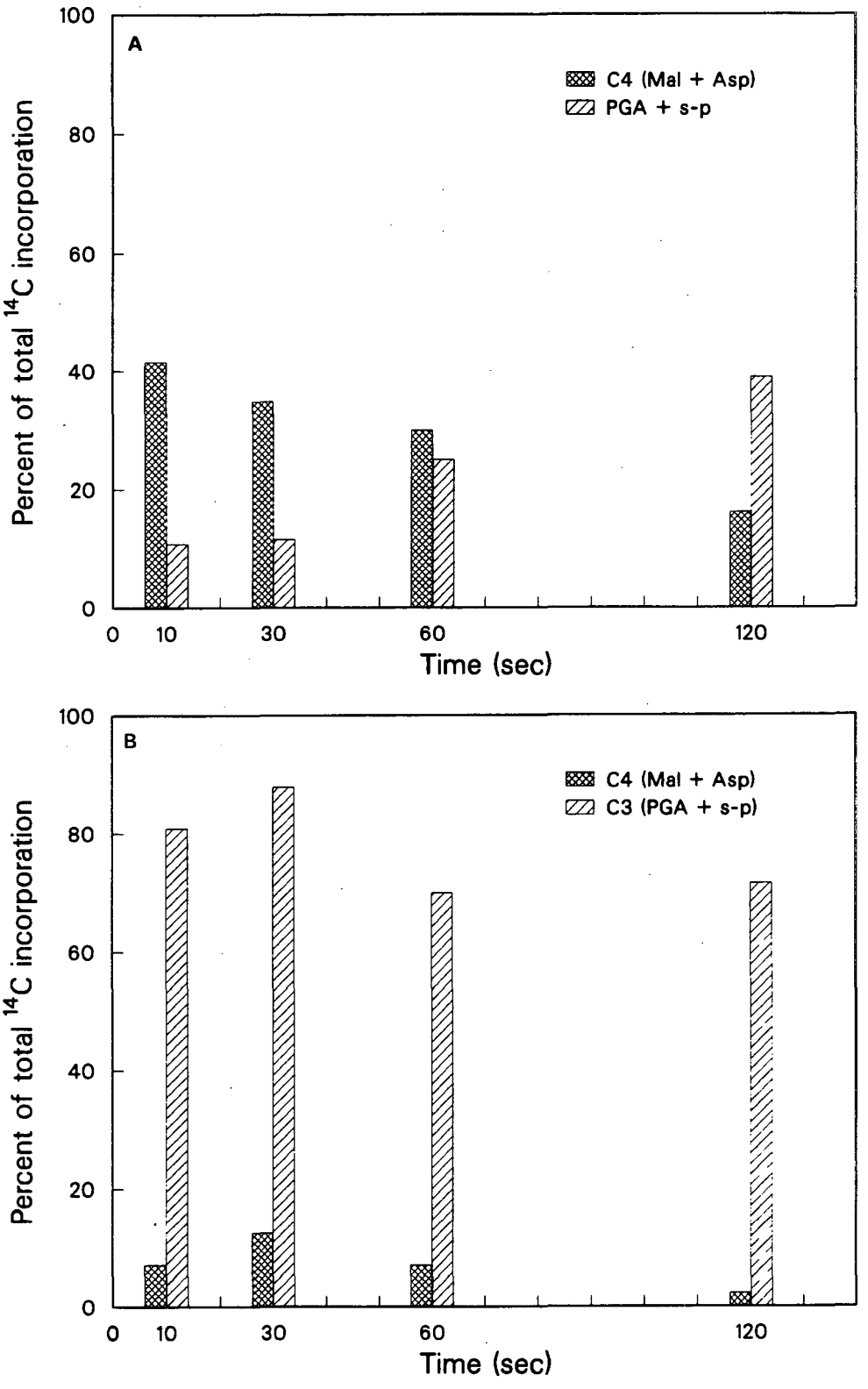

A
C4 (Mal + Asp)
PGA + s-p
Percent of total ^{14}C incorporation
100
80
60
40
20
0
0
10
30
60
120
Time (sec)
B
C4 (Mal + Asp)
C3 (PGA + s-p)
Percent of total ^{14}C incorporation
100
80
60
40
20
0
0
10
30
60
120
Time (sec)
XCG 863-86

Fig. 6

Fig. 7 Incorporation of $^{14}CO_2$ into wheat leaf base section analyzed by two-dimensional chromatography and radioautography. Malate and aspartate after 10 s in $^{14}CO_2$ made up 43 % of the total ^{14}C incorporation. o, origin; 6pglua, 6 phosphoglycerate; s-p, sugar phosphate; cit, citrate; fum, fumarate; mal, malate; glu, glutamate; ala, alanine.

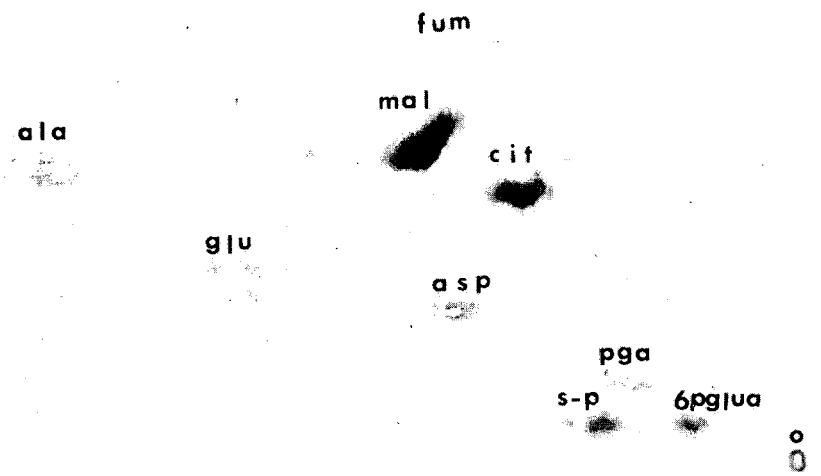
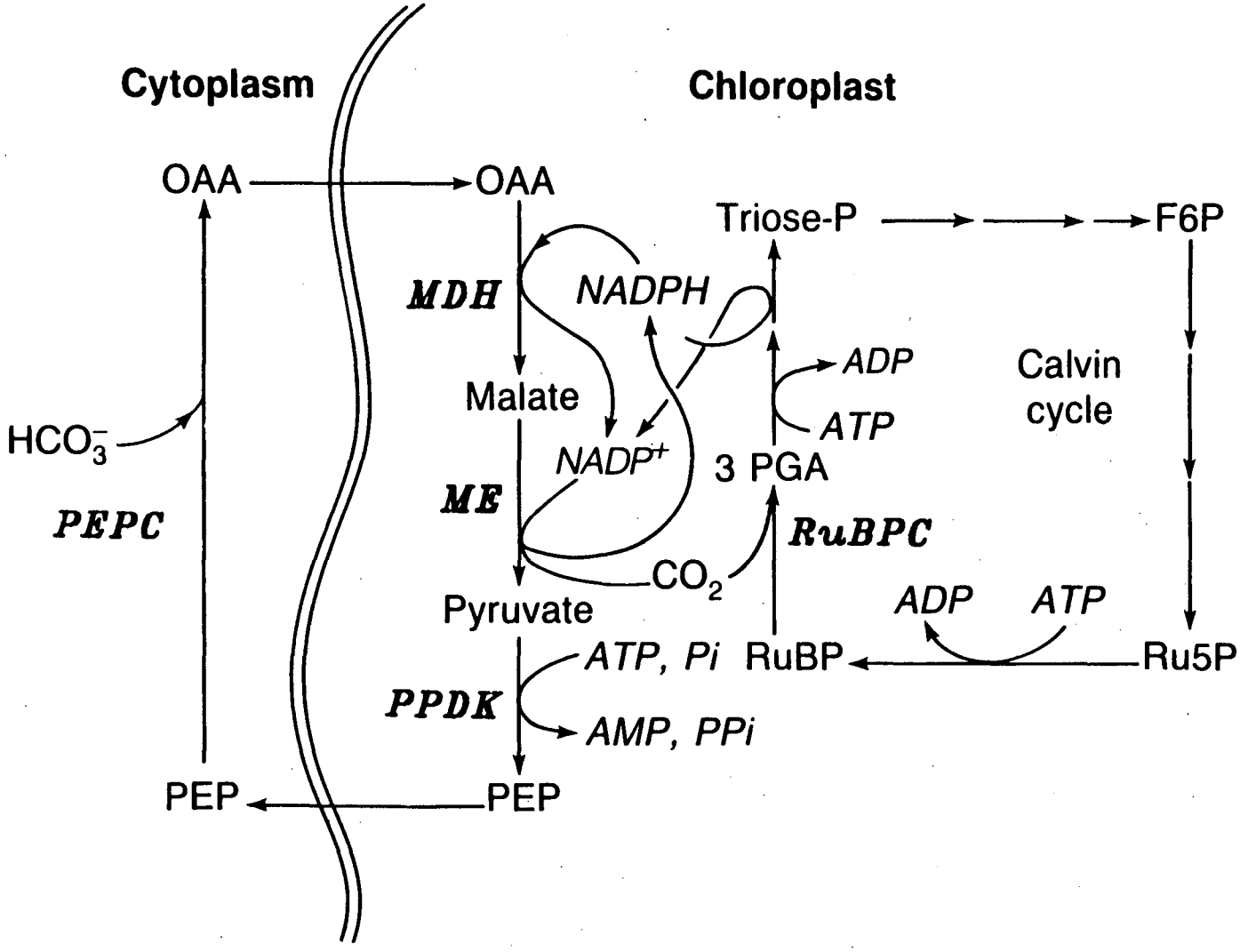


Fig. 7

XBB 851-938

Fig. 8 Hypothetical pathway for C_4 intracellular carbon transport in wheat.

Possible C_4 metabolism in single cell



XBL 842-10110A

Fig. 8

Part Four

Developmental Regulation of Gene Expression in Maize

Appearance and accumulation of C_4 carbon metabolism enzymes in developing maize leaves and A188 tissue cultures

RESULTS

Protein blot analysis of enzymes of carbon metabolism during early stages of development

PPDK and PEPC are already present in non-chlorophyllous callus at about 20% and 10% respectively of the levels (μg PPDK per soluble protein) found in the leaf, whereas NADP-ME and RuBPC are not detectable at this stage. RuBPC appears upon greening of the callus and gradually increases in amount with shoot regeneration. The appearance of ME-NADP lags behind that of the other three enzymes as it is observed for the first time only after the shoot has reached about 1 cm. The level of PPDK decreases once during the early stage of shoot formation, then increases as the shoot develops (Fig. 1).

