Photorespiratory Metabolism in C_3-C_4 Intermediate Species of *Alternanthera* and *Parthenium*

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

by

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NOVEMBER 1993

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Dedicated

To the loving memory of my sister
late **Siva**
Her sense of hope is my dearest legacy

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DECLARATION

The candidate declares that this work has been carried out by her under the supervision of Dr. A.S. Raghavendra, Associate Professor, School of Life Sciences. University of Hyderabad, Hyderabad - 500 134, India and that this thesis work has not been submitted for any degree or diploma of any other University or Institution.

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CERTIFICATE

This is to certify that the thesis entitled "Photorespiratory metabolism in C_3 -C_4 intermediate species of *Alternanthera* and *Parthenium*" is based on the results of the work done by Ms. M. Tirumala Devi for the degree of Doctor of Philosophy under my supervision. This work has not been submitted for any degree or diploma of any other University or Institution.

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ACKNOWLEDGEMENTS

It gives me immense pleasure to express my sincere gratitude to Dr. A.S. Raghavendra, Associate Professor, School of Life Sciences, for his kind guidance, valuable suggestions and constant encouragement throughout my research work.

I express my thanks to Prof. N.C. Subramanyam FNA, Dean, School of Life Sciences and Prof. A.R. Reddy, Head, Department of Plant Sciences for allowing me to use the research facilities available in the School. I also thank Prof. P.R.K. Reddy, former Dean of the School.

I am very much thankful to my brother (Mr. Rajagopalan) for his constant help and encouragement during the second half of my work.

I am also thankful to my colleagues, in particular to Dr. Vani, Mrs. Gayathri Swaminathan, Ms. Shyamala and Ms. Parvathi for their help during my work.

Special thanks are to all my friends and class mates who extended their help during the period of my work.

I am greatly indebted to my parents, whose unlimited efforts framed the backbone of my academic career, for their constant support and encouragement. I extend my thanks to my sister (Mrs. Raghava) and brother-in-law (Mr. Purnachandra Rao) for their wholehearted encouragement. Special thanks are also to my niece (Lavanya and Ragini) and nephew (Bobby) who made me to feel happy and easy at many times during my research period.

The financial assistance from UGC, DST and CSIR is gratefully acknowledged.

ABBREVIATIONS

Ala AT	L-alanine aminotransferase
Asp AT	L-aspartate aminotransferase
BHB	butyl-2-hydroxy-3-butynoate
CA	carbonic anhydrase
CCCP	carbonyl cyanide *m*-chlorophenylhydrazine
CE	carboxylation efficiency
Ches	2-(N-cyclohexylamino)ethanesulphonic acid
DCDP	3,3-dichloro-2-dihydroxyphosphinoyl-methyl-2-propenoate
DCPIP	2,6-dichlorophenolindophenol
DTNB	5-5'-dithiobis-2-nitrobenzoate
FAA	formalin-aceto-alcohol
FMN	flavin mononucleotide
GDC	glycine decarboxylase
GGAT	glutamate-glyoxylate aminotransferase
glycidate	2,3-epoxypropionate
GO	glycolate oxidase
GOGAT	glutamate:2-oxoglutarate aminotransferase (glutamate synthase)
G-6-P	glucose-6-phosphate
GS	glutamine synthetase
α-HPMS	α-hydroxy-2-pyridinemethanesulphonate
HPR	hydroxypyruvate reductase
INH	isonicotinyl hydrazide
K_I	dissociation constant of enzyme-inhibitor complex
LCP	light compensation point
MDH	malate dehydrogenase
ME	malic enzyme
MSO	L-methionine sulphoximine
OAA	oxalacetic acid
PEP	phospho*enol*pyruvate

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(Abbreviations continued)

PEPC	PEP carboxylase
PEP-CK	PEP-carboxykinase
PGA	3-phophoglycerate
PGAP	phosphoglycerate phosphatase
PGP	phosphoglycolate phosphatase
PMS	N-methylphenazonium methosulfate
PPDK	pyruvate, orthophosphate dikinase
PR	photorespiratory
QR	quantum requirement
QY	quantum yield
RuBP	ribulose-1,5-bisphosphate
RuBPC	RuBP carboxylase
RuBPO	RuBP oxygenase
SGAT	serine-glyoxylate aminotransferase
SHMT	serine hydroxymethyltransferase
TCA cycle	tricarboxylic acid cycle
	maximum velocity of an enzyme catalyzed reaction
Γ	C02 compensation point
$\delta^{13}C$	a measure of carbon isotope discrimination

All remaining abbreviations are according to those found in *Plant Physiology*, January 1993

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Chapter I

Chapter 1

GENERAL INTRODUCTION AND SCOPE OF THE PRESENT INVESTIGATION

Higher plants are grouped into three main categories based on the type of photosynthetic carbon metabolism, *viz.*, C3-, C4- and CAM plants (Zelitch 1975, Troughton 1975, Edwards and Walker 1983). C3 and C_4 plants are named after the initial stable product during primary carboxylatlon. Phosphoglyceric acid (PGA), a three carbon compound formed by Rubisco, is the initial product of photosynthesis in C3 plants (Edwards and Walker 1983, Buchanan 1992). C_4 acids (oxalacetate, malate or aspartate) are the primary products, due to the predominance of phosphoenolpyruvate carboxylase (PEPC) in C4 and CAM plants (Edwards and Walker 1983, Edwards *et al.* 1985, Winter 1985, Hatch 1987, 1992a, b, Griffith 1988, Buchanan 1992). The CO2 released from decarboxylation of C4 acids is later refixed through Calvin cycle, thus Rubisco forming the step of secondary carboxylation. The primary and secondary carboxylation reactions are spatially separated in C4 species whereas they are temporally separated in CAM plants (Edwards and Walker 1983, Hatch 1987).

C4 plants have a unique syndrome of several anatomical, physiological and biochemical characteristics. Some of them are: specialized Kranz leaf anatomy, short lntervelnal distances and high frequency of plasmodesmata, saturation of photosynthesis at

atmospheric levels of CO_2, low or non-detectable photorespiratory C02 efflux, low sensitivity of photosynthesis to O_2, high water- and nitrogen-use efficiency compared to C3 species, relatively low discrimination of ^{13}C against ^{12}C, primary assimilation of carbon into C4 dicarboxylic acids, high complement of C4 cycle enzymes, differential compartmentation of photosynthetic enzymes of C4 cycle in mesophyll and Calvin cycle in bundle sheath cells, low levels of photorespiratory enzymes and their exclusive localization in bundle sheath cells (Recent reviews: Edwards and Huber 1981, Osmond and Holtum 1981, Edwards and Walker 1983, Hatch 1987, 1992a, Furbank and Foyer 1988, Edwards and Ku 1990, Leegood and Osmond 1990, Nelson and Langdale 1992, Peisker and Henderson 1992, Dai *et al.* 1993, Iglesias *et al.* 1993, Raghavendra and Das 1993).

Several plant species with characteristics intermediate to those of C3 and C4 plants are identified in recent years (Reviews: Raghavendra 1980, Holaday and Chollet 1984, Monson *et al.* 1984, Edwards and Ku 1987, Monson 1989a, Monson and Moore 1989, Rawsthorne 1992, Raghavendra and Das 1993, Raghavendra and Devi 1993). The first identified naturally occurring C3-C4 intermediate was *Mollugo verticillata* (carpet weed) by Kennedy and Laetsch in 1974. Since then at least 24 species of 8 genera from six families were recognised as naturally occurring C3-C4 intermediate species (Rawsthorne 1992). Most of these intermediates belong to genera that also contain both C3 and C4 species (Table 1.1). However, in the genera *Parthenium* (Asteraceae) and *Moricandia* (Brassicaceae),

TABLE 1.1. Taxonomic distribution of C3-C4 intermediate species

Family/Genus	Species		
	C3 type	C3-C4 intermediate	C4 type
MONOCOTYLEDONEAE			
Cyperaceae			
Eleocharis	*E. acutangula* *E. atroparpurea* *E. jenlculata*	*E. pusilla*	*E. caespitosissima* *E. retroflexa*
Poaceae			
Neurachne	*N. alopecuroidea* *N. lanigera* *N. queenslandica* *N. tenuifolia*	*N. minor*	*N. munroi*
Panicum	*P. bisulcatum* *P. hylaeicum* *P. laxum* *P. rivulare*	*P. decipiens* *P. millioides* *P. schenckii*	*P. coloratum* *P. maximum* *P. miliaceum* *P. prionitis*
DICOTYLEDONEAE			
Aizoaceae			
Mollugo	*M. lotoides* *M. pentaphylla*	*M. nudicaulis* *M. verticillata*	*M. cerviana*
Amaranthaceae			
Alternanthera	*A. sessiles*	*A. ficoides* *A. tenella*	*A. caracasana* *A. pungens*
Asteraceae			
Flaveria	*F. cronquistii* *F. pringlei* *F. robusta*	*F. angustifolia* *F. anomala* *F. chloraefolia* *F. floridana* *F. linearis* *F. oppositifolia* *F. pubescens* *F. ramosissima* *F. sonorensis*	*F. australasica* *F. bidentis* *F. trinervia*

(Continued on next page)

(Table 1.1 continued)

Family/genus	C3 type	C3-C4 intermediate	C4 type
		F. brownii *	
		F. palmeri	
		F. vaginata	
Parthenium	*P. argentatum*	*P. hysterophorus*	
	P. incanum		
Brassicaceae			
Moricandia	*M. foetida*	*M. arvensis*	
	M. foleyi	*M. nitens*	
	M. moricandioides	*M. sinaica*	
		M. spinosa	
		M. suffruticosa	

The species were complied from the references included in the text. The important references include: Brown and Brown (1975), Keck and Ogren (1976), Sayre and Kennedy (1977), Kennedy *et al.* (1980), Hegde and Patil (1981), Rajendrudu and Das 1981, Pathan and Nimbalkar (1982), Ku *et al.* (1983, 1991), Patil and Hegde (1983), Rumpho et *al.* (1984), Hattersley *et al.* (1986), Hattersley and Stone (1986), Monson *et al.* (1986), Rajendrudu *et al.* (1986), Bruhl *et al.* (1987), Moore *et al.* (1987a), Cheng et *al.* (1988), Hylton *et al.* (1988) and Brown and Hattersley (1989).

* C4-like C3-C4 intermediates.

only C3 plants and C3-C4 intermediates are known to exist but not C4 species (Edwards and Ku 1987, Monson 1989a). Like their C4 counterparts, intermediates are also found in most advanced orders of angiosperms, such as Asterales (*Flaveria*, *Parthenium*), Caryophyllales (*Alternanthera*, *Mollugo*), Capparales (*Moricandia*) and Poales (*Neurachne*, *Panicum*). In the genus *Flaveria,* of the 21 species, ten species are C3-C4 intermediates. The reasons for such high frequency of intermediacy in this genus are not known (Edwards and Ku 1987, Monson 1989a). This genus also contains the most advanced C3-C4 intermediates which are almost C4-like (for e.g. *Flaveria brownii,* Cheng *et al*. 1989, Edwards and Ku 1987, Ku *et al*. 1991).

The important features of C3-C4 intermediates are summarized in Table 1.2. Some of the most important ones are: (i) partial or imperfect 'Kranz' anatomy, (ii) intermediate levels of CO_2 compensation points (Γ) indicating reduced photorespiration, (iii) biphasic response of Γ to O_2, (iv) sharp decrease in Γ at low light intensities, (v) sensitivity of photosynthesis to O_2 less than that in C3 species (Monson *et al*. 1984, Edwards and Ku 1987, Monson 1989a, Monson and Moore 1989, Schuster and Monson 1990, Rawsthorne 1992). The characteristics of C3-C4 intermediates are described further in the following pages with reference to those of C3 or C4 species.

Table 1.2. Important characteristics of C3-C4 intermediates.

1. Habitat

   (a) Mostly distributed in semi-arid, hot and dry environments.

2. Anatomy

   (a) A near complete or partial Kranz leaf anatomy.
   (b) Mesophyll is less impact and not totally radiate.
   (c) A number of organelles (chloroplasts, mitochondria and peroxisomes) in the bundle sheath cells.
   (d) Bundle sheath chloroplasts are arranged in a centripetal manner.
   (e) Increased plasmodesmatal frequency and shorter interveinal distances compared to C3 species.

3. Physiology

   (a) Higher photosynthetic rates than C3 species under warm leaf temperatures.
   (b) Reduced sensitivity of photosynthesis to 02.
   (c) Intermediate levels of CO2 compensation points.
   (d) Decrease in Γ under low light intensities.
   (e) Diphasic response of Γ to increase in $[O_2]$.
   (f) Mostly C3-like $\delta^{13}C$ values.
   (g) Increased water and nitrogen use efficiency.

4. Biochemistry

   (a) C3 cycle - the major route of CO2 assimilation.
   (b) Variability in C_4 cycle operation.
   (c) 2-5 fold increase in C_4-enzymes over C3 species.
   (d) Partial reduction in the activity-levels of photorespiratory enzymes.
   (e) Incomplete compartmentation of photosynthetic and photoresplratory metabolisms.
   (f) Distinct localization of glycine decarboxylase and serlne hydroxymethyltransferase in bundle sheath cells.
   (g) Decreased levels of photorespiratory metabolites compared to C3 species.
   (h) Efficient refixation of photorespired CO_2.