Enzyme activity in A188 callus

Callus PPDK has low activity and, as has been the case for others (Sugiyama and Laetsch, 1975), it was difficult to demonstrate ATP and Pi dependence. After overnight dialysis against buffer containing phosphate (see METHODS AND MATERIALS), a preparation showing pyruvate and ATP dependence was obtained, but dependence on Pi has not been demonstrated. Activity (as determined by change in absorbance at 340nm) was strictly dependent on pyruvate concentration, giving a Km (pyruvate) about 80 μM, less than 1/3 that of leaf PPDK (Fig.2). Also an assay for pyruvate kinase activity (Baysdorfer and Bassham, 1984) was negative.

Protein blot analysis of extracts from regenerating A188 tissue cultures demonstrate that A188 callus PPDK activity per mg soluble protein is about 5-16% and PEPC activity 2-5% as compared with regenerated maize leaf (Table 1). The activity ratio of PEPC/PPDK in maize leaf is about 18

whereas this ratio in callus is 5 to 8, so that PEPC activity is in great excess. The Km (PEP) of PEPC for maize leaf is 0.6 mM whereas that of callus is 0.05 mM (Fig.3). Enzyme activities are not expressed on an activity per unit of chlorophyll basis, since the amount of chlorophyll varies greatly with the samples. ME-NADP activity is present in maize callus with V_{max}= 0.06 U/g fresh wt. and Km(L-malate)= 0.5 mM (Fig.4). No NAD-ME activity was detectable.

^{14}C Incorporation in regenerating tissue cultures

In non-chlorophyllous callus, the primary labelled fixation products were malate (71%) and aspartate (14%). No discernable labelling of phosphoglycerate and other products of the reductive pentose phosphate cycle occurred (Fig. 5). The total incorporation of ^{14}C increased with regeneration of shoots (Fig. 6). The fixation pattern in callus shows a slight difference from dark to light. However, the callus cultures often showed variability depending on the tissue used. There was substantial labelling of citrate at 10 sec. Sometimes, a large incorporation into PEP was observed (not shown).

In the light with 3 cm regenerated shoots, the labelling of malate and aspartate predominated at 10 s, and then decreased with time, accompanied by an increase in PGA and sugar phosphates. In the dark, labelling of citrate was again observed. In the 8 cm shoot, the % of C_4 acids after 10 s illumination is 77%, but even after 2 min is still 65%. It may appear that this represents a step backwards in the development of C_4 carbon transport, but it should be remembered that the total fixation has increased more than three-fold between the 3 cm and 8 cm shoot. It would appear that during this development, β-carboxylation has increased faster than C_4 transport. Thus, the kinetic pattern of

^{14}C fixation is changing towards that expected for C_4 carbon transport, but increases in activity of PEPC may occur sooner than increases in transport of metabolites between cells. The amount of ^{14}C fixation into other compounds such as alanine, citrate, fumarate, phosphoenolpyruvate, 2-ketoglutarate and some unknowns is minor and the total is not significant.

Protein blot analysis of enzymes of carbon metabolism during seedling development: seedling sections

In maize seedling first leaves, the enzyme polypeptides for C4 metabolism increased concurrently from the 3-5 cm section to the tip (Fig. 7A). In the basal 0-2 cm section, RuBPC, PEPC and ME-NADP were not detectable. In maize second leaf sections, RuBPC is present in the basal 0-1 cm region at about 0.5% of the final level found in the distal section (Fig. 7B). The accumulation of RuBPC becomes noticeable in 4-6 cm section where the level reaches about 10% of the final level. Then the amount of RuBPC polypeptide stays constant or decreases slightly toward the tip. PPDK is also present in the basal 0-1 cm region at about 0.5% of the final level of accumulation and increases concurrently with RuBPC. PEPC and ME-NADP follow roughly a similar pattern.

The increase of total amount of these polypeptides is due both to the increase in their proportion of total soluble protein with development and also to an increase in the amount of soluble protein. The increase can be seen either on a soluble protein basis or fresh weight basis. The pattern of enzyme accumulation is closely related to that of soluble protein (Table 2), whereas there was little correlation with chlorophyll content. The chl a/b ratio increased with development to a value typical for C_4 leaves.

Leaf age

The 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, RuPBC, PEPC, and PPDK levels were similar at the two ages, but the level ME-NADP was very different, increasing about several fold from 7 d to 9 d (Fig. 8).

Effect of leaf position on the profile

The pattern of appearance and accumulation was basically the same for upper maize leaves, but the level of enzymes was higher (Table 3). In the mature maize leaf, the portion of soluble protein allocated to these enzymes did not increase. When an equal amount of soluble protein was loaded into each lane, there was no increase in the band intensity of these enzymes. Since equal amounts of plant extract contained increasing amounts of soluble protein toward the tip, an increase of the band intensity for each protein was observed (Fig. 9). Compared with the first leaves, the relative accumulation of enzymes for the mature leaf sections is less. It should be remembered that only the blade of the mature leaves was studied.

PEPC activity of maize second leaves

The enzyme activity in the tip 3 cm of maize second leaf (11 d after planting) was 0.58U/g fresh weight whereas that of the basal 3 cm section was only 0.075U/g fresh weight. When the activity is expressed per mg of chlorophyll, however, the values were 0.58 and 0.45 respectively.

Light microscopy of callus and regenerated shoots:

Non-chlorophyllous callus tissue (Fig. 10a) can vary from loose aggregates of irregularly shaped cells (Fig. 10b) to a compact tissue (meristemoids) and thence to root-like structures apparently developing

from these meristemoides. Root-like structures (Fig. 10c) apparently have the apical organization of roots (root caps, lineages of cells originating from a meristematic area) and a central vascular cylinder (Fig 10d). These structures lack the highly organized anatomy of a mature corn root, however. No obvious plastids are evident, but amyloplasts may be present in the root-like structures.

Chlorophyllous tissue (Fig. 11) has groups of tracheoids associated with chlorenchyma which are several layers thick, individual cells with large vacuoles. Under direct microscopic observation, the chloroplasts appear to be agranal and to contain starch granules.

Short shoots (Fig. 12c) develop from embryoids in the embryogenic callus (Fig. 12a). There are islands of meristematic areas and vascular elements. Chloroplasts are scattered throughout the tissue. A section of a 5 mm regenerated shoot shows an apical meristem (Fig. 12b). The shoots which are 1-3 cm do not have Kranz anatomy (Fig. 12d). The interveinal distance is about 270 μm and there are 2-8 mesophyll cells between a pair of vascular bundles. Mesophyll cells are about 60 μm across, are irregular in size, and they contain few chloroplasts. There is one small vascular bundle between a pair of large vascular bundles. Intercellular spaces occur frequently.

In shoots 8 cm or more in length (Fig 13a), the interveinal distance (ID) is 150-200 μm and the number of mesophyll cells between a pair of vascular bundles is about 2 which is the typical value reported for C_4 grass (Miranda et al 1981). The ID of the control maize leaf obtained from a green house is about the same also. Two types of vascular bundles, large and small, have been found in the maize leaf (Miranda et al 1981). There are 8 small vascular bundles

between a pair of large vascular bundles.

In the regenerated maize leaf, however, the mesophyll cells are arranged in a less orderly way than in typically mature maize leaves (Fig 13c). The mesophyll consists mostly of palisade parenchyma and is unifacial. They are 30-40 μm across in size which is the same as in the maize leaf of the seedling. Not all the mesophyll cells contain chloroplasts so that only a fraction of the mesophyll cells may be capable of photosynthesis.

Hattersley and Watson (1976) have shown that the condition where there are no cells between the metaxylem vessel elements and the laterally adjacent sheath cells of large bundles, i.e. the 'Xy-MS-condition', is a characteristic of NADP malic enzyme species. This condition is present (Fig. 13b) in older regenerated shoots. This anatomical evidence suggests that C_4 metabolism should be present. Bundle sheath chloroplasts are centrifugally arranged, however, in contrast to the control maize leaf which is not as regular (Fig. 13d). Sometimes chloroplasts are missing or they are randomly scattered thorughout the cell. The number of chloroplasts is only 1/4 to 2/3 of that found in the control leaf tissue, however. In summary, the regenerated shoot has Kranz anatomy but lacks some structures which are present in the normal maize leaf control.

Since the section of the regenerated plant used was obtained from the 4th leaf whereas the section of the control was obtained from the 3rd leaf (see Materials and Methods), a direct comparison cannot be drawn.

DISCUSSION

Appearance and properties of enzymes in A188 callus

Protein blot analysis of the appearance and accumulation of enzymes of carbon metabolism such as PEPC, RuBPC, PPDK and ME-NADP in non-chlorophyllous and regenerating callus suggests that there may be an earlier stage of appearance than has been seen in sections of a leaf (Mayfield and Taylor 1984). In maize leaf sections, all these enzymes appear at about the same time and increase in coordinance with each other from position at which bundle sheath and mesophyll cells have differentiated. In contrast, PPDK appears at relatively high levels in the undifferentiated callus before the appearance of RuBPC and NADP-ME. The temporary decrease of PPDK at the shoot-forming stage may be due to the transition from a cytoplasmic form characteristic of heterotrophic tissue to a chloroplast form found in leaves.

Although mature leaves contain poly(A)RNA translating only to 110 kilodalton PPDK polypeptide (when probed with PPDK antibody), both the embryoid-forming callus and the young first maize leaves have poly(A)RNA which yields both 110 and 94 kilodalton PPDK polypeptide (Fig.2 Part Two.) It thus appears that the change from cytoplasmic to chloroplastic PPDK occurs during an early stage of leaf ontogeny, beginning even before the appearance of Kranz anatomy. However *in vivo*, the leaf-type PPDK polypeptides may not accumulate in the embryoid-forming callus. Since the mol wt of the mature polypeptide is the same for the heterotrophic type, I could not measure the fraction of the leaf-type PPDK in callus. The first leaves used for mRNA isolation contained the sheath sections. The heterotrophic type PPDK polypeptide may be attributable to the sheath section. Isolation of mRNA from

this section followed by an analysis of the translation products may prove this hypothesis.

PEPC found in callus has different kinetic properties from the leaf type. Both the Km (PEP) and V_{max} values we find for callus PEPC are closer to the values reported by Ting and Osmond (1973) for the PPDK in C_3 species than for the C_4 leaf type. The kinetic properties of maize callus PEPC are consistent with the those reported by Ruzin (1984) for the enzyme in regenerating tissue culture of a C_4 dicot, Euphorbia degeneri. Ruzin found that there are two isoenzymes of PEPC in leaf, but only one isoenzyme was present in callus. I find that maize callus PEPC has a lower Km (about 0.05 mM) than the leaf PEPC (Km = 0.55 mM). The properties of PEPC seem to undergo changes upon establishment of autotrophy in a C_4 plant according to Deleens and Brulfert (1983) who found that in maize seedlings PEPC activity during days 5 to 7 was low and had low sensitivity to glucose-6-phosphate, but after 7 days shifted progressively towards the properties of the C_4 photosynthetic type.

The ratio PEPC/PPDK in callus is much lower that that of C_4 leaf type. However, PEPC activity is still 5 to 8 times in excess of PPDK so that PPDK may be the rate limiting enzyme in callus for the conversion of pyruvate to oxalocetate as is reported to be the case in C_4 leaves (Sugiyama et al 1984).

NADP-ME is present in callus according to activity assay but not by immunoassay. The enzyme has Km (L-malate) = 0.5 mM which is similar to the value reported for etiolated maize leaf NADP-ME (Pupillo and Bossi, 1979). The activity we found in callus is 1/3 to 1/4 of the enzyme activity reported in etiolated maize leaf. NAD-ME was not detectable in callus.

Since antibody prepared against maize leaf ME-NADP did not cross react with NADP-ME in callus, the level of enzyme must be very low and also the protein structure may be immunologically different. Another explanation might be that the enzyme activities were artifactual due to some other component in the reaction mixture than ME.

Pattern of ^{14}C incorporation in A188 callus

The ^{14}C incorporation products in callus are as expected in tissue with low ME-NADPH activity for decarboxylation. In these callus tissues, malate and aspartate are not converted to CO_2 for subsequent incorporation into sugar phosphates, but might be used for anaplerotic synthesis of other amino acids, chlorophyll, etc. The properties and quantities of PEPC also suggest a different role from C_4 photosynthetic carbon transport. Considering the lack of an efficient vascular system in these tissues, translocation of the C_4 acids would not be expected. Kennedy et al (1977) did not find any net increase in end products of the C_3 cycle during the pulse chase in C_4 callus, Portulaca oleracea consistent with my result.

Appearance and accumulation of C_4 enzymes in sections of maize seedlings

The profile of appearance and accumulation of these enzymes I find in maize seedlings is mostly consistent with reports by Williams and Kennedy (1978), Perchorowicz and Gibbs (1980), and Mayfield and Taylor (1984). Mayfield and Taylor (1984), however, did not see any RuBPC, PEPC and PPDK polypeptide in basal 0-2 cm sections, their appearance occuring first in 2-4 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 my objective was to determine the order of expression 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, I was able to detect polypeptides which are present in less than 1% of the amount present in the leaf tip section.

In maize second leaf sections, the first 0-2 cm region is insensitive to CO_2 concentration (Miranda et al 1981) suggesting that the stomata are non-functional. According to the activity assay, PEPC activity in the basal section is only 10% of that of the leaf tip. The bottom 3 cm of leaf tissue exhibits a capacity for photosynthetic electron transport but not for CO_2 fixation. PEPC in the basal section, therefore, must be refixing respiratory CO_2 and may not be of the C_4 type.

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/b ratio of this young first leaf tissue is less (2.7) than that of more mature leaf (3.2) indicating that the younger leaves have not fully developed at this stage, since the developed maize leaves usually have Chl a/b ratio of about 3.0-3.5 in our studies. Since ME-NADP accumulates 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.

It is only in tissue above 3 cm from the leaf base that the maize leaf exhibits high photosynthetic rates. Deleens and Brulfert (1983) reported a shift of properties of PEPC towards the C_4 type upon establishment of autotrophy in maize seedlings. This shift occurs 7 days after planting. This is about the same time as the appearance

of an appreciable amount of RuBPC and PPDK under our maize seedling growth conditions (4-7 cm section in our study). This is also the same time as light dependent onset of accumulation of PEPC and RuBPC (Nelson et al 1984). Also 3.5 cm from the base, the characteristic dimorphic chloroplasts of maize are found (Miranda et al 1981). The centrifugal arrangement of bundle sheath cell chloroplasts is found 3 cm from the base. Above 4 cm, interveinal distance decreases to 125-140 μm, a more typical value for the C_4 leaf. This is where I see a noticeable accumulation of the four enzymes studied, and also where Mayfield and Taylor (1984) observed their appearance. As they suggested, cell differentiation may be an important factor for an accumulation of C_4 carbon fixtion enzymes in maize leaf. After that, all four enzymes increased rapidly up the length of the leaf, reaching a plateau by the 10-11 cm section.

While these analyses of enzyme appearance in sections of the developing leaf provide a clear picture of the development of C_4 characteristics from base to tip, examination of leaves appearing later on the plant have suggested other developmental relationships. Crespo et al (1979) reported that leaves number 1 and 2 had a PEPC/RuBPC ratio of less than 1 while leaves 3-5 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. Waygood and Law (1976) claimed that the first leaves utilized the Calvin Cycle. Mayfield and Taylor (1984) found no evidence for C_3 fixation in sections

from the 3rd leaf. I find that from the first section on, in either first or later leaves, both RuBPC and PEPC appear and increase in concert with one another.

A possible explanation for such discrepancies might be that Crespo et al (1979) used maize leaves in positions 1 to 5 from three week old plants. With maize seedlings, leaves in position 1 and 2 are already becoming senescent by the third week in our studies, as we find both chlorophyll content and soluble protein, including polypeptides as measured by protein blot, reduced to about half the levels of younger mature leaves. Khanna et al (1973) reported that in old sorghum leaves the levels of C_4 enzymes such as PEPC decrease more rapidly than that of RuBPC, which would lead to more C_3 type CO_2 fixation.

Conclusions

My results indicate that, in maize, Kranz anatomy and certain fine structures (such as the arrangement of chloroplasts in cells) are essential for C_4 intercellular photosynthetic carbon transport, commonly termed C_4 photosynthesis. In callus tissues, C_4 acids are formed, and their formation may be a part of anaplerotic metabolism or biosynthesis but not of intercellular carbon transport. Such anaplerotic metabolism can be stimulated by light.

While the heterotrophic type PPDK (which I propose is cytoplasmic) is present in white callus, both heterotrophic and autotrophic (chloroplastic) PPDK (as well as RuBPC) already are present in greening callus and in basal sections of seedlings. During differentiation of A188 callus, there is a dip in the level of PPDK polypeptide which could be a consequence of the transition from cytoplasmic to chloroplastic type.

In second and in later leaves, the increases in levels of RuBPC, PPDK, PEPC, and ME-NADP are well coordinated with each other and with the development of Kranz anatomy. In the first leaves, however, PPDK appears slightly before the other polypeptides, according to protein blots, and the percentage of of soluble protein allotted to PPDK polypeptide synthesis is greater in younger leaves than in older leaves. ME-NADP, in contrast to PPDK, appears to be limiting in the young leaves, as has been suggested earlier (Perchorowitz and Gibbs 1980).

In the base to 3 cm region of maize first leaves, heterotrophic type PEPC is present. The formation of C_4 acids mediated by PPDK and PEPC in this tissue may be both for anaplerotic synthesis and to build up a level of malate required for the later onset of C_4 carbon transport.

In maize seedlings, C_3 photosynthetic carbon fixation <u>does not</u> precede C_4 photosynthesis. A possible explanation for C_3 photosynthesis in maize reported previously (Crespo, et al 1979) may be that the leaves used were three weeks old and could already have been undergoing senescence.

Table 1. Comparison of PEPC and PPDK activities, chlorophyll content, and chlorophyll a/b ratios in maize callus and regenerated shoots.

Tissue	Total Chl µg/ g FW	Chl a/b	Enzyme activities and K_m values PEPC U/ g FW	PEPC K_m µM	PPDK U/ g FW	PPDK K_m µM	PEPC/ PPDK
Leaf[a]	780	3.2	4.4	600	0.24	250	18
Green callus	38	2.8	0.08	-	0.01	80	8
White callus	N.D.	-	0.17	50	0.035	80	5
Regenerated leaf[b]	92	3.0	-	-	-	-	-
Regenerated leaf[c]	1490	3.2	-	-	-	-	-

N.D., not detactable; -, not measured; a, a second leaf of a seedling was used; b, a regenerated shoot (5 mm in length) was used; c, a regenerated shoot (200 mm in length) was transferred from the petri dish to a flask and was grown in atmospheric CO_2.

Table 2. Content of chlorophyll and of soluble protein in second maize leaf sections.

Distance from base cm	Total Chl µg/g FW	Chl a/b	Soluble protein mg/g FW
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

Table 3. Effect of position in maize leaf on levels of C_4 enzymes

Leaf position	Relative enzyme protein level			
	PPDK	PEPC	RuBPC	ME-NADP
1st (tip)	60	60	60	45
3rd (base)	3	1	1	0.5
3rd (tip)	100	100	100	100
mature (max)	110	110	120	110

The level of polyeptide in the 3rd leaf tip section was used for comparison (100 %).

Fig. 1 Appearance and accumulation of PPDK, PEPC, RuBPC and ME in early stages of differentiation of maize 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 indicates the length of the regenerated shoots in cm.