Leaf anatomy

Leaves of C4 species are characterized by typical Kranz anatomy (Laetsch 1974, Edwards and Walker 1983, Hatch 1987) with two distinct layers of cells each containing numerous chloroplasts: the inner thick-walled bundle sheath cells around the vascular tissue and the outer radially arranged mesophyll cells. The leaves of C3 plants lack this type of leaf anatomy and their bundle sheath cells, even if present, contain very few or no organelles. The number of plasmodesmata on bundle sheath cells of C4 plants is several times greater than that on normal cells in C3 leaves (Hatch and Osmond 1976, Edwards and Walker 1983; a recent ref. Hatch 1992b).

All the C3-C4 intermediates have Kranz-like leaf anatomy though not as typical as in C4 (Monson *et al.* 1984, Edwards and Ku 1987, Brown and Hattersley 1989) plants. A marked variation occurs among the C3-C4 species in the degree of Kranz-cell development. Their leaf anatomy can be categorized into three types: Kranz, Kranz-like and poorly developed Kranz (Holaday and Chollet 1984, Edwards and Ku 1987). Examples for such gradation in leaf anatomy are: (i) typical Kranz in *Flaveria brownii* (Brown and Hattersley 1989) and *Neurachne minor* (Hattersley *et al.* 1986, Brown and Hattersley 1989), (11) Kranz-like in *F. floridana* and *F. chloraefolia* (Holaday *et al.* 1984) and (iii) poorly developed Kranz in *F. pubescens* and in *M. verticillata* (Holaday and Chollet 1984).

The bundle sheath cells of *Neurachne minor* are suberized as in C4 plants (Hattersley *et al.* 1986). The bundle sheath cells of intermediates are enriched with not only chloroplasts but also mitochondria. In C_3-C_4 intermediates the arrangement of chloroplasts in bundle sheath cells is usually centripetal, a feature characteristic of NAD-ME type C_4 species (Edwards and Ku 1987). In *Panicum* intermediates some mitochondria in bundle sheath cells are completely enclosed by chloroplasts (Brown *et al.* 1983b).. There are a large number of plasmodesmata on bundle sheath cells in *Panicum* intermediates, similar to that of C_4 *Panicum* species (Brown et *al.* 1983a).

CO_2 compensation point - a measure of photorespiration

CO_2 compensation point (Γ - CO_2 concentration where net photosynthesis becomes zero) is regarded as a measure of photorespiration (Andrews and Lorimer 1987, Sharkey 1988). Photorespiration is a process of light dependent O_2 uptake/CO_2 evolution and plays an important role in carbon/nitrogen metabolism of the leaf (Reviews: Zelitch 1982, Ogren 1984, Artus et *al.* 1986, Husic *et al* 1987, Givan et *al* 1988, Sharkey 1988, Canvin 1990, Lea *et al.* 1990, Lea and Blackwell 1990, 1992). Photorespiratory process is closely linked to Calvin cycle and involves the coordination of three organelles; viz., chloroplast, peroxisome and mitochondrion (e.g. Lea and Blackwell 1992).

The C02 compensation points are quite high (30-70 $\mu l\ l^{-1}$) in C3 plants but are close to zero (0-5 $\mu l\ l^{-}$) in C4 species (e.g. Krenzer *et al.* 1975), while the values of C3-C4 intermediates fall in between those of C3 and C4 plants (Holaday and Chollet 1984, Edwards and Ku 1987, Monson and Moore 1989). The decreased level of Γ (5-35 $\mu l\ l^{-1}$) in C3-C4 intermediates reflects the reduction in magnitude of photoresplration (Holaday and Chollet 1984, Edwards and Ku 1987, Canvin 1990).

In C4 plants the 'Kranz' (leaf) anatomy facilitates the differential localization of primary carboxylation reactions in mesophyll cells and Calvin cycle enzymes in bundle sheath (Reed and Chollet 1985, Hatch 1987, Sheen and Bogorad 1987, Schaffner and Sheen 1992). The decarboxylation of C4 acids in the bundle sheath leads to the concentration of C02 (nearly 7-10 fold higher than the atmospheric levels) and results in the suppression of oxygenase function of Rubisco, and thereby photorespiration (Hatch and Osmond 1976, Edwards and Huber 1981, Hatch 1987). Because of this, all C4 species exhibit low Γ values and photosynthesis is not affected by O_2 concentrations.

Photosynthetic features

Photosynthetic characteristics were studied in detail only in a few C3-C4 intermediates, namely *Flaveria* (Bauwe et *al.* 1987,

Apel *et al.* 1988, Wessinger *et al.* 1989, Ku *et al.* 1991), *Panicum* (Brown and Brown 1975, Morgan *et al.* 1980, Edwards *et al.* 1982, Holaday *et al.* 1982, Hattersley *et al.* 1986), *Moricandia arvensis* (Holaday *et al.* 1982, Bauwe 1984, Toriyama *et al.* 1988) and *Parthenium hysterophorus* (Moore *et al.* 1987a). The differences observed in the photosynthetic/photorespiratory CO_2 exchange characteristics between C3 and C3-C4 intermediates are pronounced at low $[CO_2]$ but show little or no difference at ambient or high $[CO_2]$ (Edwards and Ku 1987). The rates of apparent photosynthesis by C3-C4 intermediates at atmospheric CO_2 are similar to those of C3 plants (Holaday and Chollet 1984). These characteristics are discussed further in the following pages.

The studies of Krall and Edwards (1990, 1993) indicated a tight linkage between PS II activity and CO_2 fixation in C4 plants under variable $[CO_2]$, which is in contrast with C3 plants, where ratio of Q_{YPSII}/Q_{YCO2} dramatically increases with decrease in CO_2 (because of elevated photorespiration). However, no information is available on the relation between PS II activity and CO_2 fixation in C3-C4 intermediates.

Photosynthesis and photorespiration in higher plants are regulated by several external and internal factors (Edwards and Walker 1983). Light, temperature, CO_2 and O_2 are some of the important external factors. Among the major internal factors are metabolite pools and enzyme activities. The pattern of regulation

of photosynthesis/photorespiration by the external factors in C3 and C4 plants is discussed below in relation to C3-C4 species.

Light

Light plays a key role in regulating the photosynthetlc carbon assimilation (Zelltch 1971, Björkman 1981, Berry and Downton 1982, Edwards and Walker 1983, Evans 1987, Walker 1992). The effect of light on photosynthesis depends on the mode of CO2 fixation in higher plants (Björkman 1972, Buchanan 1992). In C3 plants photosynthetic rates increase with light intensity and saturate at about 400 μmol m s (about 1/5 of full sun light), whereas C4 photosynthesis is not saturated at even full sun light (Edwards and Walker 1983, Hatch 1992a).

The light compensation point (where net CO2 assimilation is zero) of C4 plants is normally higher than those of C3 plants (Walker 1992). However LCP depends on also the light intensity during plant growth. Low light grown plants show low LCP (about 20% of that in high light grown plants). The alteration of LCP is believed to be due to variation in the rates of dark- and photo-respiration (Heath and Meidner 1967). The dark respiration varies with previous intensity and duration of illumination (Bjorkman *et al.* 1972, Sharp *et al.* 1984).

Quantum yields (QY) are defined as the amount of oxygen evolved or C02 assimilated (in the absence of photorespiratlon) per quanta of light absorbed (Edwards and Walker 1983, Evans 1987). QY is taken as a measure of photosynthesis in higher plants (Björkman 1981, Osmond 1981, Evans 1987). Since the operation of C4 pathway requires additional amount of energy (ATP and NADPH), the QY in C4 plants are reduced, compared to C3 plants (Osmond et *al.* 1981, Bassham and Buchanan 1982, Berry and Downton 1982, Hatch 1987). Similar QYs are reported for representative C3 and C4 species of *Flaveria* under atmospheric CO_2 concentrations (Monson et *al.* 1986, Monson 1987). The low QYs in some of the C3-C4 intermediates compared to C3 and C4 species is attributed to the possible operation of a futile C4 cycle due to the absence of distinct compartmentation of Rubisco (Monson et *al.* 1986, Monson 1987).

Photosynthesis is inhibited in leaves of C3 plants when illuminated at high light intensities particularly under limiting carbon dioxide. This phenomenon of decrease in carbon assimilation at supra-optimal light is called as photoinhibition (Osmond 1981, Osmond et *al.* 1981, Berry and Downton 1982, Leegood et *al.* 1988, Barber and Anderson 1992). Photoinhibltlon is due to the over excitation of photosystems, under sub-optimal conditions of carbon metabolism and results in the damage to the photosynthetic machinery. The C4 plants have the ability of CO_2 concentration within bundle sheath cells due to the unique C4-photosynthesis (Hatch 1987, 1992a). Not surprisingly, 10 ppm CO_2 in 21% 02 is

effective in preventing almost completely the photoinhibition in C4 plants. In illuminated leaves of C3 plants, CO2 at concentrations much higher than the atmospheric levels are necessary to avoid photoinhibition (Osmond 1981). There is no information regarding the photoinhibition in C3-C4 intermediates.

Like photosynthesis, photorespiration is also modulated by light intensity in C3 plants (Sharkey 1988, Canvin 1990). Photorespiration increases at high photosynthetic active radiation (PAR) levels in several C3 species (Heath 1969, Whiteman and Koller 1967, Zelitch 1971). In *Panicum schenckii* (C3-C4 intermediate species), photorespiration was reduced by 1.0 mg dm h by lowering the PAR to 100 $\mu E\ m^{-2}\ h^{-1}$ (Brown and Morgan 1980, Byrd and Brown 1989).

Similarly, Γ (a measure of photorespiration) changes with light intensity (up to a threshold intensity) in C3 plants, while staying unaffected in C4 plants (Ku et *al.* 1991). Γ decreases sharply at low intensities but remains constant at high light intensities in C3 species (Monson *et al.* 1984, Ku *et al.* 1991). At low photon flux densities (PFD), Γ in the C3-C4 intermediates of *Alternanthera*, *Flaveria*, *Moricandia* and *Panicum* decreases to the values of C3 plants (Brown and Morgan 1980, Holaday and Chollet 1984, Rajendrudu *et al.* 1986, Ku *et al.* 1991). The decreased Γ at high light intensities is an indication of refixation of photorespired C02 through photosynthetic process which responds to light intensity (Monson *et al.* 1984, Ku et *al.* 1991).

Temperature

Both photosynthesis and dark respiration increase along with temperature between 0 and 35 C, but the extent of increase in dark respiration is less compared to that of photosynthesis (Zelitch 1971). The pattern of photosynthetic response to temperature is different in C3 and C4 species (Bjorkman 1981). The optimum temperature spans between 15 and 25 ^{o}C for C3 species, 25-30 °C for C3-C4 intermediates and 30-40 °C for C4 plants. In parallel, the quantum yield of C4 plants remain constant within a temperature range of 10 to 40 C, whereas in C3 plants, quantum yield increases as the temperature is raised from 10 to 25 °C but decreases above 25 °C (Berry and Downton 1982, Oberhuber and Edwards 1993). Thus, C4 species are superior to C3 species under normal environmental conditions (Ehleringer and Bjorkman 1977, Berry and Downton 1982, Ehleringer and Pearcy 1983). This may be due to the internal CO_2 concentrating mechanism in C4 species. At high CO2 levels (suppressing photorespiration in C3 species), the response of C3 plants to varying temperature is similar to C4 species (Berry and Bjorkman 1980).

Photorespiration plays a dominant role in temperature regulation of photosynthesis, particularly in C3 plants (Osmond et al. 1981). Photorespiratory rates are low at low temperatures (<20 ^{o}C), but increase when temperature exceeds 25 °C (Sharkey 1988)

Related phenomena like C02 compensation point, oxygen inhibition of photosynthesis and post illumination burst also increase with temperature in C3 plants resulting in a decrease in net photosynthesis. Some of these effects can be partly attributed to changes in the solubilities of C02 and 02 (Ku and Edwards 1977, Berry and Björkman 1980, Edwards and Walker 1983).

The increase in photorespiration with temperature is less in C3-C4 intermediates than that in C3 species. Increase in temperature between 20-35 C elevated photorespiration from 2.5 to 3.3 mg dm h (32% increase) in *Panicum schenckii*, a C3-C4 intermediate species, while elevating from 7.3 to 10.2 mg dm h (40% increase) in tall fescue *{Festuca arundinacea)*, a C3 species (Brown and Morgan 1980). Accordingly, increased temperature between 20 and 35 °C had little effect on Γ in C3-C4 intermediates of *Panicum {P. millioides* and *P. schenckii)* whereas Γ in C3 species *{Festuca arundinacea)* was very sensitive to temperature changes.

The response of C3-C4 intermediates to temperature appears to depend on the extent of C4 pathway operation. Γ in C4-like C3-C4 intermediate species of *Flaveria brownii* showed very little response to changes in temperatures which was similar to that in C4 species (Ku *et al.* 1991). But Γ in some of the intermediates (for e.g. *F. floridana)* showed similar response to that of C3 species (Ku *et al.* 1991).