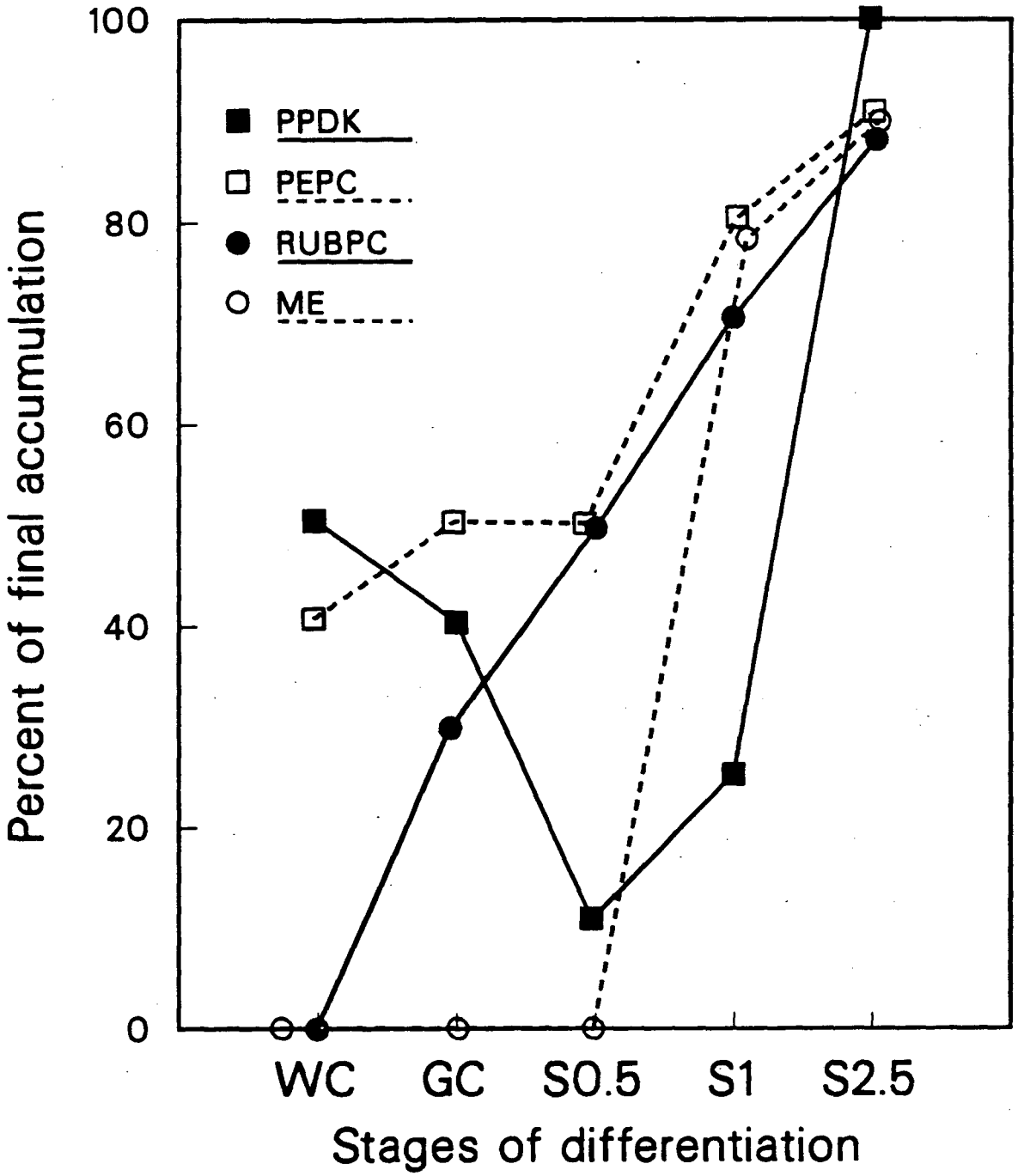

100
80
60
40
20
0
Percent of final accumulation
PPDK
PEPC
RUBPC
ME
WC
GC
S0.5
S1
S2.5
Stages of differentiation
XCG 853-146

Fig. 1

Fig.2 Determination of Km (pyruvate) of callus PPDK. Km=0.08mM.

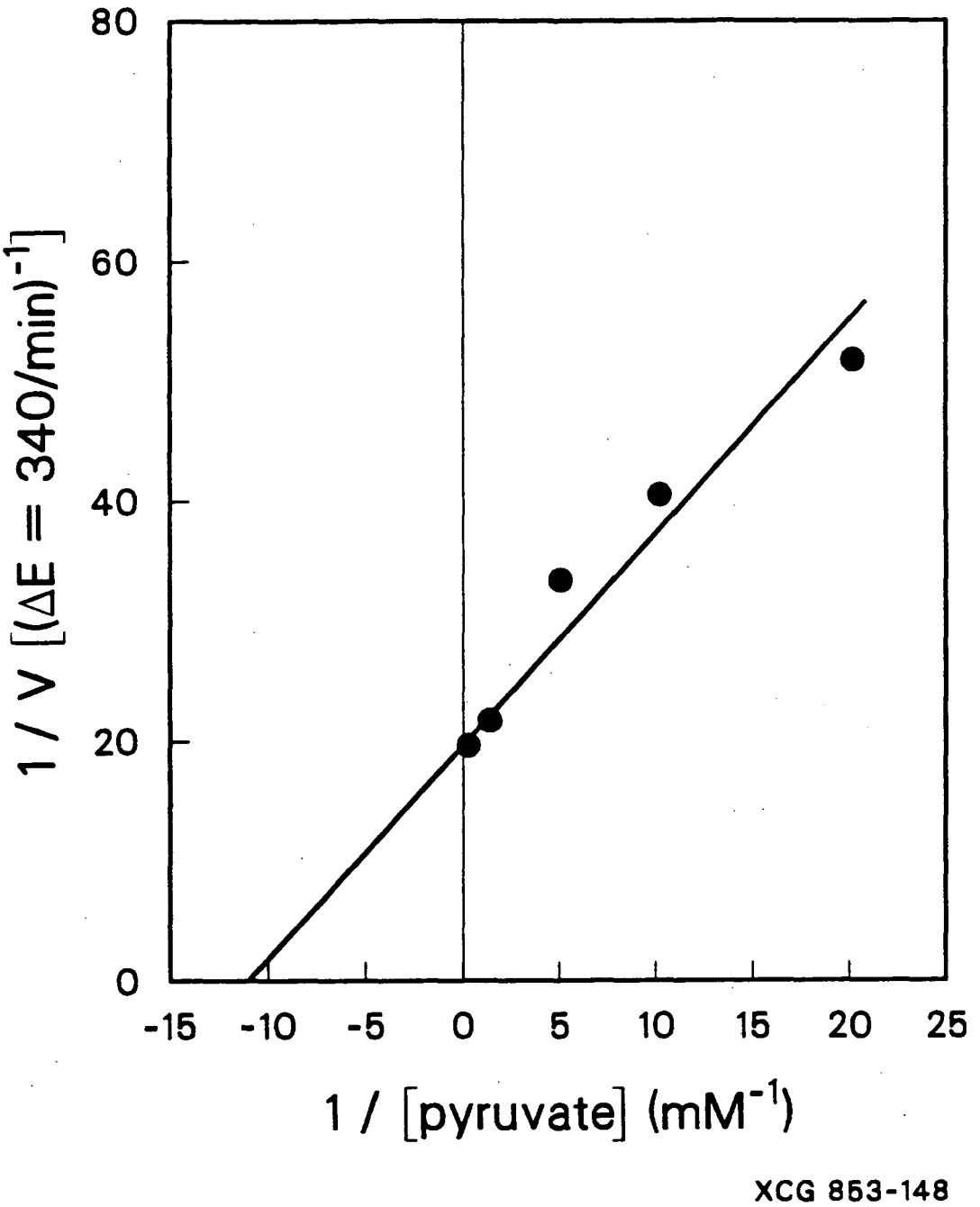
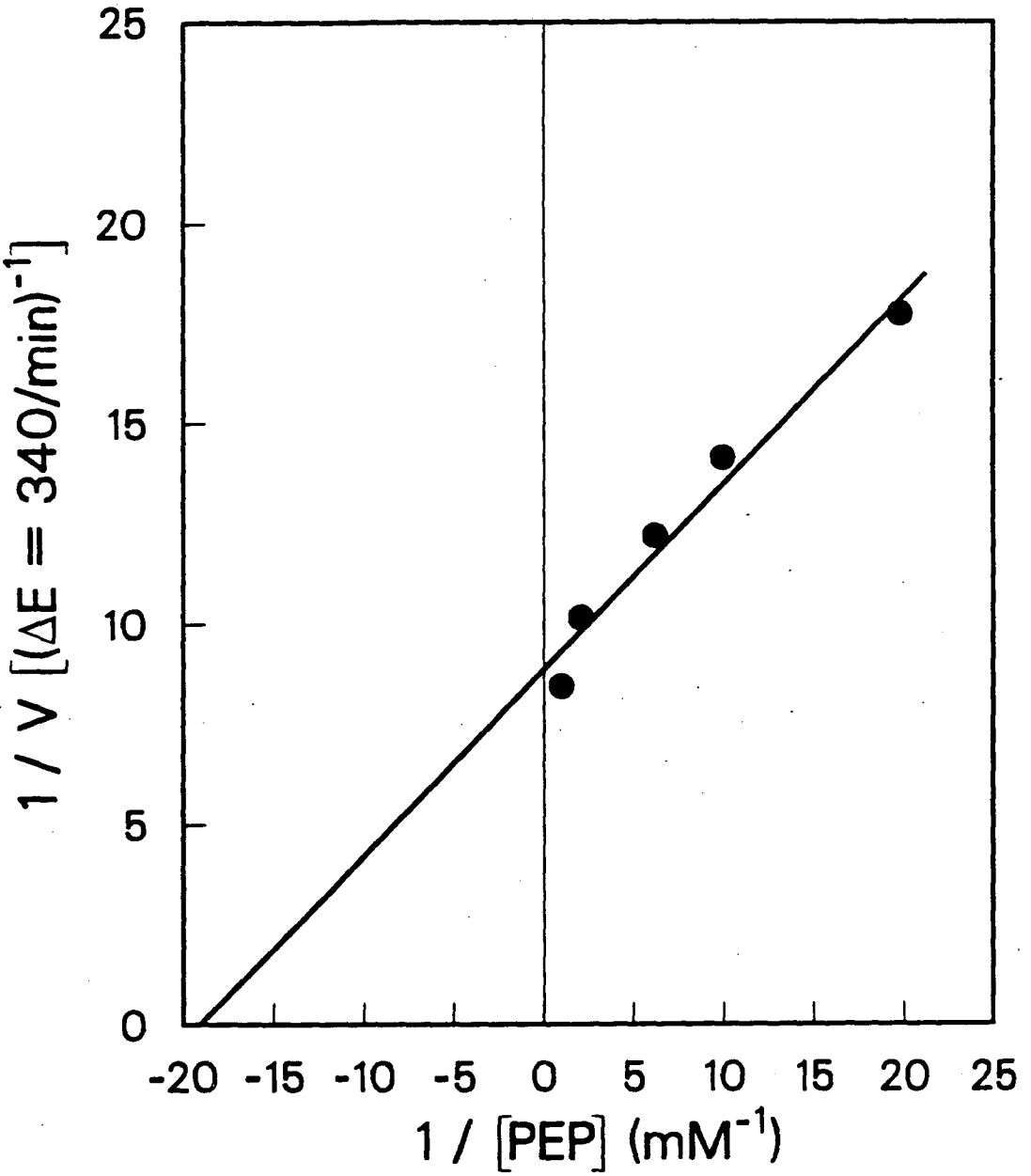

80
60
40
20
0
1 / V [(ΔE = 340/min)$^{-1}$]
-15
-10
-5
0
5
10
15
20
25
1 / [pyruvate] (mM^{-1})
XCG 853-148

Fig. 2

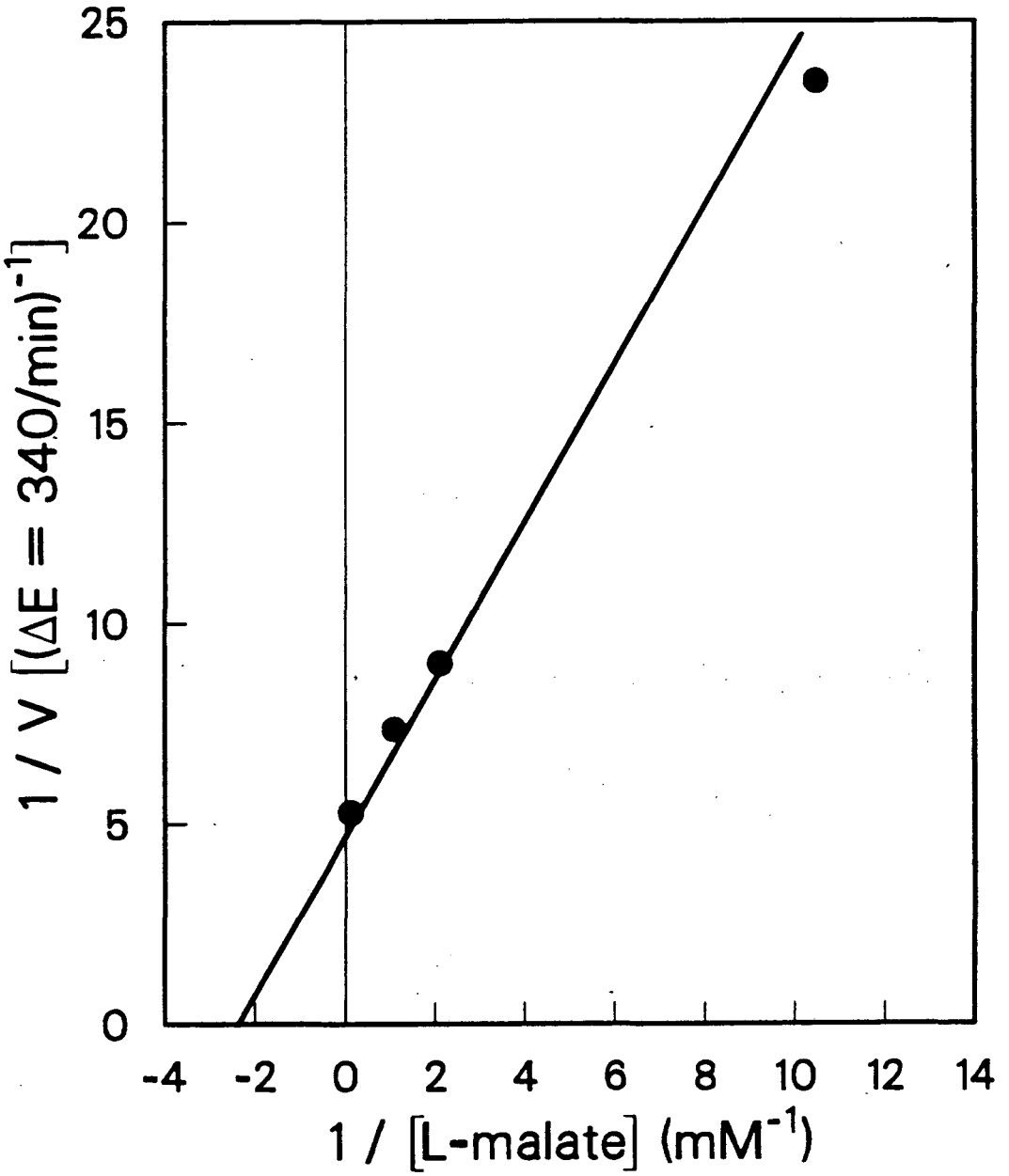
Fig.3 Determination of Km (PEP) of callus PEPC. Km=0.05mM.



XCG 853-152

Fig. 3

Fig.4 Determination of Km (L-malate) of callus NADP-ME. Km=0.6mM.



XCG 853-147

Fig. 4

Fig. 5 Incorporation of ^{14}C into C_4 acids after 10 sec of exposure to [^{14}C]-bicarbonate in regenerating maize plant. WC, white callus; GC, green callus; S1, 1 cm regenerated shoot; S3, 3 cm regenerated shoot; S7, 7 cm regenerated shoot.

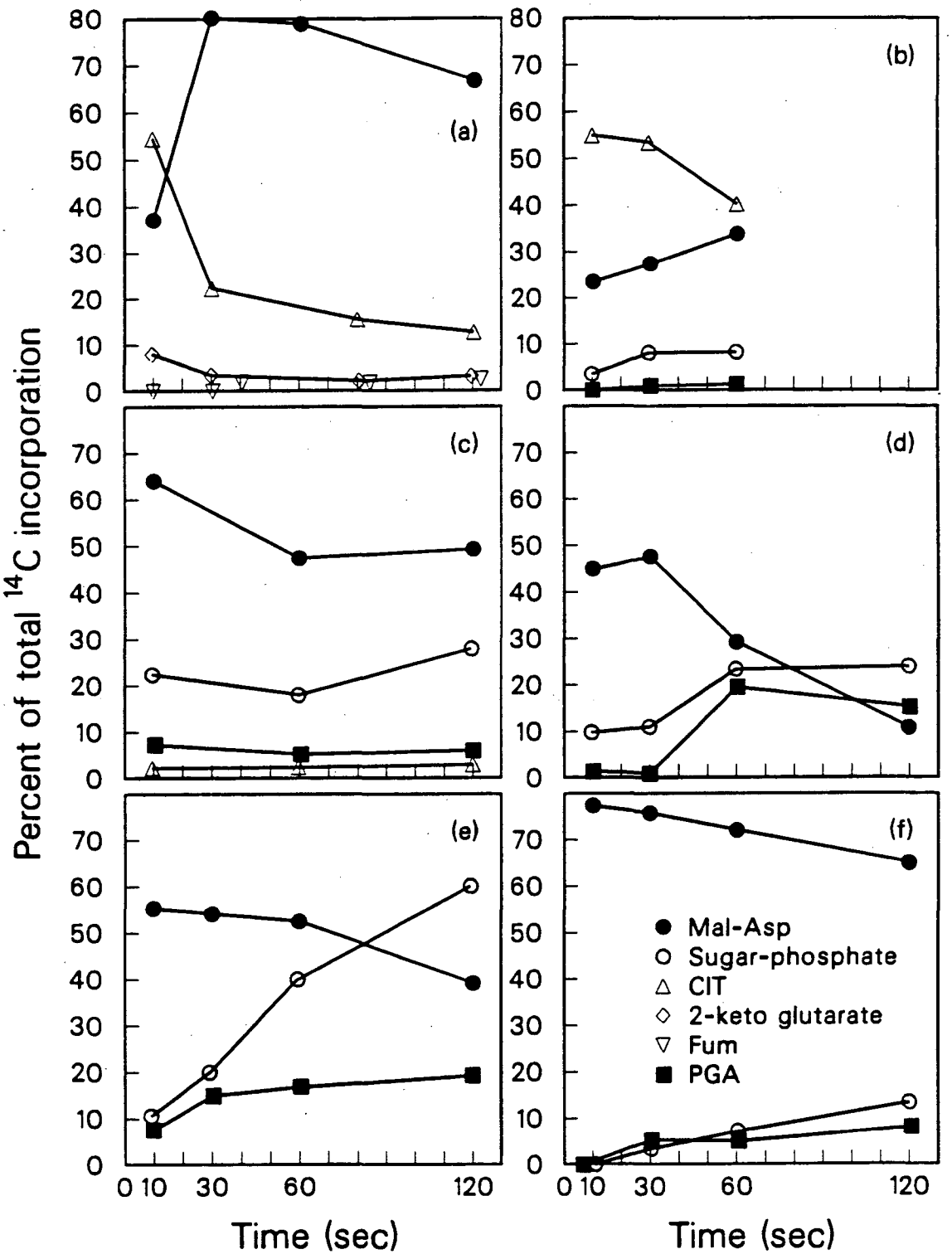


Fig. 5

XCG 863-150

Fig. 6 Total incorporation of ^{14}C into regenerating callus.