The reduced responses of photorespiratlon and Γ to temperature in C3-C4 intermediates appears to be due to the CO2 concentration/recycling due to the operation of a partial or nearly complete C4 pathway (Brown and Morgan 1980). Thus the reduced photorespiration bestows an advantage on C3-C4 species for a better performance at high temperature than that of C3 plants (Henning and Brown 1986, Monson 1989b, Monson and Jaeger 1991). The studies of Schuster and Monson (1990) have indeed shown that the C3-C4 species possess an advantage of photosynthesis at warm leaf temperature over C3 plants.

Carbon dioxide

CO_2 is a major limiting factor for photosynthetic carbon reduction in C3 plants, since the atmospheric (350 ppm) CO2 concentration is not sufficient to saturate their primary carboxylation by Rubisco (Leegood *et al.* 1985, Woodrow and Berry 1988, Jenson 1990, Bowes 1991, Long 1991). However, photosynthesis gets saturated at atmospheric concentrations of CO_2 in C4 plants because of the low $K_{m(CO2)}$ of PEP carboxylase (responsible for primary carboxylation) in these species (Hatch and Osmond 1976, Edwards and Walker 1983). For e.g. Sorghum, a NADP-ME type C4 species, has higher CE than wheat, a C3 plant, because of the CO2 concentrating ability of C4 photosynthesis (Edwards and Walker 1983, Monson *et al.* 1984). In fact, photosynthesis in wheat saturates at a CO2 concentration less than the expected level due

to the photochemical/substrate (RuBP) limitation on photosynthesis (Monson *et al.* 1984). Carboxylation efficiency and r in leaves of *Flaveria floridana* (a C3-C4 intermediate) under different CO2 concentrations were in between those of *F. pringlei* (C3) and *F. trinervia* (C4) (Byrd and Brown 1989).

Since photosynthesis in C3 plants is limited by CO2 concentration, increasing the COz concentration results in an increase in photosynthetic rates (until photosynthesis becomes saturated i.e. about 900 ppm [COz]) (Bowes 1991, 1993, Long 1991). Such increase in net photosynthetic rates by elevated COz appear to be due to two main reasons: (i) increased availability of the substrate, *i.e.* CO2 for Rublsco, and (11) decrease in oxygenation of RuBP since CO2 competes with O2 (Andrews and Lorimer 1987, Salvucci 1989, Portis 1992). Consequently the energy requirement for the operation of Calvin cycle is lowered at higher CO2 concentrations in C3 plants (Canvin 1990). High CO2 does not alter photosynthetic efficiency or QYs of C4 plants, as carbon assimilation in these plants is already saturated at atmospheric CO2 levels (Berry and Downton 1982).

Both dark respiration (DR) and light compensation points (LCP) decrease at high CO2 concentrations in both C3 and C4 plants (Heath and Meidner 1967, Downton *et al.* 1980, Berry and Downton 1982). Light compensation point is high in C4 plants because of their high light requirement. Yet, dark respiration and light

compensation point decrease with increased carbon dioxide concentration in C4 plants.

Oxygen

In C3 plants there is 30-35"/. inhibition of photosynthesis by 21% 02 (Ku and Edwards 1977, Edwards and Walker 1983, Edwards and Ku 1987, Sharkey 1988) and no such inhibition was observed in C4 plants (Rathnam 1978, Ogren and Chollet 1982, Canvin 1990). In C3-C4 intermediates, photosynthesis is inhibited by 21V. O_2 but the extent of inhibition is relatively small (20-25%) (Monson *et al.* 1984, Rawsthorne 1992).

The oxygen sensitivity of photosynthesis in C3 plants depends on the intercellular concentration of CO_2 (Edwards and Ku 1987). In *Flaverla brownii*, a C4-llke species, the 02 inhibition of photosynthesis is 8-12%, where as in *Neurachne minor* (C3-C4 intermediate) it is about 31% (Edwards and Ku 1987). In *Panicum prionitis* and *P. maximum* (C4 species) apparent photosynthesis is insensitive to oxygen, while in *P. hylaeicum*, *P. rivulare*, *P. laxum* and tall fescue (C3 species) photosynthesis is inhibited by 28-36% at 21% oxygen. In C3-C4 intermediate species of *P. millioides*, *P. schenckii* and *P. decipiens* the inhibition is moderate (21-23%) (Morgan *et al.* 1980). The 02 inhibition of photosynthesis is high (31-36%) under low light but is slightly relieved (16-24%) at high light intensities in C3-C4 intermediates. This is proposed to be

due to the increased recycling of photoresplred CO_2 under high light in these intermediates (Brown 1980).

Photosynthesis is insensitive to low 02 (<5%) in some of the C3-C4 intermediates of *Flaveria* (*Flaveria anomala, F. linear is, F. pubescens* and *F. ramosissima*) but gets inhibited at relatively high 02 concentration. For e.g. at 21% O2, photosynthesis was inhibited by about 20-25% in these species compared to 28/4 in C3 species of *Lycopersicon esculentum* (Ku *et al.* 1983). The extent of O_2 inhibition of photosynthesis in some representative C3, C3-C4 intermediate and C4 species are: 254 in *Flaveria trinervia* (C_4), 53% in *L. esculentum* (C3), 17% in *F. ramosissima*, 20-46% in *F. anomala, F. linearis F. floridana, F. oppositifolia* and *F. pubescens* (C3-C4 species) (Ku *et al.* 1983, Brown *et al.* 1986).

Carboxylation efficiency (CE - the initial slope of photosynthesis in response to varying [CO_2] at low levels) of C3 species is inhibited under ambient CO_2 and 02 concentrations due to the competitive nature of 02 with CO_2 while reacting with Rubisco (Edwards and Walker 1983). The O_2 inhibition of CE varies with species. Because of the efficient internal CO2 concentrating mechanism, the CE of C4 plants is unaffected by 02. In C3-like intermediates of *Panicum* and *Flaveria,* the degree of 02 inhibition of CE is similar to that of C3 species indicating the absence of C02 concentrating mechanism in these species (Monson *et al.* 1984, Edwards and Ku 1987). On the other hand, O_2 exerts a moderate

effect (less than that in C3 species) on CE of C_4-like C3-C4 intermediates (*Flaveria ramosissima* and *Neurachne minor*) indicating the operation of partial C4 cycle in these species (Monson *et al.* 1984, Edwards and Ku 1987).

Photorespiratlon increases along with 02 concentration (up to 44%) in both C3 and C3-C4 intermediates of *Panicum* and similar observations were made in other C_3 species (Forrester *et al.* 1966, Morgan *et al.* 1980). As mentioned already, Γ is taken as an indicator of extent of photorespiratlon and the degree of refixation of photoresplred CO_2. In C_4 species, the rise in 02 concentration up to even 50% does not influence Γ whereas in C3 plants Γ increases linearly with rise in 02. The Γ in C4 species of *Panicum prionitis* and *P. maximum* is little affected with O_2, but in C3 species of *P. hylaeicum*, *P. rivulere*, *P. laxum* and tall fescue Γ was high and increased curvilinearily up to 487. 02. In C3-C4 species of *Panicum* the Γ is intermediate and increases linearly with O_2 (Morgan *et al.* 1980). Most of the C3-C4 intermediates have a biphasic response of Γ to change in 02 concentration (Apel 1980, Morgan *et al.* 1980, Holaday *et al.* 1982, 1985, Hattersley et *al.* 1986, Edwards and Ku 1987, Moore *et al.* 1987a, Rawsthorne 1992). The apparent break point of 02 concentration varies with the intermediate species: *Panicum* - 10 to 25%, *Moricandia* - 10 to 15%, *Parthenium hysterophorus* 10%, *N. minor* and *F. brownii* - >50% (Edwards and Ku 1987). The exact biochemical basis of such biphasic response to 02 in the intermediates is not known.

Pattern of $^{14}CO_2$ assimilation

The CO_2 pulse- CO_2 chase experiments help to identify the principal type of photosynthetic carbon metabolism, operating in a plant. Further, these experiments can demonstrate also the pattern of photoresplratlon. In C4 plants, after a 3-sec $^{14}CO_2$ pulse, **about** *907.* of the C occurs in malate or aspartate, where as up to 80% label enters PGA 1n C3 plants (Edwards and Walker 1983). In C3-C4 intermediates the percentage of C appearing in C4 acids during short term pulse experiment varies widely, with a range from as low as *57.* to as high as *757.* (Monson *et al.* 1986, Edwards and Ku 1987). On short term exposure to $^{14}CO_2$, the % of label found in malate/aspartate within leaves of different C3-C4 intermediates were: 3-4°/. 1n *Partheniumhysterophorus* (Moore *et al.* 1987a), 1-8% 1n *Panicum* (Chastin and Chollet 1988), 4-40% in *Moricandia* (Holaday and Chollet 1984, Chastin and Chollet 1988), 10-40% in *ttollugo* (Sayre and Kennedy 1977), and 40-42% 1n *Flaveria.* In *Flaveria brownii*, a C4-like intermediate species, about 75% of the label was in C4 acids (Rumpho *et al.* 1984).

The extent of photorespiratory cycle operation is elucidated **from** the extent of label in glycine and serlne, key photorespiratory intermediates. After 3-10 sec pulse, the label of C in glycine and serine, the two photorespiratory metabolites, ranged from 3-20% 1n Intel mediates of *Mollugo*, *Horicandia* and ***Parthenium***

(Kennedy et *al.* 1980, Moore *et al* 1987a, Chastin and Chollet 1988). In *F. floridana*, another C3-C4 intermediate, the extent of label in glycine (and serine) was 14%, which was low compared to that in C3 species (Holaday and Chollet 1984). In *Parthenium hysterophorus* about 6% of the initial label was in glycolate against 177. in *P. incanum* (C3), indicating the reduced glycolate metabolism in these species (Moore *et al.* 1987a). Similarly the extent of photoresplratory glycolate in *Panicum millioides* was less than that in barley (C3) but was much higher than that in a C4 species, *P. miliaceum* (Servaites *et al.* 1978).

Inter- and intracellular compartmentation of enzymes is an integral biochemical component of C_4 photosynthesis. The atmospheric CO_2 is initially fixed by PEP carboxylase yielding OAA in mesophyll cells (O'Leary 1982, Andreo *et al.* 1987, Devi *et al.* 1992a). C_4 acids formed in the mesophyll cells are rapidly transported into the bundle sheath cells and are decarboxylated. The decarboxylation occurs via three mechanisms, *viz.,* NADP-malic enzyme, NAD-malic enzyme and PEP-carboxy kinase type. Malate is decarboxylated to CO_2 and pyruvate mediated by NADP-ME enzyme. Aspartate is converted into OAA or malate to be decarboxylated through PEP-CK or NAD-ME, respectively (Edwards and Walker 1983, Edwards *et al.* 1985, Hatch 1987, 1992a, b, Edwards and Ku 1990, Raghavendra and Das 1993). There is no clear demonstration of such intercellular compartmentation of photosynthetic enzymes in C3-C4 intermediates, although a partial compartmentation of C4 cycle

enzymes (PPDK in the mesophyll and NAD-ME in bundle sheath cells) was suggested in case of *Panicum milioides* (Rathnam and Chollet 1978).

C discrimination

Though all plants discriminate against $^{13}CO_2$ during photosynthesis, the extent of discrimination is greater in C3 plants than that by C4 plants, and hence the values of $^{13}C/^{12}C$ in leaf samples of C3 plants are relatively high (Reviews: O'Leary *et al.* 1992, Peisker and Henderson 1992).

The principal discrimination against C02 occurs during RuBP carboxylation. When stomata are completely open, offering little resistance to entry of CO_2, a discrimination (5 C) close to -27%. is expected in C3 plants (Smith and Turner 1975, O'Leary 1981, 1988, Farquhar *et al.* 1989). Since Rubisco is capable of marked discrimination against $^{13}CO_2$, all the C3 species show higher (negative) values (-25 to -37%.) of $\delta^{13}C$. The basis of $^{13}CO_2$ discrimination in C4 plants is more complex. CO_2 is initially fixed by PEP carboxylase in the mesophyll cytosol, then the C02 released in the bundle sheath cells as a result of C4 acid decarboxylation is refixed by Rubisco. The net discrimination of gaseous C02 as a result of such two-step carboxylation initiated by PEP carboxylase results in low values of *S* C (-11 to -18%.) in these species (von Caemmerer 1989, O'Leary *et al.* 1992).

The 5 C values for C3-C4 intermediates range from -21 to -30%. (Hattersley and Roksandic 1983, Hattersley *et al.* 1986, von Caemmerer 1989, 1992}. Some of the C3-C4 species (e.g. *Panicum milioides*) show 5 C values close to those of C3 species (Hattersley *et al.* 1986). The C3-llke $\delta^{13}C$ values in intermediate species suggests that most of the carbon is routed primarily through Rubisco at ambient CO_2. Only in certain cases (for e.g. *Panlcum milioides*) the $\delta^{13}C$ values are more negative in C3-C4 intermediates than those in C3 species (Hattersley *et al.* 1986). According to the model of Peisker (1986), the C3 like $\delta^{13}C$ values in C3-C4 intermediates are the result of restriction on C4 cycle imposed by the limitation in PEP regeneration. Though carbon discrimination may not have an immediate physiological significance, it reflects an important relationship between carboxylation reaction, CO2 transfer processes including CO2 leakage from bundle sheath and assimilation into organic matter (Peisker 1985).

Biochemical basis of reduced photorespiration in C3-C4 intermediates

The reduced levels of photorespiration in C3-C4 intermediates may be due to either (1) an efficient refixation of photoresplratory CO_2 or (11) a CO2 concentrating mechanism due to the operation of a partial C4 cycle (Edwards and Ku 1987).

(1) Efficient refixation of photorespired CO2: CO2 released from photorespiration or dark respiration is refixed before it escapes into the intercellular spaces of the leaf, in plants like *Moricandia arvensis* or *Panicum milioides* (Holaday and Chollet 1984, Rawsthorne 1992). Immunogold localization studies showed that glycine decarboxylase was confined to mostly the bundle sheath cells in *Moricandia arvensis* (Rawsthorne *et al.* 1988a), a situation resembling to the one in C_4 plants (Hatch 1987). The large abundance of chloroplasts, mitochondria and peroxisomes along with predominant localization of key photorespiratory enzymes (e.g. GDC and SHMT) in bundle sheath cells facilitates the physical proximity of decarboxylation and carbon fixation reactions. This leads to the enhanced refixation of photorespired C02 by Rubisco in bundle sheath chloroplasts.

Studies on light/dark metabolism of exogenously supplied [1-^{14}C]glycine or [1-^{14}C]glycolate by leaf discs of C_3-C_4 species *Moricandia arvensis* and *Panicum milioides* also support this hypothesis. $^{14}CO_2$ evolution from [1-^{14}C]glycine in C3 species was reduced on illumination by about 607., while in C_3-C_4 intermediates such reduction was as high as 90%. The relatively high light/dark ratios of CO2 release from these photorespiratory metabolites suggest that the intermediates have a greater capacity than the C3 species to refix photorespired CO_2 in the light (Holbrook et *al.* 1985, Edwards and Ku 1987). If the same process occurs during

photorespiratlon *in vivo,* it could account, at least in part, for the reduced rates of photorespiratory C02 evolution in C3-C4 intermediates (Holbrook et *al.* 1985). The rate of $^{14}CO_2$ evolution from [1-^{14}C]glycine is inversely related to the rate of $^{14}CO_2$ fixation by leaf discs of *Moricandia arvensis,* demonstrating that the photorespiratory CO2 loss is reduced as the rate of photosynthesis increases since more CO_2 is refixed principally by Rubisco (Holbrook *et al.* 1985, Kumar and Abrol 1990).

(ii) Partial C4 cycle operation: A partial or nearly complete C4 cycle operation was detected in some of the *Flaveria* intermediate species (*F. brownii*, *F. ramosissima*, *F. linearis, F. anomala*). The first indication that C_4 photosynthesis may occur in these intermediates was the detection of substantial activities of the C4 cycle enzymes - PEP carboxylase, PPDK, NADP-malic enzyme, and NADP-malate dehydrogenase - in leaves of these species (Ku *et al.* 1983, 1991). PEP carboxylase in *F. linearis* and *F. pubescens* had intermediate characteristics in maximum velocity on a Chl basis and Km for PEP, compared to the C3 and C4 *Flaveria* species examined (Nakamoto *et al.* 1983). Pulse-chase experiments with CO2- CO2 confirmed the functioning of C4-like cycle in *Flaveria ramosissima* (Rumpho *et al.* 1984), *F. pubescens* (Bassüner et *al.* 1984), *F. anomala, F. floridana*, *F. linearis* (Monson et *al.* 1986). The limited C4 pathway operation can be sufficient for reducing photorespiration by elevating the CO_2 concentration at the site of

Rubisco in the bundle sheath cells (Rumpho *et al.* 1984, Monson *et al.* 1986, Edwards and Ku 1987, Rawsthorne 1992).

(iii) Possible third alternative: Along with the above factors, reduction in the photorespiratory enzymes may also play an additional role in reducing photorespiration in C_3-C_4 intermediate species. The activity-levels of photorespiratory enzymes in the intermediates are reduced by varying degrees compared to those of C3 species. In *Flaveria* and *Panicum* intermediates the photorespiratory enzymes like glycolate oxidase, NADH-HPR, and SHMT were reduced relative to that of C3 species (Ku *et al.* 1976, 1991). Low levels of photorespiratory enzymes like glycolate oxidase and GDC are reported in *Flaveria ramosissima* and *Moricandia arvensis*, respectively (Moore *et al.* 1988, Kumar and Abrol 1990). However it is not certain if a reduction in photorespiratory enzymes can form a major biochemical basis for reduction in photorespiration in these species.

Studies on photorespiratory metabolites like ammonia in C3-C4 species are very few. The levels of photorespiratory NH_3 in the C_3-C_4 intermediates of *Parthenium* and *Moricandia* were less than those in C3 plants (Kumar and Abrol 1989, 1990). This situation is similar to that of C4 species, where low PR-NH_3 (Kumar *et al.* 1984, Kumar and Abrol 1989, 1990) and glycolate (Zelitch 1973, 1979) accumulation are reported. The reduced levels of photorespiratory metabolites in C4 species can be attributed to the concentration of

C02 at the site of rubisco in bundle sheath cells due to highly efficient C4 pathway operation. Similarly the decrease of photorespiratory metabolites (NH3, glycolate and glyoxylate) could probably be due to the high C02 concentration near the site of Rubisco due to either an efficient refixation of photorespiratory/ respiratory CO2 or partial C4 cycle operation. The high CO2 near Rubisco reduces its oxygenase function and decreases the formation of glycolate, the photorespiratory substrate.

Evolution

The C3-C4 intermediate species offer a good model to study the biochemical and molecular aspects of C4 pathway development, since, the intermediates represent possibly a 'transitory stage' during the evolution of C4 plants from C3 species (Raghavendra 1980, Moore 1982, Pelsker 1986, Monson and Moore 1989, Hatch 1992a, b, Brown and Bouton 1993, Raghavendra and Das 1993, Raghavendra and Devi 1993). C3-C4 intermediates have an advantage over C3 species in their adaptation to warm environments, improved water and nitrogen use efficiencies, and reduced levels of photorespiration (Monson 1989a, Monson and Moore 1989, Schuster and Monson 1990, Brown and Bouton 1993).

Four alternative mechanisms were proposed for possible origin of C3-C4 intermediates as indicated below (Monson 1989a, Monson and Moore 1989):

(i) Natural hybrids of C3 and C4 species: Many hybrid plants with Kranz anatomy and intermediate photosynthetic characters were obtained during the hybridization experiments with *Atriplex* species (Björkman *et al.* 1970) and other genera of different photosynthetic types (Review: Brown and Bouton 1993). However, none of them showed typical 'C4 photosynthesis'.

(ii) Plants in the process of 'reverse evolution', *i.e.* evolution of C3-C4 intermediates from C4 plants: Since there is a lack of any apparent benefit from this evolutionary pattern, this hypothesis seems to be invalid for the evolution of the C3-C4 intermediates. Only a limited reduction in photorespiratory mechanism in C3-C4 intermediates clearly suggest that this mode of evolution is not a favorable one.

(iii) 'Dead ends' of evolution: The observed gradation in the photosynthetic and photorespiratory characteristics in *Flaveria* intermediates indicates the progressive evolution and rules out this possibility.

(iv) 'Transitory stage' during the evolution of C4 plants: The hypothesis of intermediates as 'evolutionary links' is quite appealing at this juncture but additional evidence is needed.

Scope for future work

Despite the considerable work already done, there are still marked gaps in our knowledge of C3-C4 intermediates. Some of the areas which require further attention include: (a) the mechanism of reduction in photorespiratory metabolism particularly in species without a perfect Kranz-syndrome and functional C_4 pathway; (b) possible existence of marked variation in kinetic/regulatory properties of one or more photorespiratory/photosynthetic enzymes; (c) the extent of inter- and intracellular compartmentation of enzymes; (d) operation of a unique CO2 concentrating mechanism other than C4 cycle (e.g. as in submerged aquatic plants, see reviews by Bowes 1991, 1993); (e) modulation by environmental factors (light and temperature) or internal factors (e.g. leaf age) of C_3-C_4 intermediacy; (f) physiological and biochemical basis of biphasic response of Γ to ambient 02; (g) a critical reexamination of carboxylation efficiency at different light/temperature regimes, and (h) molecular biological aspects of transcription and translation of key enzymes such as PEP carboxylase, NAD-malic enzyme or glycine decarboxylase.

Objectives of the present investigation

The major objective of the present work was to study and assess the mechanism of reduced photorespiration in C_3-C_4 intermediates of *Alternanthera ficoides*, *Alternanthera tenella* and

Parthenium hysterophorus. An attempt was therefore made to compare the photosynthetic, photorespiratory and respiratory metabolism in leaves of three C3-C4 intermediates with those of two C3 species *[Alternanthera sessiles* and *Pisum sativum*) and three C_4 plants (*Alternanthera pungens*, *Amaranthus hypochondriacus* and Zea *mays)*.

1. As a first step, the maximum catalytic activities of several photosynthetic and photorespiratory enzymes were assayed in leaf extracts of the C3-, C4- and C3-C4 intermediate species.

2. In the next phase, accumulation of photorespiratory metabolites (ammonia, glycolate and glyoxylate) on illumination was examined.

3. Later on the decarboxylation of externally added glycine by leaf discs was determined in light and dark so as to assess the extent of refixation of photorespiratory CO_2.

4. A partial reduction in photorespiratory metabolism was noticed, as indicated by reduced activity-levels of enzymes and related metabolites. It was necessary to check if there were any major changes in the characteristics of key photosynthetic/photorespiratory enzymes in leaves of these intermediates.

Two key enzymes were chosen for this purpose: glycolate oxidase and PEP carboxylase which are important enzymes of photoresplration and C4 photosynthesis, respectively. However, the properties of these two enzymes from C3-C4 intermediates were similar to those of C3 rather C4 species, suggesting that there was no alteration in enzyme-protein.

5. During our attempts to characterize the comparative pattern of light activation of PEP carboxylase in leaf discs, leaf homogenates and protoplasts, very interesting observations were made with maize mesophyll protoplasts.

   Although the topic does not directly concern C3-C4 intermediates, the data obtained with maize mesophyll protoplasts were also incorporated into this thesis. These results provided a novel demonstration of light activation of PEP carboxylase in C4 mesophyll protoplasts for the first-time and suggested that pH could be an important factor in such light activation.

6. Most of the work on C3-C4 intermediates focused on their photorespiratory metabolism. An attempt was therefore made to reexamine the response to ambient CO2 of photosynthetic or respiratory oxygen exchange in leaf discs of intermediates, in comparison with C3 and C4

species. While the photosynthetic properties of intermediates were similar to C3 species, the response of dark respiration to ambient CO2 differed significantly. The inhibition of dark respiration by moderate or high concentrations of CO2 was much more pronounced in C3 species than that in C4 plants or C3-C4 intermediates.

7. In the last phase, mesophyll protoplasts and bundle sheath strands were isolated from leaves of a C3-C4 intermediate species, *Alternanthera tenella*. The activities of important enzymes involved in photosynthesis, photorespiration and respiration were determined in these mesophyll and bundle sheath preparation so as to assess the differential localization/distribution, if any. A significant compartmentation into bundle sheath cells of this intermediate could be noticed only in case of enzymes involved in glycine decarboxylation and TCA cycle pointing out that mitochondrial metabolism is an important feature in C3-C4 intermediates.

Chapter 2

Chapter 2

MATERIALS AND METHODS

Plant Material

Plants of *Amaranthus hypochondriacus* L. (cv. AG-67), *Amaranthus viridis* L., *Pisum sativum* L. (cv. Arkel), and Zea *mays* L. (cv. Ganga 5) were raised from seed. *Alternanthera ficoides* L.R.Br.R, *A. pungens* (L.) H.B & K., *A. sessiles* (L.) R.Br.*ex* DC., *A. tenella* Colla and *Parthenium hysterophorus* L. were collected *from* campus and were multiplied by transplantation of cuttings/ seedlings.

The **seedlings/plants** were grown on soil, supplemented with farm-yard manure, in 30-cm diameter plastic tubs or earthen pots (Fig. 2.1 A to E). The tubs/pots were kept in the field under a natural photoperiod of approximately 12 h and an average temperature of 30°C day/20°C night. The plants were watered daily.

Third and fourth leaves (counting from the fully developed youngest one) were excised from 3-4-week-old plants between 8.00 and 9.00 a.m. and were used for the experiments.

Leaf anatomy

The leaf tissue was fixed in FAA (Formalin-Aceto-Alcohol) (consisting of 50% (v/v) alcohol, 40% (v/v) formaldehyde and 5% (v/v) glacial acetic acid) for 10 h. Thin hand cross sections of

Fig. 2.1. Photographs of experimental plants grown in the field. A: *Alternanthera sesslles* (a C3 plant), B: *Alternanthera pungens* (C4), C: *Alternanthera tenella*, D: *Alternanthera ficoides*, and E: *Parthenium hysterophorus*. C to E are C3-C4 intermediate species.


A


C


B


D


E

about 100 μm thickness were prepared with free hand using new razor blades. The sections were stained with methylene blue and observed under a research microscope (Wolfe, Japan).

Photographs of leaf cross sections were taken from the microscope using a black and white negative film (ISO 125/22° ASA-ORWO, Germany).

Chlorophyll and protein estimations

Chlorophyll was estimated as per Arnon (1949).