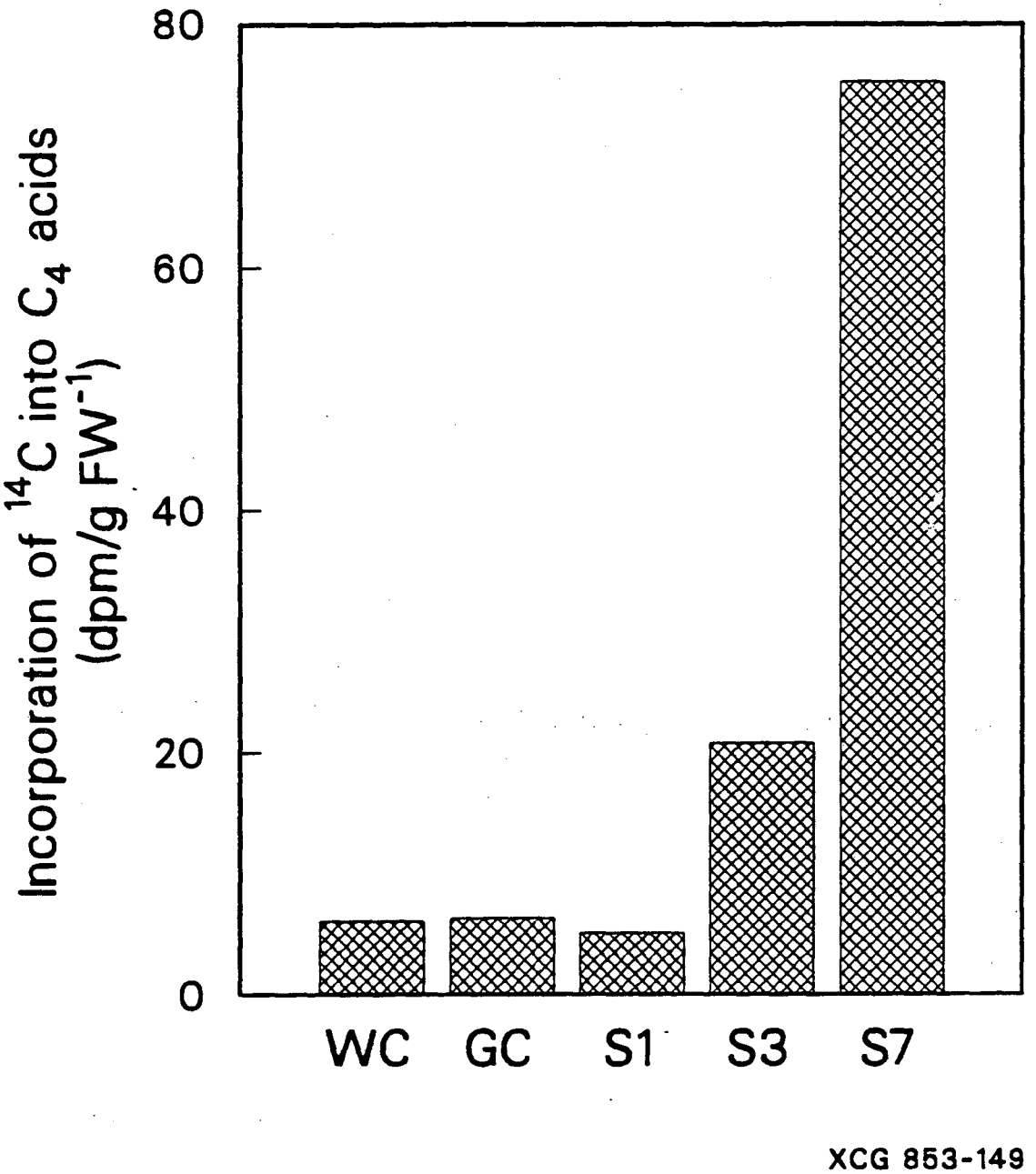

80
60
40
20
0
Incorporation of ^{14}C into C_4 acids
(dpm/g FW^{-1})
WC
GC
S1
S3
S7
XCG 853-149

Fig. 6

Fig. 7 Protein blot analysis of PPDK, PEPC, RuBPC, and ME-NADP in maize leaf sections.

A: The first leaf

B: The second leaf

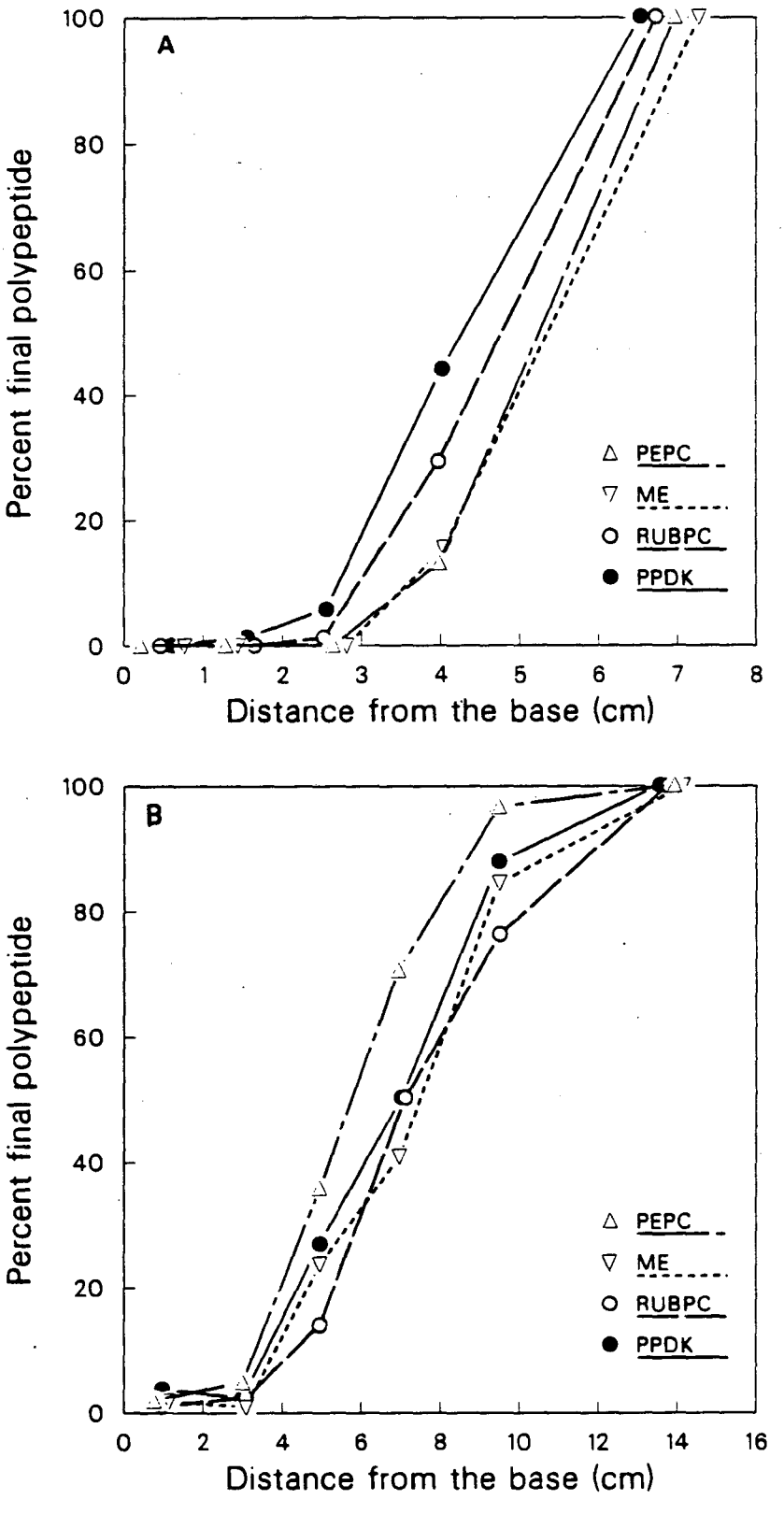
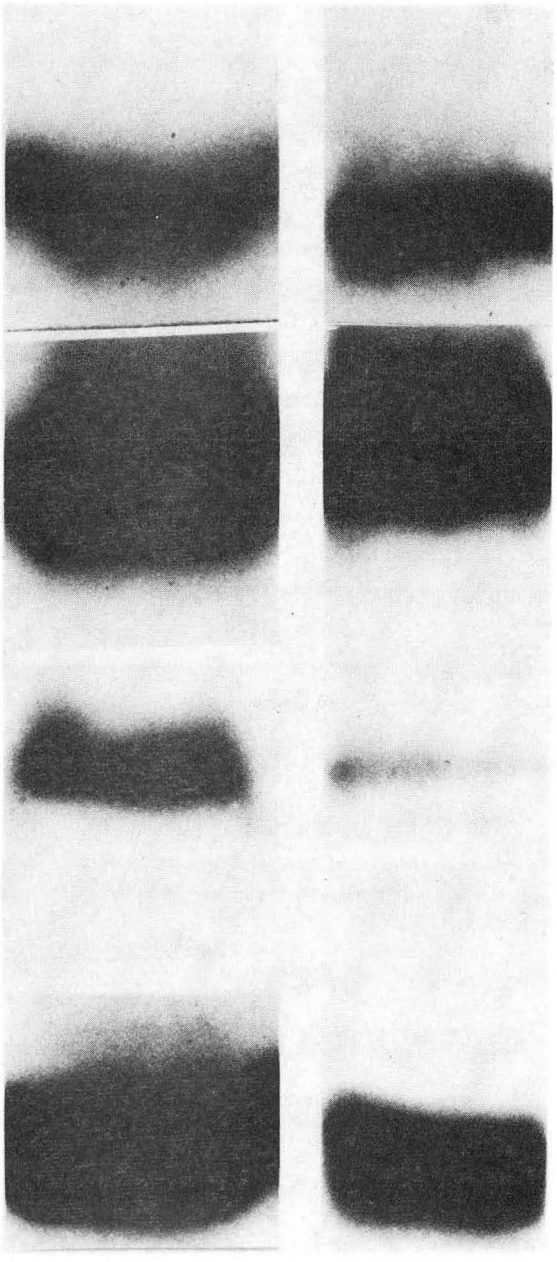

A
Percent final polypeptide
Distance from the base (cm)
PEPC
ME
RUBPC
PPDK
B
Percent final polypeptide
Distance from the base (cm)
PEPC
ME
RUBPC
PPDK
XCG 853-83

Fig. 7

Fig. 8 Change in the level of ME-NADP with cell differentiation in the first leaf.

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.