500 mg of freshly collected leaf tissue was homogenized with 80% (v/v) acetone using a mortar and pestle. The homogenate was centrifuged at 1,000 g for 5 min in a table-top centrifuge (Remi R8C). The clear supernatant was made up to 25 ml with 80% acetone. The absorbance of the acetone extract was measured at 645, 663 and 710 nm using 807. acetone as the blank.

The following equations were used to calculate the concentration of total chlorophyll, Chl a and Chl *b*.

Total chlorophyll ($\mu g\ ml^{-1}$) = (20.2 x A645) + (8.02 x A663)

Chlorophyll a ($\mu g\ ml^{-1}$) = (12.7 x A663) - (2.69 x A645)

Chlorophyll *b* ($\mu g\ ml^{-1}$) = (22.9 x A645) - (4.68 x A663)

The absorbance at 645 and 663 nm were corrected for turbidity, if any, by subtracting the absorbance of acetone extract at 710 nm.

In some of the experiments, a small aliquot of the leaf homogenate (or protoplasts) was mixed with acetone, to make a final concentration up to 80% (v/v). The mixture was cleared by centrifugation and chlorophyll was estimated as described above.

Protein was estimated by using either Lowry method (Lowry *et al.* 1951) or the dye binding (Coomassie Brilliant Blue G-250) method of Bradford (1976).

Lowry method is based on the colour reaction of Folin-Ciocalteu phenol reagent with tyrosine residues of protein. To each 0.2 ml of protein sample, 1 ml of alkaline copper sulfate solution (50 ml reagent A [2% w/v Na_2CO_3 in 0.1 M NaOH] mixed with 1 ml reagent B [0.5% $CuSO_4.5H_2O$ in 1% sodium-potassium tartrate]), was added and the mixture was allowed to stand for 10 min. Then 0.1 ml of Folln's reagent (commercial preparation diluted with water in a ratio of 1:1) was added and mixed immediately. After 30 min incubation at 25°C, the absorbance was read at 750 nm. A standard curve was prepared with BSA (10-100 μg ml).

The Bradford method (1976) of protein estimation was done by reacting the protein with a dye (Coomassie Brilliant Blue G-250). To the 0.1 ml of the sample (made with 0.15 M NaCl solution), 5 ml of the dye (prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% (v/v) ethanol followed by addition of 100 ml of 85% (w/v) phosphoric acid and the volume made up to 1 1 with

distilled water) was added and vortexed. After incubation at 30°C for 1 h the absorbance was read at 595 nm. A standard curve was prepared by using 1 to 10 μg of BSA ml^{-1}.

Extraction and Assay of Enzymes

The extraction and assays of photosynthetic, photorespiratory and respiratory (mitochondrial) enzymes were done by using the published procedures. However several of them were modified so as to achieve maximal activity in leaf extracts. The details are described below.

Unless otherwise mentioned, leaf samples (1 to 1.5 g) were rapidly homogenized with 4 vols of prechilled extraction medium, using a mortar and pestle. The crude homogenate was filtered through four layers of muslin cloth and cleared by centrifuging at 10,000 *g* (Hermle Z 320 K centrifuge) for 10 min at 4°C. An aliquot was kept aside prior to centrifugation for chlorophyll estimation (described above). The supernatant was used for enzyme assays. Deviations from this standard extraction procedure are mentioned wherever relevant. The details of extraction medium which depends on the enzyme being assayed are described below.

The assays were done usually with enzyme extracts equivalent to 1 to 2 μg Chl in the assay medium of 1 ml. All the spectrophotometric assays were conducted in a Shimadzu UV-Vis Spectrophotometer (Model UV-160A). Radioactivity was monitored using a liquid scintillation counter (Model Beckman LS 1800).

The maximum catalytic activities were determined by optimizing conditions during assay for individual enzyme in each species. Further experiments were done to ensure that low levels of the photoresplratory enzymes were not due to any endogenous inhibitory substances. For this, enzyme activities were determined in extracts prepared by cohomogenizing the leaves or by mixing the leaf extracts of intermediate species with pea (typical C3 plant with very low phenolics).

PHOTOSYNTHETIC ENZYMES

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31):

Extraction

The extraction medium was 50 mM Hepes-KOH pH 7.2, 10 mM MgCl2, 2 mM K_2HPO_4, 1 mM EDTA, 20% (v/v) glycerol, 1% (w/v) insoluble PVP, and 10 mM β-mercaptoethanol.

Assay

PEPC was assayed spectrophotometrically at 25°C by coupling oxalacetate formation with NAD-malate dehydrogenase (Igleslas and Andreo 1989). The assay mixture (1 ml) contained 20 mM Tricine-KOH (pH 7.8), 5 mM MgCl2, 10 mM NaHC03, 2 units of NAD-MDH and 0.2 mM NADU. Following a 30-sec incubation of the enzyme in the assay mixture, the reaction was initiated by the addition of 50 µl of 50 mM PEP. The reaction was followed for 3 min by monitoring the oxidation of NADH at 340 nm. The activity was calculated by using the molar extinction coefficient of NADH (6.2 mM^{-} cm).

Pyruvate, orthophosphate dlklnase (PPDK, EC 2.7.9.1):

Extract ion

The extraction medium was 100 mM Tris-HCl pH 7.5, 10 mM MgCl2, 5 mM sodium pyruvate, 2 mM K_2HPO_4, 1 mM EDTA, 5 mM DTT, 1% (w/v) insoluble PVP and 1% (w/v) sodium ascorbate.

Assay

The enzyme assay was done within 10 min after extraction of the enzyme at 25°C by following the change in absorption at 340 nm by a coupled reaction using PEPC and NAD-MDH (Aoyagi and Bassham 1983). The assay mixture (1 ml) contained 100 mM Tris-HCl pH 7.5, 10 mM MgCl2, 50 mM NaHC03, 5 mM DTT, 2.5 mM K_2HPO_4, 0.2 mM NADU, 2 units of PEPC, 2 units of MDH and 1.25 mM ATP. The reaction was started by adding pyruvate (final concentration 1.25 mM). The activity was calculated by using the molar extinction coefficient of NADH (6.2 mM^{-1} cm^{-1}).

NAD-malate dehydrogenase (EC 1.1.1.37)

Extraction

Extraction medium contained 50 mM Hepes-KOH (pH 7.5), 2.5 mM EDTA, 5 mM MgCl2 and 5 mM DTT.

Assay

The enzyme was assayed spectrophotometrically by following the oxidation of NADH at 340 nm (Osmond and Avadhani 1970). The assay mixture (1 ml) contained 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 0.25 mM NADH and enzyme extract. The assay was started by adding oxalacetate to a final concentration of 5 mM.

NADP-malate dehydrogenase (EC 1.1.1.82)

Extraction

The extraction medium contained 50 mM Tricine-KOH (pH 8.0), 5 mM $MgCl_2$ and 5 mM DTT.

Assay

The assay was done spectrophotometrically by monitoring the oxidation of NADPH at 340 nm (Kanai and Edwards 1973). An aliquot of the enzyme was preincubated in 25 mM DTT in a volume of 0.1 ml for 15 min at room temperature. The reaction mixture (1 ml) contained 50 mM Tricine-KOH (pH 8.0), 0.2 mM NADPH and preactivated enzyme extract (equivalent to 1 fig chlorophyll). The reaction was started by adding OAA to a final concentration of 3 mM.

NADP-malic enzyme (EC 1.1.1.40)

Extraction

The extraction medium consisted of 50 mM Hepes-KOH (pH 7.5), 2.5 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT and 0.5% (w/v) insoluble PVP.

Assay

The enzyme was measured spectrophotometrically by following the reduction of NADP (Kanai and Edwards 1973). The assay medium contained 25 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 20 mM $MgCl_2$, 5 mM DTT, 0.4 mM NADP and 5 mM malate. The reaction was initiated by adding malate. A control reaction was run without magnesium in order to account for any activity due to NADP-mallc dehydrogenase. The activity was calculated by using the molar extinction coefficient of NADPH (6.2 mM^{-1} cm^{-1}).

NAD-malic enzyme (NAD-ME, EC 1.1.1.39):

Extraction

The extraction medium contained 50 mM Hepes-KOH (pH 7.6), 2 mM MnCl2, 10 mM DTE (freshly prepared), 1% (w/v) insoluble PVP and 0.5% (v/v) Triton X-100.

Assay

The enzyme was assayed as per the procedure described by Hatch *et al.* (1982). The reaction mixture (1 ml) consisted of 25 mM Hepes-KOH (pH 7.6), 0.25 mM EDTA, 5 mM DTT, 5 mM MnCl2, 5 mM NAD, 75 μM CoA and 25 μM acetyl CoA. After two min of incubation, the assay was started by adding malate to make a final concentration of 5 mM. The reaction was measured by monitoring the increase in absorption (formation of NADH) at 340 nm. The activity was calculated by using the molar extinction coefficient of NADH ($6.2\ mM^{-1}\ cm^{-1}$).

Aspartate aminotransferase (Asp AT. EC 2.6.1.1):

Extraction

Leaf pieces (1.0 g) were powdered in liquid N2, and later homogenized in 6 vols of prechilled extraction medium containing 100 mM Hepes-KOH (pH 7.5), 10 mM MgCl2, 10 mM DTE, 1 mM EDTA, 2.5 mM pyruvate, 0.5% (w/v) BSA, 1% (w/v) casein, 0.05% (v/v) Triton X-100 and 2% (w/v) insoluble PVP. The extract was filtered through 4 layers of muslin cloth and cleared by centrifuging at 15,000 *g* for 15 min and the supernatant was used for the assay (Moore *et al.* 1987a).

Assay

The assay was done at 25°C by monitoring the oxidation of NADH at 340 nm in a coupled reaction (Edwards and Gutierrez 1972). The assay medium (1 ml) consisted of 50 mM Tricine-KOH (pH 8.0), 2.5 mM 2-oxoglutarate, 0.03 mM pyridoxal 5-phosphate, 0.1 mM NADH, 2 mM EDTA and 2 units NAD-MDH. The reaction was initiated by the addition of L-aspartate to make a final concentration of 2.5 mM. The activity was calculated by using the molar extinction coefficient of NADH (6.2 mM^{-1} cm^{-1}).

Alanine aminotransferase (Ala AT. EC 2.6.1.2):

Extraction

The extraction procedure for ala AT was similar to that of asp AT.

Assay

The assay was done by monitoring the oxidation of NADH at 340 nm (Edwards and Gutierrez 1972). The assay medium of 1 ml contained 50 mM Tricine-KOH (pH 8.0), 2.5 mM 2-oxoglutarate, 0.03 mM pyridoxal 5-phosphate, 0.2 mM NADH, 2 mM EDTA and 5 units of lactate dehydrogenase. The reaction was started by the addition of 50 µl of 50 mM L-alanine. The activity was calculated by using the molar extinction coefficient of NADH (6.2 mM" cm").

RuBP carboxylase (RuBPC, EC 4.1.1.39):

Extraction

The extraction medium was 50 mM Hepes-KOH pH 7.8, 2 mM EDTA, 5 mM MgCl2, 5 mM DTT and 1% (w/v) insoluble PVP. A pinch of acid-

washed sand was added during grinding in order to break the bundle sheath tissues effectively.

Assay

RuBP carboxylase was assayed by monitoring the incorporation of [C]NaHCO3 into acid stable products. The reaction mixture of 500 µl (100 mM Tris-HCl (pH 8.0), 25 mM MgCl2 and 1 mM DTT) was prepared in CO_2-free distilled water. Assays were performed in eppendorf tubes. The enzyme was preactivated at 30°C for 10 min in 10 mM NaIIC03 and 10 mM MgCl2. The reaction was started by injecting 50 µl of preactivated enzyme and 50 µl of 5.0 mM RuBP to the reaction mixture containing 100 µCi [^{14}C]NaHCO3 (specific activity 51 Ci mol). The reaction was stopped by adding 400 µl of 10 N formic acid after different time intervals (15, 30, 45 or 60 sec) at 30°C.

The reaction mixture was then transferred to small glass beakers and evaporated to dryness. The resulting residue was resuspended in 50 or 100 µl of deionized water, and was added to scintillation vials containing 10 ml of Bray's mixture (contains 200 mg POPOP, 4 g PPO, 60 g naphthalene, 100 ml methanol and 20 ml ethylene glycol, volume made up to 1 1 with 1,4-dioxan). Radioactivity was counted using a liquid scintillation counter.

Carbonic anhydrase (CA, EC 4.2.1.1):

Extraction

The extraction medium was 40 mM Hepes-KOH (pH 8.0) and 10 mM DTT.

Assay

The enzyme was assayed by monitoring the rate of CO_2 hydration at 4°C as a change of pH (Hatch and Burnell 1990). The 1 ml reaction mixture contained 25 mM barbitone-KOH buffer (pH 8.2) and the enzyme extract. The reaction was initiated by adding 250 µl of C02-saturated distilled water at 4°C and gently stirring the mixture. The pH of the medium was continuously recorded. The nonenzymatic reaction rate was measured by adding 250 µl saturated CO2 solution to the buffer without the enzyme extract. The change of pH was converted to equivalent µmol H generated (and hence equivalent CO_2 hydrated assuming complete dissociation of H_2CO_3) by titrating the reaction mixture with a standard solution of H_2SO_4 in the range from pH 8.3 to 7.6. The activity at 25°C was calculated from the values measured at 4°C.

PHOTORESPIRATORY ENZYMES

RuBP oxygenase (RuBPO, 4.1.1.39):

Extraction

The extraction medium was 50 mM Hepes-KOH (pH 8.5), 20 mM MgCl2, 5 mM DTT and 1% (w/v) insoluble PVP.

Assay

The assay was done as per Lorimer *et al*. (1977) by measuring the oxygen uptake in a Clark type oxygen electrode (Hansatech, UK). The assay medium consisted of 50 mM Hepes-KOH (pH 8.5) and 1 mM MnCl2. The enzyme was preactivated at 30°C for 10 min in 10 mM NaHCO3 and 10 mM MgCl2. The reaction was run without adding the enzyme (with substrate RuBP, final concentration 0.5 mM) for 2 mln

and then the preactivated enzyme (equivalent to 2 μg Chl) was added. The reaction was continued for 60-90 sec. The background activity (02 consumption in the absence of the preactivated enzyme) was subtracted from the 02 consumption rates obtained in presence of the preactivated enzyme.

Phosphoglvcolate phosphatase (EC 3.1.3.18):

Extraction

The extraction medium was 50 mM Tris-acetate (pH 6.3).

Assay

The enzyme was assayed by measuring the inorganic phosphate formed. The assay medium (1 ml) consisted of 50 mM Mes-KOH (pH 5.5), 8 mM $MgCl_2$ and enzyme extract (Osmond and Harris 1971). The reaction was started by adding phosphoglycolate (final concentration 5 mM) and was stopped after 15 min by adding 0.2 ml of 40% (w/v) TCA.

Inorganic phosphate was measured by the method of Fiske and Subbarow (1925). An aliquot (1 ml) of the reaction mixture was treated with 1 ml 5 N H_2SO_4 followed by 1 ml of ammonium molybdate and 0.1 ml of reducing agent (0.2 g amlnonaphthalene sulphonlc acid, 1.2 g sodium bisulphite, 1.2 g sodium sulphite dissolved in 100 ml distilled water). The absorbance of the blue colored complex at 660 nm was determined and compared to the standards (100-1000 μmoles ml^{-1} KH_2PO_4).

Phosphoglycerate phosphatase (EC 3.1.3.20):

Extraction

The extraction was done in 50 mM Tris-acetate buffer pH 6.3.

Assay

The enzyme was assayed by measuring the inorganic phosphate formed. The reaction medium (1 ml) consisted of 50 mM Mes-KOH, 8 mM MgCl2 and enzyme extract. The reaction was started by adding 3-PGA to make a final concentration of 5 mM. The reaction was continued for 15 min and terminated by adding 0.2 ml of 40% (w/v) TCA. The phosphate content in the reaction mixture was measured as described above (for phosphoglycolate phosphatase).

Glycolate oxidase (GO. EC 1.1.3.1):

Extraction

The extraction medium was 100 mM Hepes-KOH (pH 8.3), 2 mM MgCl2, 2 mM MnCl2, 1 mM EDTA, 2 mM DTE, 1% (w/v) insoluble PVP and 0.05V. (v/v) Triton X-100.

Assay

The enzyme was assayed by monitoring spectrophotometrically the conversion of phenylhydrazine to phenylhydrazone (due to the formation of complex with glyoxylate) at 324 nm. The assay medium (1 ml) consisted of 50 mM Hepes-KOH (pH 8.3), 2.5 mM MgCl2 and 3 mM phenylhydrazine. The reaction was started by the addition of 50 µl of 100 mM glycolate. The activity during the initial lag period of three minutes was ignored. The activities were calculated based on the molar extinction coefficient of phenylhydrazone at 324 nm (16.95 mM^{-1} cm^{-1}) (Felerabend and Beevers 1972).

Catalase (EC 1.11.1.6):

Extraction

The extraction medium was 40 mM Tricine-KOH (pH 7.8), 2 mM $MgCl_2$ and 1 mM EDTA (Tolbert *et al.* 1969).

Assay

The enzyme was assayed by monitoring the decomposition of H_2O_2 as a decrease in absorbance at 230 nm. The assay medium (1 ml) consisted of 50 mM potassium phosphate buffer (pH 7.0). Leaf extract (equivalent to about 1 µg Chl) was added to the assay buffer and the reaction was started by adding 50 µl of 0.2 M H_2O_2. The reaction was followed by recording change in absorption at 230 nm (Tolbert *et al.* 1969). The molar extinction coefficient of H_2O_2 (0.067 mM^{-1} cm^{-1}) was used for calculations.

NADH-hydroxypyruvate reductase (NADH-HPR, EC 1.1.1.29):

Extraction

The extraction medium contained 50 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 3 mM DTE and 1% (w/v) insoluble PVP.

Assay

The assay was done by monitoring the oxidation of NADH at 340 nm in presence of hydroxypyruvate according to Kleczkowski and Randall (1988). The assay medium (1.0 ml) consisted of 50 mM Mes-KOH (pH 6.5) and 0.2 mM NADH. The reaction was started by adding hydroxypyruvate to make a final concentration of 1 mM. The molar extinction coefficient of NADH at 340 nm (6.2 mM^{-1} cm^{-1}) was used for calculations.

Glycerate kinase (EC 2,7.1.31):

Extraction

The extraction medium was 50 mM Hepes-KOH pH 7.5, 5 mM EDTA and 5 mM DTE.

Assay

Glycerate kinase was assayed by coupling the PGA formed by glycerate kinase to the enzymatic reactions of PGA kinase and glyceraldehyde-3-phosphate dehydrogenase (Osmond and Harris 1971, Usuda and Edwards 1980). The assay medium (1 ml) contained 50 mM Hepes-KOH (pH 8.0), 5 mM MgCl2, 5 mM ATP, 5 units 3-PGA kinase, 5 units glyceraldehyde 3-phosphate dehydrogenase, 10 mM β-mercapto-ethanol and 0.25 mM NADH. The reaction was started by adding glycerate (final concentration 5 mM) to the assay mixture. The oxidation of NADH was monitored at 340 nm. The molar extinction coefficient of NADH (6.2 mM^{-1} cm^{-1}) was used for calculating the activity.

Glutamate-glyoxylate aminotransferase (EC 2.6.1.4):

Extraction

The extraction medium was 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 10 mM $MgSO_4$ and 10% (v/v) ethanediol.

Assay

The assay was done by measuring the formation of the product [1-^{14}C]glycine from [1-^{14}C]glyoxylate. The assay medium consisted of 20 mM phosphate buffer pH 7.5, 25 mM glutamate, 0.1 mM pyridoxal 5-phosphate and 20 mM glyoxylic acid. The reaction was started by

adding 20 µl (10 µCi) of [1-^{14}C]glyoxylate (specific activity 4.75 Ci mol). Reaction was carried out at 30°C for 15 min and terminated by boiling in water bath for 3 min. Unreacted [1- C]glyoxylate was removed by passing the reaction mixture through a column (5.0 cm X 1.0 cm) of Dowex-1 (acetate) and the column was washed with 2 ml of water (Kisaki and Tolbert 1969). The effluents were combined (3 ml) and an aliquot (500 µl) of effluent kept in 10 ml of Bray's mixture (components as described for RuBP carboxylase) in a scintillation vial and the radioactivity for [1- C]glycine was measured in a liquid scintillation counter.

Serine-glyoxylate aminotransferase (EC 2.6.1.45):

Extraction

The enzyme was extracted as per Murray *et* aJ. (1987). The extraction medium contained 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 10 mM $MgSO_4$ and 10% (v/v) ethanediol. The homogenate was passed through 4 layers of muslin cloth and centrlfuged at 10,000 *g* for 20 min. 5 ml of the supernatant was dialysed for 6 h against 5 mM Tris-HCl (pH 7.5). The dialysed homogenate was used for the enzyme assay.

Assay

The enzyme was assayed by measuring the conversion of [^{14}C]serine into [^{14}C]hydroxypyruvate (Smith 1973). The reaction mixture (1 ml) contained 60 mM Hepes-KOH (8.0), 1 mM [^{14}C]serine (Specific activity 135 Ci mol^{-1}; 0.5 µCi in assay mixture) and 10 mM glyoxylate. The reaction was started by adding 10-20 µg Chl equivalent leaf extract and incubated for 30 min at 30 C. The

reaction was stopped by adding 0.2 ml of 1.5 M TCA. Precipitated protein was removed by centrifugation and discarded.

The supernatant was applied on to a column (5.0 cm X 1.0 cm) of Dowex-50 (H) to remove unreacted [^{14}C]serine. The column was washed with 2 ml of distilled water. 0.5 ml aliquot of the effluent was added to 10 ml Bray's mixture (components described as for RuBP carboxylase) and the radioactivity was measured using a liquid scintillation counter.

Glycine decarboxylase (GDC. 2.1.2.10) and

Serine hydroxymethyltransferase (SHMT. 2.1.2.1):

These two enzymes were determined in mitochondrial pellets (instead of crude leaf extracts), prepared as described below:

Preparation of crude mitochondrial pellet

Mitochondria were prepared from leaves as described by Bergman *et al.* (1980). Small pieces (ca. 20 mm) of leaves were ground vigorously (to ensure complete breakage of bundle sheath strands) in a mortar and pestle at 4°C using 4 vols of prechilled extraction buffer (25 mM Hepes-KOH, pH 7.8, 0.3 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 4 mM cysteine, 0.1% (w/v) BSA and 0.6% (w/v) soluble PVP). The homogenate was filtered through two layers of muslin cloth and centrifuged at 5,000 *g* for 3 min. The supernatant was centrifuged again at 20,000 g for 7 mln. The pellet constitu- the crude mitochondrial preparation. The pellet was resuspended in a medium similar to the one used for the extraction except that BSA was omitted.

Assay of GDC

The mitochondrial pellet was examined for the activity of glycine decarboxylase by using [1-^{14}C]glycine (specific activity 14.5 Ci mol). The reaction was performed in small (ca. 10 ml capacity) injection vials with centre wells. Filter paper wicks wetted with hyamine hydroxide were placed in the centre wells (for trapping the released CO_2).

The vials were sealed with rubber septa. The assay medium (1 ml), modified from that of Oliver (1979), consisted of 50 mM Tris-HCl (pH 7.8), 2 mM NaN03, 0.3 M sorbitol, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM MgCl2, 0.5 mM KH_2PO_4, 2 mM DTT, 0.1 mM pyridoxal 5-phosphate, 1 mM NAD, 10 mM glycine and crude mitochondria (corresponding to 2.5-5.0 µg protein). The reaction was initiated by adding [1-^{14}C]glycine (specific activity of 14.5 Ci mol^{-1}, final 0.5 µCi), allowed for 30 min in a shaker water bath at 30°C, and was stopped by adding 0.2 ml of 40% (v/v) perchloric acid. The injection vials having the reaction medium were left in shaker water bath for a further period of 30-60 min. The centre wells along with the filter paper wicks were kept in scintillation vials containing 10 ml Bray's mixture and the radioactivity was measured in a liquid scintillation counter.

Assay of SHMT

The mltochondrlal preparation was further processed (Woo 1979) for assaying SHMT. The initial mltochondrlal pellet was resuspended in 20 mM phosphate buffer (pH 7.5) and 1 mM DTT. Then

the fraction was solubulized by preincubation with 0.05% (v/v) Triton X-100 at 0°C for 10 min before use.

The enzyme was assayed at 25°C by using [U-^{14}C]serine (specific activity 135 Ci mol^{-1}) and determining the incorporation of radioactivity into N,N-bismethylene tetrahydrofolate. The assay medium (1 ml) consisted of 20 mM phosphate buffer (pH 7.4), 1 mM β-mercaptoethanol, 1 mM EDTA, 0.1 mM pyrldoxal 5-phosphate, 2 mM tetrahydrofolate, 2 mM DTT and mitochondrial fraction, equivalent to 2.5-5.0 μg protein (Taylor and Weissbach, 1965). The reaction was started by the addition of [U-^{14}C]serine (0.5 μCi) and terminated after 5 mln by adding 0.5 ml of 0.4 mM dimedone in 50% (v/v) ethanol. The samples were heated in a boiling water bath for 5 mln, then cooled in ice bath for 5 min. The radiolabelled dimedone derivative was extracted with 5 ml of toluene. After centrifugation, 3 ml of the top toluene phase was added to 10 ml of scintillation fluid (0.5% PPO in a mixture of toluene and methanol, 1:3 v/v) and the radioactivity was measured using a liquid scintillation counter.

MITOCHONDRIAL (RESPIRATORY) ENZYMES

Extraction

The leaf pieces (1 g) were homogenized in 4 ml of prechilled extraction medium containing 25 mM Hepes-KOH (pH 7.2), 0.3 M sucrose, 1 mM EDTA, 1 mM MgCl2, 0.1% (w/v) BSA and 0.6% (w/v) insoluble PVP. The homogenate was passed through two layers of

cheese cloth and centrlfuged at 15,000 *g* for 15 min. An aliquot was kept aside prior to centrlfugatlon for chlorophyll (Arnon 1949) and protein (Lowry *et al*. 1951) estimations.

Citrate svnthase (EC 4.1.3.7)

Citrate synthase was assayed according to the method of Srere (1969). The enzyme was measured by monitoring the formation of the free -SH groups from acetyl CoA, by using 5,5'-dithiobis-2-nitrobenzoate (DTNB).