XBB 853-2346A

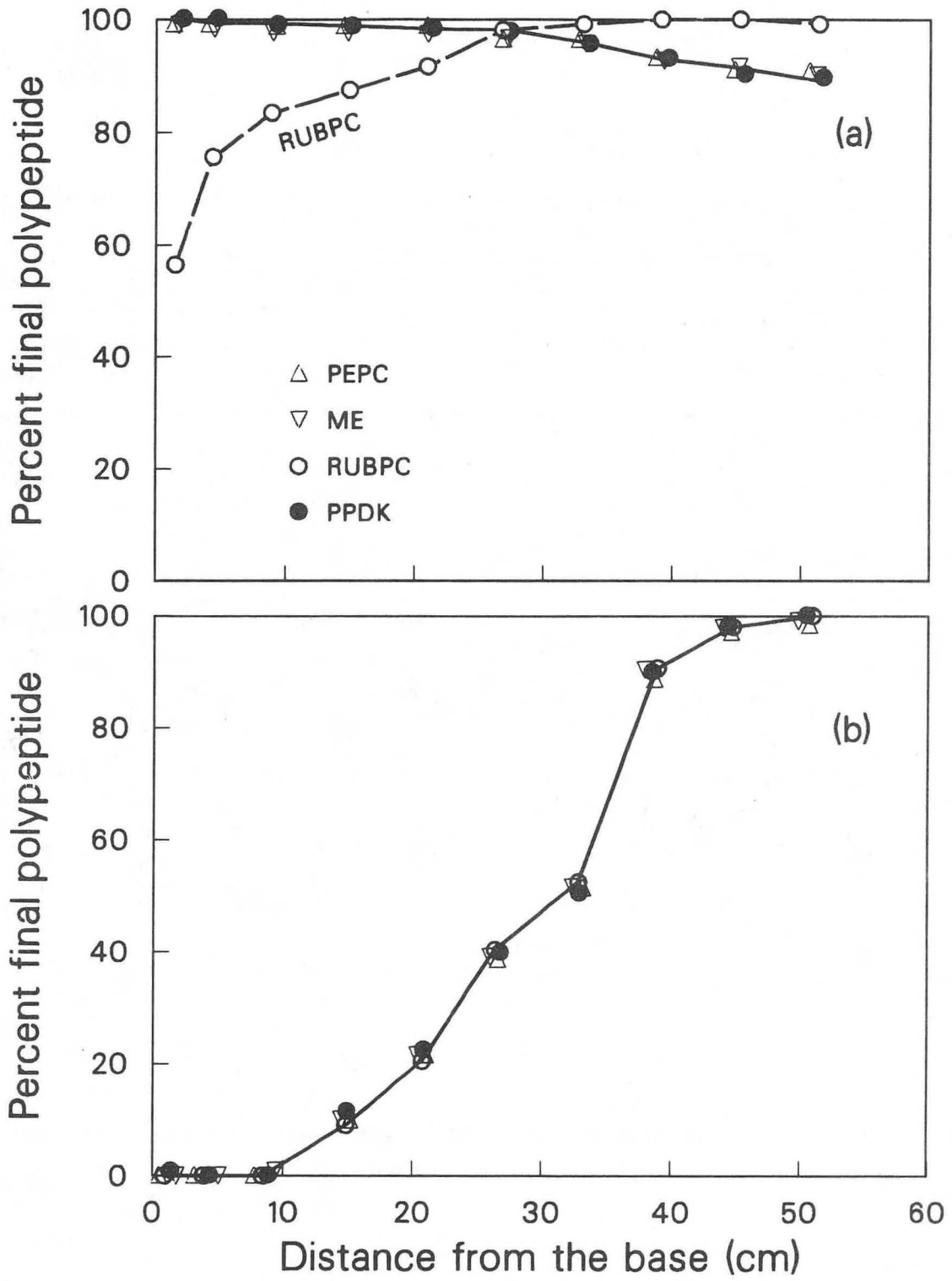
1 2

Fig. 8

Fig. 9 Appearance and accumulation of PPDK, PEPC, RuBPC, and ME-NADP in mature leaves.

a: An equal amount (50μg) of soluble protein was loaded into each lane.

b: Extract 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.



XCG 853-85

Fig. 9

Fig. 10 Non-chlorophyllous 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) (x 50). The callus has loose aggregates of irregular shapes.

c: Root-forming callus.

d: Root-like structure. Pl, plerome; Pe, periblem; Ca, calyptrogen (x100).

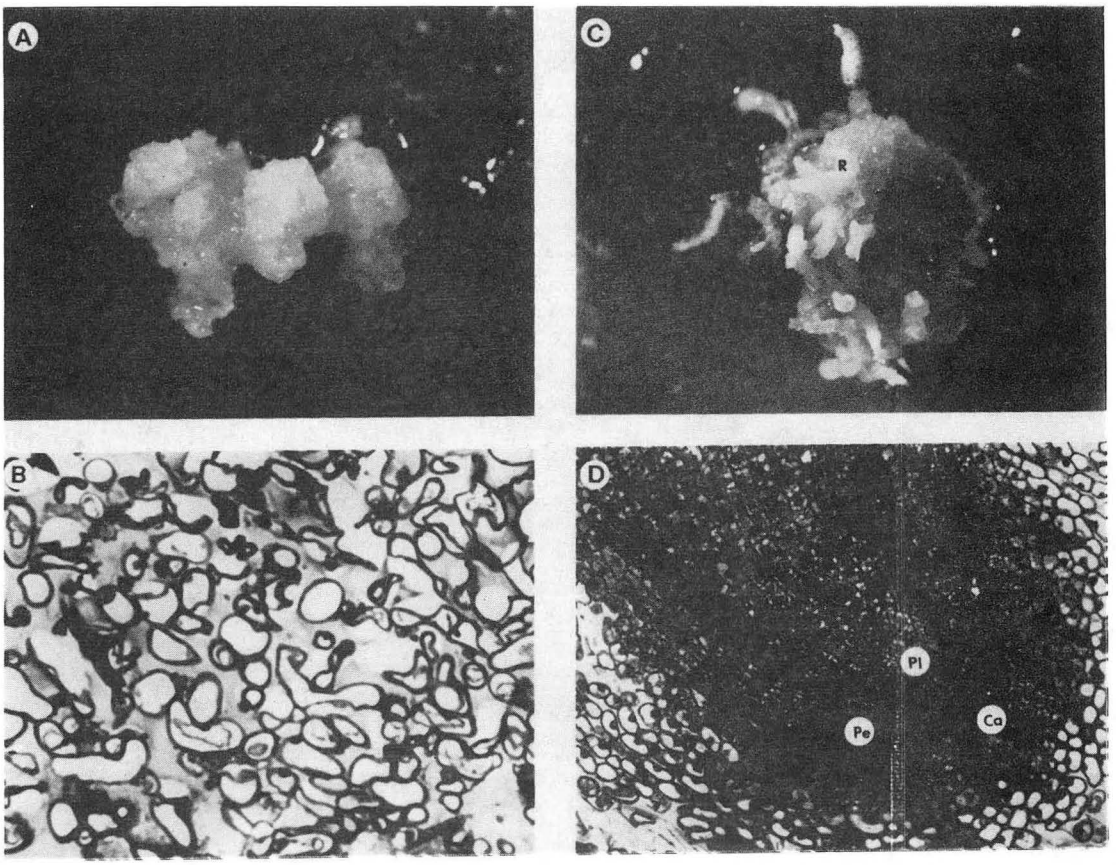


Fig. 10

XBB 847-4697A

Fig. 11 Chlorophyllous callus.

a: Cross section of the callus showing cells containing chloroplasts and occasional areas of tracheoid (x 50).

b: Enlarged view of the cell seen in (a) (x 300).