The reaction mixture of 1 ml contained 50 mM Tris-HCl (pH 8.1), 0.1 mM DTNB, 0.03 mM acetyl CoA and enzyme extract (equivalent to 5 µg Chl or mesophyll protoplasts or bundle sheath cell extract). The absorption at 412 nm was followed for three min to measure possible acetyl CoA deacylase activity. The reaction was started by the addition of 50 µl of 10 mM OAA. The activity was calculated by using the molar extinction coefficient (13.6 mM" cm) of mercaptide ion.

Isocitrate dehvdrogenase (EC 1.1.1.41)

The enzyme was assayed by monitoring the reduction of NAD at 340 nm (Cox 1969). The reaction medium contained 50 mM Hepes-KOH (pH 7.6), 1 mM $MnSO_4$, 0.67"/. NAD and 0.025% (v/v) Triton X-100. The reaction was started by adding L-isocitrate to a final concentration of 4 mM. The molar extinction coefficient of NADH (6.2 mM cm^{-1}) was used for calculating the activity.

Succlnate dehydrogenase (EC 1.9.3.1)

The assay was done spectrophotometrically by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm and using PMS to couple electron transfer between succlnate and 2,6-DCPIP. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.6), 0.005V. (w/v) PMS, 25 μM DCPIP, 3 mM KCN and 0.025% (v/v) Triton X-100. The reaction was started by the addition of sodium succinate to make a final concentration of 40 mM (Veeger *et al*. 1969). The activity was calculated by using the molar extinction coefficient of DCPIP (21 mM^{-1} cm^{-1}).

Fumarase (EC 4.2.1.2)

The enzyme was assayed by following the conversion of L-malate to fumarate (Hill and Bradshaw 1969) as the increase in absorption at 250 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5) and either enzyme extract or mesophyll protoplasts or bundle sheath cells. The reaction was started by adding L-malate (final concentration 50 mM). The activity was calculated by using the molar extinction coefficient of fumarate (2.6 mM^{-1} cm").

Cytochrome c oxidase (EC 1.9.3.1)

Cytochrome c oxidase was assayed according to the method of Cooperstein and Lazarow (1951). Reduced cytochrome was prepared by adding sodium dithionate to 8 mg of cytochrome c in 20 ml of 10 mM potassium phosphate buffer (pH 7.0) till the colour turned to pink. The reduced cytochrome was dialysed against 10 mM potassium

phosphate buffer overnight. The reaction medium (1 ml) contained 100 mM potassium phosphate buffer (pH 7.0), 0.025% (v/v) Triton X-100 and either leaf extract or bundle sheath cells or mesophyll protoplasts. The assay was started by the addition of reduced cytochrome (35 μM) to the sample cuvette. The decrease in absorbance at 550 nm was monitored. The activity was calculated by using the molar extinction coefficient of reduced cytochrome c at 550 nm (28.0 mM^{-1} cm^{-1}).

Extraction, estimation and **metabolism** of photorespiratory metabolites

AMMONIA

Leaf discs accumulate ammonia upon illumination, particularly in the presence of L-methionine sulphoximine (MSO), an inhibitor of glutamine synthetase (GS). However, a variety of sources contribute to ammonia accumulation in presence of MSO (Singh *et al*. 1985). Photorespiratory inhibitors like INH (inhibits glycine decarboxylase) and α-HPMS (inhibits glycolate oxidase) are used to distinguish the extent of non-photorespiratory ammonia (Kumar and Abrol 1989, 1990).

Leaf discs of *ca.* 20 mm^2 were cut (using a sharp paper punch) under water from freshly collected leaves. Twenty discs were washed in distilled water and 20 discs were floated in petri-dishes containing 5 ml of test solution (water, 2.5 mM MSO, 35 mM INH, 20 mM a-HPMS, 15 mM glycine or other test combination) and

illuminated at 1000 μmol m^{-2} s^{-1} for 2 h at 30°C. Care was taken to avoid heat during illumination.

At the end of illumination period (for 1 h, unless otherwise mentioned), the leaf discs were washed quickly with water, blotted dry and extracted in 3 vol of 2% (w/v) boric acid (Kumar *et al.* 1984). The extract was centrifuged at 12,000 *g* for 20 min at 4°C.

Ammonia estimation was done in the supernatant by phenol-hypochlorite method (Solarzano, 1969). To 5 ml of the sample, 0.2 ml of phenol solution (10'/. (v/v) phenol in 95V. (v/v) ethanol), 0.2 ml of sodium nitroprusside and 0.5 ml of oxidizing solution (100:25 (v/v) mixture of alkaline sodium citrate and sodium hypochlorite) were added and incubated at 30 C for 60 mln. The blue complex formed in the presence of sodium nltroprusslde was measured at 640 nm in a spectrophotometer. A standard curve prepared with 10-100 nmoles ml^{-1} NH4C1 was used for calculating the NH3 content.

The extent of PR-NH3 accumulation was calculated from the difference in the NH3 levels of samples with MSO and those with (INH + MSO) or (α-HPMS + MSO).

GLYCOLATE AND GLYOXYLATE

10 discs (*ca.* 40 mg) were incubated in different test solutions (water, glycidate, a-HPMS, NaHC03, or a combination of two) and illuminated at 800 μmol m^{-2} s^{-1} for either 1 (glycolate)

or 2 h (glyoxylate). After the incubation the discs were washed quickly in distilled water and frozen in liquid nitrogen. The photorespiratory metabolites were extracted by grinding the leaf discs in 4 vols of prechilled extraction buffer containing 0.1 M phosphate buffer, pH 7.0 and 1 mM EDTA. The extract was cleared by centrifuging at 10,000 *g* for 15 min at 4°C. The supernatant was used for the estimation of either glycolate or glyoxylate.

Glycolate was measured using Calkins' reagent (Calkins 1943). To the 1 ml of test sample or leaf extract, 2 ml Calkins' reagent (18.5 mg of 2-7 dihydroxynaphthalene in 100 ml conc. H_2SO_4) were added and the mixture was incubated for 40 min at 100°C. Glycolate was measured as the absorption at 525 nm (Calkins 1943). Calculations were based on a calibration curve prepared from authentic potassium glycolate (0.1-1 mg ml).

Glyoxylate was determined by treatment with phenylhydrazine, oxidation in an acid medium with ferricyanide, extraction into mixture of chloroform/isoamyl alcohol and monitoring the absorption of formazan derivative (Nirmala and Sastry 1972).

One ml of leaf extract was made up to 3 ml with water and then 1 ml of 400 mM phosphate buffer (pH 7.0) and 1 ml phenylhydrazine-HCl solutions were added. The reactants were mixed well and incubated at 30°C for 5 min. The tubes were cooled to 0°C by immersion in ice-water bath. Then 1 ml of prechilled conc. HC1 was added, followed by 1 ml of freshly prepared 0.15% (w/v) potassium

ferrlcyanide. The tubes were allowed to stand at 30°C for exactly 10 min, following which 7 ml of a 3:2 (v/v) mixture of chloroform/ isoamyl alcohol were added. The two phases of solution were mixed well by vigorous agitation of the tubes by hand for 1 min. The lower layer of chloroform/lsoamyl alcohol which had separated was drawn off, centrifuged briefly at 1,000 g to clarify and the absorbance of this layer was read against a blank (omitting glyoxylate) at 520 nm. Stock solution of 100 μg ml^{-1} of glyoxylate (monosodium salt) was freshly prepared in 0.1 mM EDTA. 1-10 μg ml sodium glyoxylate was used as standard.

Photoresplratory glycolate or glyoxylate were calculated by subtracting the level of glycolate or glyoxylate in the absence of inhibitors from that in the presence of inhibitors (α-HPMS for glycolate and glycidate for glyoxylate).

Validity of methods

These two methods (glycolate and glyoxylate estimations) are reasonably specific for glycolate and glyoxylate (Calkins 1943, Vogels and van der Drift 1970) if proper precautions are taken. The interference due to EDTA was avoided by preparing standards (glycolate) in presence of EDTA. The accuracy and efficiency of the extraction/assay methods were cross-checked by spikes of glycolate or glyoxylate, added to crude leaf extracts. The recovery of such glycolate or glyoxylate was 94-97%.

The Calkins' method (1943) was earlier used to estimate the levels of glycolate in tobacco leaves (Salin and Homann 1973) and in cells of *Chlamydomonas reinhardtii* (Husic and Tolbert 1987). Similarly phenylhydrazine was employed in both plant and animal tissues (Nirmala and Sastry 1972, Creach and Stewart 1982). In order to ensure that these methods do not make an under- or over-estimation, we have checked the possible interference of several organic acids on the estimation of glycolate or glyoxylate. There was no interference of glycolate during the estimation of glyoxylate and *vice versa.* The small interference of other compounds during glycolate estimation (in increasing order of interference) was: glutamate < malate < glycine < succinate < serine < glycerate) did not exceed 8%. The extent of interference during estimation of glyoxylate (in increasing order of interference: glutamate < serine < malate < glycerate < glycine < succinate) was only marginal (<2%).

DECARBOXYLATION OF [$1-^{14}$C]GLYCINE

Leaf discs of ca. 20 mm were punched from freshly collected leaves under water. The leaf discs were blotted dry and floated with adaxial surface upwards in flat injection vials (ca. 10 ml), containing centre wells (5 discs, equivalent to 30-90 µg Chl in each vial). The vials contained 2.5 ml of the buffered medium (0.3 M sorbitol, 0.3 M Mes-KOH pH 5.5, 1 mM KH_2PO_4 and 1 mM $MgCl_2$). The centre wells contained filter paper wicks, wetted with hyamine hydroxide. The vials were sealed with rubber septa.

[1-^{14}C]glycine (specific activity 14.5 Ci mol^{-1}, final 0.5 mCi) was added to the vials at zero time to give a final concentration of 50 mM glycine. The reaction was terminated after 1 h (either in light of 1000 μmol m^{-2} s^{-1} or darkness) at 25°C, by adding 0.5 ml 3 N HC1 (Holbrook *et al.* 1985). After 30-60 min of stopping the reaction, the centre wells along with the filter paper wicks were kept in 10 ml scintillation vials containing Bray's mixture (components as described for RuBP carboxylase) and the radioactivity was measured using a liquid scintillation counter.

Measurement of photosynthetic 02 evolution or respiratory O_2 uptake

Leaf discs (ca. 20 mm) were cut under water from freshly collected leaves and were immediately used. Their photosynthesis and respiration were determined by measuring the evolution/uptake of 02 at 25 °C in a leaf disc electrode (Hansatech Ltd., Kings Lynn, UK). The details of this leaf electrode system are described in literature (Delieu and Walker 1981, Walker 1988).

Eighteen leaf discs were arranged in two concentric layers (six in the inner and twelve in the outer ring) on the upper most capillary mat. Leaf discs were blotted semi-dry before transferring on to the mat. The two capillary mats were each moistened with 100 μl of bicarbonate buffer. The leaf discs were preilluminated for 5 mln at 350 μmol m^{-2} s^{-1} and left in darkness for 5 min. They were then exposed to an increasing series of light intensities ranging from 0 to 900 μmol m^{-1} s^{-1}. The illumination was provided by an array of ultra bright light emitting diodes

modulated by a light source (Model No. LS3, Hansatech, UK) through a computer. The acquisition and processing of data on O_2 exchange by leaf discs was done through computerized software, as described in detail elsewhere (Walker 1988, 1990).

An illustration of the use of computer for acquisition and processing of data on photosynthetic oxygen evolution by leaf discs at increasing photon flux densities is presented in Fig. 2.2.

The concentration of bicarbonate buffer used for moistening the capillary mat was varied to change the levels of C02 in the leaf chamber, for e.g. 0.01 mM and 1 M bicarbonate buffer were used to provide C02 concentrations of 0.03 and *5%* (v/v), respectively.

Purification of glycolate oxidase

Glycolate oxidase was partially purified from leaves of *Pisum sativum* (C3 species), *Parthenium hysterophorus* (C_3-C_4 intermediate) and *Amaranthus hypochondriacus* (C_4 plant).

The purification procedure was based on the method of Ernes and Erismann (1984). All the steps during the purification were done in cold (4 C).