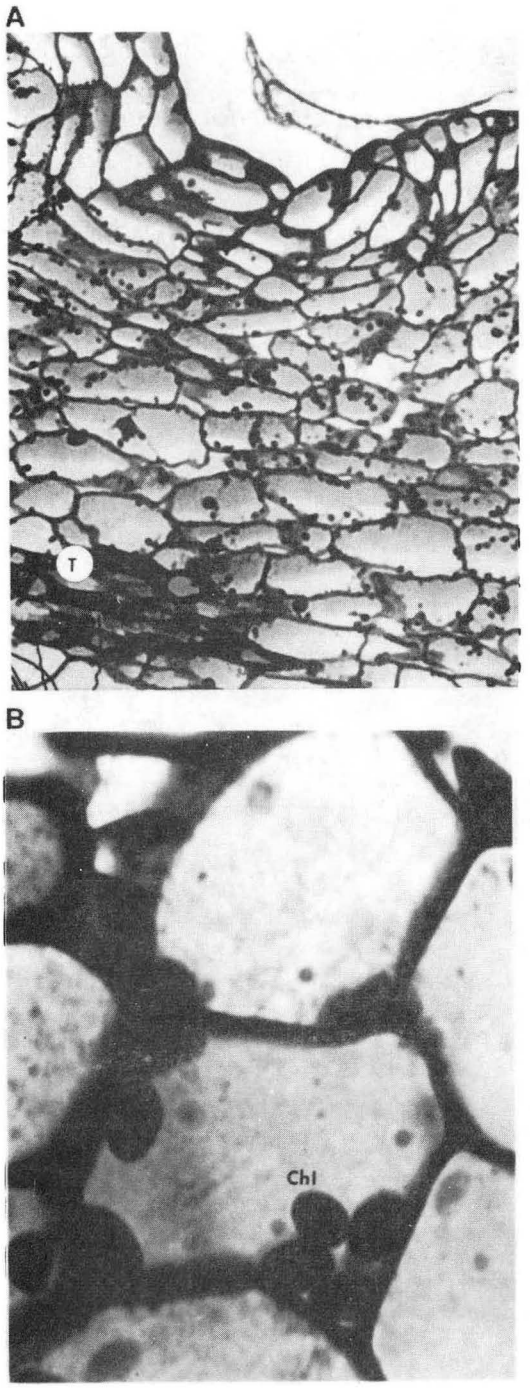


Fig. 11 XBB 847-4975A

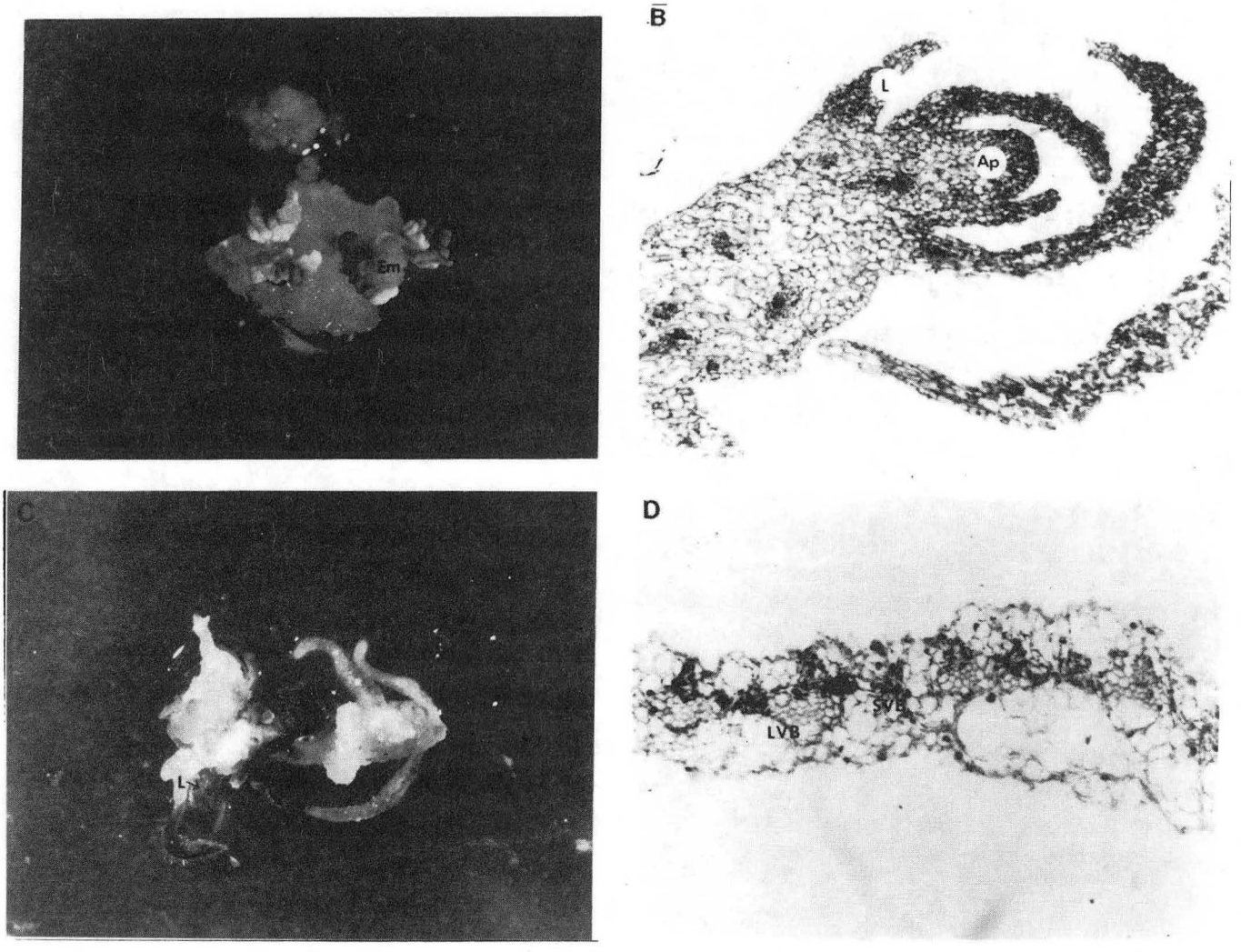
Fig. 12 Young regenerated shoot.

a: Embryoid (Em)-forming callus.

b: Transverse section of the apical meristem (Ap) and leaf initials (L) of the 5 mm regenerated shoot (x 40).

c: Regeneration of shoots from the embyoids. As many as 7-8 shoots may form in 1 cm^2 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.



XBB 852-1357

Fig. 12

Fig. 13 Older regenerated shoot.

a: Regenerated shoots with four leaf blades.

b: Transsection of a regenerated leaf(12 cm in length) (x80). "Xy-Ms" condition is present as indicated by the arrow. X, metaxylem.

c: Transsection of a regenerated shoot 7 cm in length. Occasionally, the arrangement of bundle sheath cells (BSC) is irregular and the chloroplasts are not centrifugally arranged as they were in the control leaf taken from the seedlings.

d: Transsection of a control leaf (x 80). Large chloroplasts are centrifugally arranged in the bundle sheath cells (BSC) as indicated by the arrow. MC, mesophyll cells.

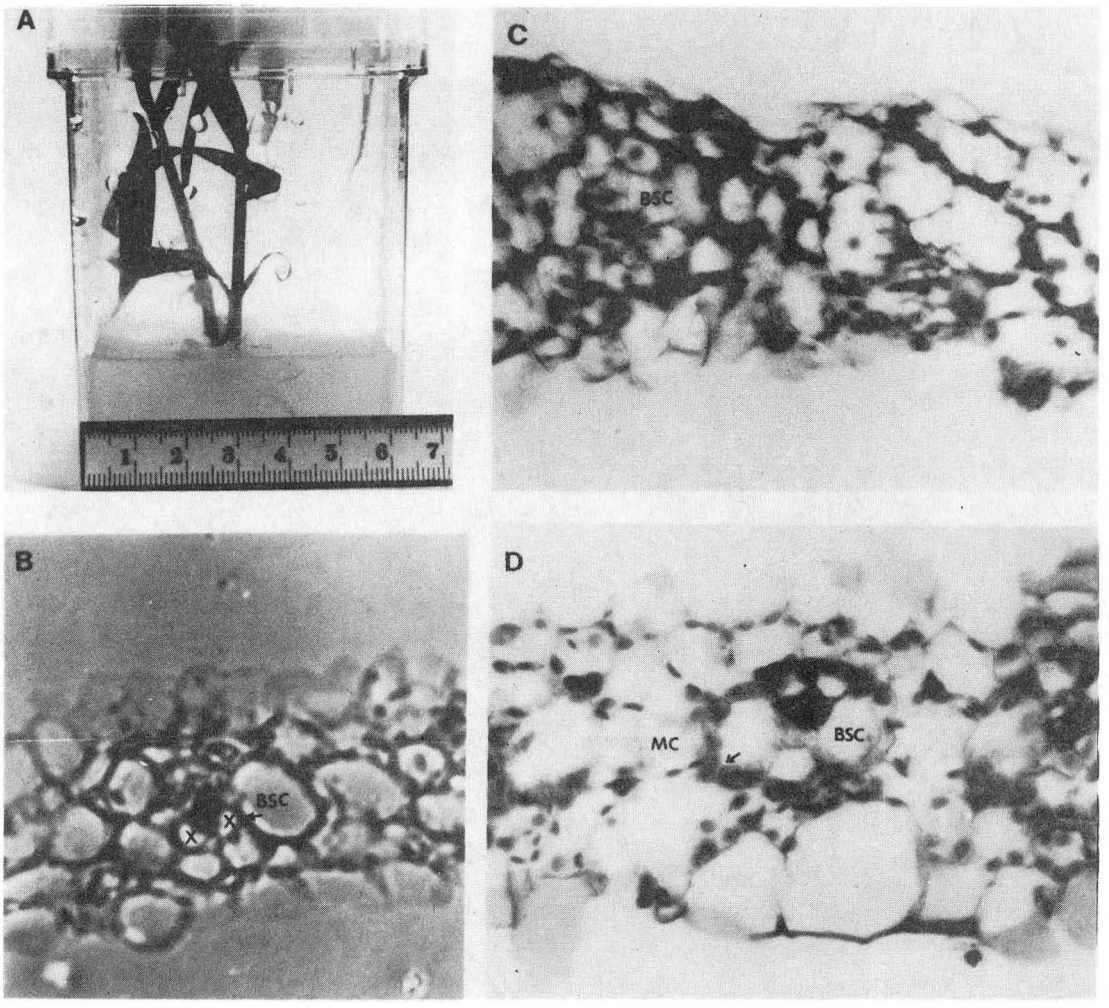


Fig. 13

XBB 852-1358A

CONCLUSION

PPDK is present not only in C_4 mesophyll cells, but also in bundle sheath cells and other photosynthetic and non-photosynthetic organs of both C_3 and C_4 plants. There seem to be at least two isoenzymes, one which is present in green leaves and the other which is present in non-photosynthetic tissues, such as seed and root. *In vivo*, these two forms are identical in mol wt (94kD) by SDS-PAGE analysis. However the leaf type is translated as a precursor with a transit sequence, while the other is translated into mature size polypeptide. The processing of the precursor takes place upon uptake by the chloroplast in the light. The light dependence might be replaced by ATP if it is a simple energy-dependent process, but I could not demonstrate it in this study.

Enzyme activity is regulated by light in two ways. Long term regulation is due to *de novo* synthesis of enzyme protein which may change in days (Hatch et al 1969). Short term regulation is due to phosphorylation of the enzyme which may occur in minutes (Hatch 1978, Ashton et al 1984). Since the PPDK reaction requires high energy for regeneration of ATP used in the reaction, this strict regulation mechanism is necessary to prevent a futile cycle under unfavorable environmental conditions. Other C_4 enzyme activities, such as NADP-ME and PEPC are light regulated, through increased protein synthesis (Collins and Hague 1983, Hague and Sims 1980), however only PPDK is regulated on a time scale of minutes. When PPDK is inactivated, however, all the subsequent reactions of the C4 transport pathway are stalled so

that the other enzymes need not be as strictly regulated as PPDK.

Although PPDK has its unique role in C_4 mesophyll cells, it has other roles in non-photosynthetic tissues such as seeds. The isoenzyme found in seeds and callus has a lower Km for pyruvate than the leaf type, lower V_{max}, and does not get inactivated by darkness. This form of isozyme may be similar to the PPDK found in bacteria (Evans and Wood 1977) and may have preceeded the photosynthetic type in evolution. Further studies employing molecular biology techniques are necessary to determine if these isoenzymes are the product of a single gene or multiple genes. Are PPDK genes in C_3 and C_4 plants the same? There are many questions remained to be answered. The in vitro transcription system can be used to answer some of the questions.

Due to the frame of this research, I focused on PPDK. However regulation of C_4 PEPC gene could have been very interesting to study along with PPDK. Multiple forms of PEPC are known (Ting and Osmond 1973), and C_4 type PEPC is unique to green plants possessing C_4 pathway. Is C_4 PEPC gene present in C_3 plants, but not detectable? If so, what regulates the gene expression?

Another interesting aspect is the mutual exclusiveness of expression of PPDK and RuBPC-ss. In C_4 bundle sheath cells, PPDK is present only at about 10% (μg PPDK/mg soluble protein) of the level found in mesophyll cells. RuBPC on the other hand is detectable only in bundle sheath cells.

The study with maize sections of seedling has demonstrated clearly the importance of Kranz anatomy and cell differentiation as well as coordinated expression of C_3, C_4 enzymes.

It was observed in the light micrographs that the bundle sheath chloroplasts were arranged in a centrifugal pattern in an orderly manner. What causes the chloroplast to move toward the mesophyll cells? Is there some gradient to which bundle sheath chloroplasts are sensitive? There may be some factors which are partly responsible for the compartmentalization of enzymes in two cell types. The factors could be intermediates of metabolism, energy charge, CO_2 gradient, etc. This requirement for both biochemical and cytological competence for C_4 pathway makes future genetic enginering aimed at imparting "C_4" characteristics into C_3 plants by introducing a specific C_4 gene seem complicated. However genetic engineering plus breeding for C_3 plants with higher photosynthetic rate as seen in the primitive wheat seems feasible. If such C_3 plants are able to adapt to a wide range of environments, they might be more beneficial than present C_4 species which show limited photosynthesis under cool and light-limiting climatic conditions.

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