Step 1 (Crude extract)

10 g of freshly collected leaves were homogenized in 30 ml of prechilled 50 mM Tris-HCl buffer (pH 8.3) in a blender for one min in case of pea and *Parthenium* or 3 min in case of *Amaranthus*

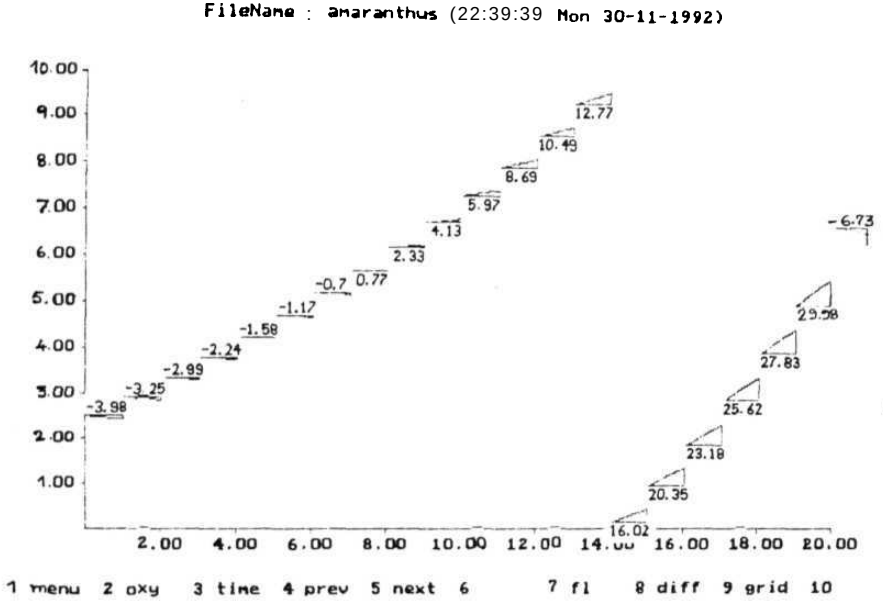


Fig. 2.2. A print-out of computer screen showing recorder traces of oxygen measurements of **a** typical experiment. During each period of 90 **sec, oxygen uptake/evolution by leaf** discs **was** recorded and computed. **Light intensity was** increased progressively (from left to right) by the computer **itself by a** previously determined program. In this experiment 20 **light intensities were** used ranging from 0 to 900 μmol m s and then 0 again. **The measurement** at **'zero'** light intensity represents dark respiration.

hypochondriacus (in order to ensure complete breakage of bundle sheath cells). The medium contained 1% (w/v) insoluble PVP for *Parthenium* and a few mg of acid-washed sand for *A. hypochondriacus*. The homogenate was filtered through two layers of cheesecloth and centrifuged at 15,000 g for 15 min.

Step 2 (Acetic-acid precipitation)

The pH of the supernatant was brought to 5.3 using freshly prepared 10% (v/v) acetic acid, and the mixture was stirred gently for 5 min. The acetic acid extract was clarified by centrifugation at 15,000 *g* for 10 min.

Step 3 (Precipitation with 25% ammonium sulphate)

The pH of the supernatant obtained was adjusted to 8.3 with 1 N NaOH. Finely powdered salt of ammonium sulphate was added to supernatant to reach a 25% saturation of $(NH_4)_2SO_4$. After 30 min of gentle stirring, the suspension was centrifuged at 15,000 g for 15 min and the pellet discarded.

Step 4 (Precipitation with 45% ammonium sulphate)

The supernatant fraction of the Step 3 was adjusted to 45% saturation in $(NH_4)_2SO_4$ by adding the salt. After 30 min of gentle 6tlrrlng, the suspension was centrifuged at 15,000 g for 20 min. The pellet was dissolved in 1 ml of 25 mM Tris-HCl buffer (pH 8.3).

Step 5 (Protamlne sulphate treatment)

Four hundred µl of freshly prepared 2% (w/v) protamlne sulphate solution was added to 1 ml of the enzyme preparation obtained in Step 4, stirred for 10 min, and centrifuged at 15,000 *g* for 10 mln. The pellet (consisting of nucleic acids) was discarded.

Step 6 (Column chromatography on Seralose-6B)

The supernatant from Step 5 was loaded on to Seralose-6B column (5.5 X 2.0 cm), preequilibrated with 5 mM Tris-HCl (pH 8.3). The enzyme was eluted (1.5 ml fractions) using the same buffer at a flow rate of 20 ml h^{-1}. The fractions were assayed for activity and the enzyme was found to appear in the void volume.

Step 7 (65% Precipitation with ammonium sulphate)

The combined fraction containing the enzyme activity (from Step 6) was brought to 65% $(NH_4)_2SO_4$ saturation and stirred gently for 30 min. The suspension was centrifuged at 15,000 g for 20 min. The pellet was dissolved in 500 µl of 25 mM Tris-HCl buffer (pH 8.3).

Step 8 (Chromatographv on Sephadex G-200)

The enzyme preparation obtained in the Step 7 was loaded on to a column of Sephadex G-200 (5.3 X 1.5 cm) equillibrated previously with 5 mM Tris-HCl buffer (pH 8.3). The enzyme was eluted (1.0 ml fractions) using the same buffer at a constant flow rate of 20 ml h^{-1}).

The fractions were assayed for the enzyme activity and fractions containing GO were pooled (Fig. 2.3). The enzyme was transferred into a dialysis bag and concentrated by leaving in finely powdered polyethylene glycol for 2-3 h. The purified enzyme was stored at -20 °C after mixing with an equal volume of glycerol.

The absorption spectrum of purified glycolate oxidase was determined in a UV-Vis 160A Shimadzu Spectrophotometer. The K_M (glycolate), K_I(α-HPMS) and V_{max} were calculated by Eddie-Hofstee method using a BASIC program.

Light activation of PEP carboxylase: Comparative studies

ILLUMINATION OF LEAF DISCS/LEAF HOMOGENATES

Leaf discs

Twenty leaf discs (*ca*. 80 mg) were prepared from freshly collected leaves and were left in darkness for 2 h at 30 C. After the predark treatment, the leaf discs were illuminated at an intensity of 1000 $\mu mol\ m^{-2}$ s~ . An extract was prepared from prilluminated or darkened leaf discs in 4 vols of prechilled extraction buffer and the activity of PEPC was assayed (as indicated for the enzyme in the section on "Extraction and assay of enzymes").

Leaf homogenate

The homogenate prepared by grinding the leaf tissue in 4 vols of prechilled extraction buffer (50 mM Hepes, pH 7.2, 0.3 M sorbitol, 2.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT and 1 mM KH_2PO_4) was illuminated at an intensity of 1000 $\mu mol\ m^{-2}$ s" for 20 min at

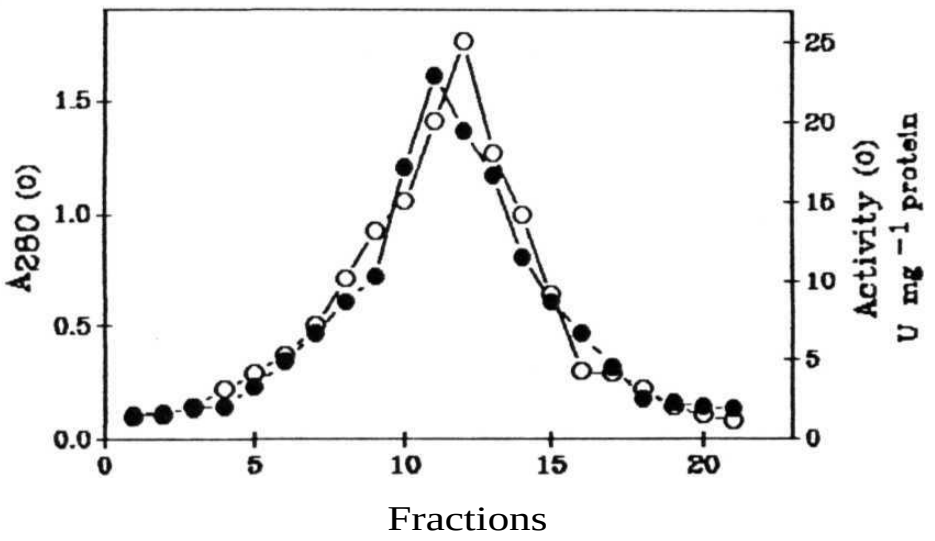


Fig. 2.3. Elution profile of glycolate oxidase from Sephadex G-200 with 5 mM Tris-HCl (pH 8.3) at a flow rate of 10 ml h^{-1} Both specific activity (U mg^{-1} protein h^{-1}) and A280 of the fractions are represented.

30 i 1 C. A water filter was kept between the light source and the leaf homogenate in order to prevent heating.

MAIZE MESOPHYLL PROTOPLASTS

Isolation

leaves were cut into 1 mm thick pieces and kept in freshly prepared digestion medium containing *ZV.* (w/v) cellulase, 0.2'/. (w/v) macerozyme, 10 mM Mes-KOH pH 5.5, 1 mM CaCl2, 1 mM MgCl2, 0.2% (w/v) BSA, 0.5 M sorbitol, and 0.2 mM KH_2PO_4 in a petri dish for 1 h at 30 t 1°C. After incubation, the digestion medium was carefully removed with the help of a Pasteur pipette. A few ml of fresh washing medium, consisting of 20 mM Hepes-KOH, pH 7.2, 0.5 M sorbitol, 1 mM CaCl2, 1 mM MgCl2 and 1 mM KH_2PO_4, were added to the petri dish to submerge the leaf pieces. The petri dish was agitated gently to liberate protoplasts into the medium. The medium was passed successively through 500-, 85- and 60 µm nylon filter cloths. The final filtrate was centrifuged at 100 *g* for 5 min (Remi R8C model) and the pellet was suspended in a small volume of resuspension medium.

Characteristics

The number of protoplasts in the preparation was counted using a haemocytometer. The size of the protoplasts was measured with a precalibrated occular micrometer using a microscope (Wolfe, Japan). The viability and intactness of protoplasts were checked using neutral red and Evans blue, respectively.

Oxygen uptake/evolution

The uptake or evolution of oxygen by protoplasts was monitored at 30°C using a Clark type oxygen electrode (Model DW2, Hansatech Ltd., Kings Lynn, UK). Calibration of oxygen content in the electrode chamber was made with air saturated water, assuming a concentration of 230 nmoles of O_2 per ml at 30°C.

Incubation/illumination

Protoplasts were washed and resuspended in a low (2 mM) or high (20 mM) Hepes-buffered resuspension medium in such a way so as to have the equivalent of 200 µg of Chl ml" . The suspension was kept in darkness for at least 1 h at 0°C and then either maintained in darkness at 30 C or illuminated with tungsten lamps (Philips comptalux 75 W) to provide an intensity of 1000 µmol m s . A water filter was placed between the light source and the protoplast suspension to minimize heating. At the required time, a 50 µl aliquot of protoplasts, equivalent to 10 µg chlorophyll was added to the reaction mixture to assay PEPC.

Mesophyll protoplasts and bundle sheath cells from *Alternanthera tenella,* a C3-C4 intermediate

Isolation

Freshly collected leaves were made into small pieces (*ca*.1 mm) with a sharp blade and were placed in plasmolysis medium containing 10 mM Mes-KOH (pH 6.0), 0.3 M sorbitol and 1 mM $CaCl_2$. After 30 min the leaf pieces were transferred to a digestion medium of 10 mM Mes-KOH (pH 5.5), 0.5 M sorbltol, 2.5% (w/v) Cellulase

'Onozuka' R-10, 0.3% (w/v) Macerozyme R-10, 0.5% (w/v) BSA, 0.2 mM CaCl2, 0.2 mM KH_2PO_4, 1 mM MgCl2, 0.25 mM EDTA and 10 mM sodium ascorbate. The digestion was carried out in petri dishes for 90 min at 30°C in light (200 $\mu mol\ m^{-2}\ s^{-1}$).

After incubation, the digestion medium was removed slowly with a pipette. A few ml of washing medium containing 10 mM Hepes-KOH (pH 7.2), 0.5 M sorbitol, 1 mM CaCl2, 1 mM MgCl2 and 1mM KH_2PO_4 were added to the digested leaf pieces. The petri dishes containing the digested leaf pieces and washing medium were gently tapped, so as to release the protoplasts into the medium. The washing medium containing the protoplasts was filtered through a tea strainer (a pore-width of ca. 500 μm). The leaf pieces were washed again with a few additional ml of washing medium and the protoplasts released into the medium were collected after filtering. This procedure was repeated twice. The combined filtrates were passed through a 60 μm nylon filter (Saryu Textiles, Bombay, India).

The filtrate obtained through 60 μm nylon filter contained mesophyll protoplasts and was further processed. The filtrate was passed through a 20 μm nylon filter. The protoplasts retained on the 20 μm nylon filters were washed into washing medium (as described above) and were collected by centrifugation at 100 *g* for 2 min. The pellet contained mesophyll protoplasts.

Bundle sheath strands were retained on 60 μm nylon filter while preparing mesophyll protoplasts. These were collected by washing into the washing medium (as described above). The medium was centrifuged briefly at 200 *g* for 2 Bin and the pellet contained the bundle sheath strands.

Size and purity

The size of the protoplasts was measured with a precalibrated occular micrometer using a research microscope (Wolfe, Japan).

Contamination of mesophyll protoplasts with bundle sheath cells or *vice versa* (discernible distinctly by their size) was very low as observed under the microscope.

Measurement of photosynthesis or respiration

The assay medium consisted of 10 mM Hepes-KOH (pH 7.2), 0.6 M sorbitol, 1 mM CaCl2, 1 mM MgCl2 and 2 mM KH_2PO_4. The uptake or evolution of 02 in mesophyll protoplasts/bundle sheath cells was monitored at 30°C using a Clark type oxygen electrode (Model DW2, Hansatech Ltd., UK). During measurement of photosynthesis protoplasts were illuminated with red light at intensity of 1000 μmol m^{-2} •" .

Calibration of O2 content in the electrode chamber was made with air saturated water at 30°C (Walker 1988).

Enzyme assays in mesophyll protoplasts and bundle sheath cells

The mesophyll protoplasts were incubated in the assay mixture (without the substrate) prior to the initiation of the reaction for 5 min in order to ensure the complete breakage of the protoplasts. The bundle sheath strands were gently ground in washing medium prior to assay of the enzymes. Unless otherwise mentioned, the enzymes were assayed in a reaction medium of 1 ml.

Replication/Statistical analysis of variation

The experiments were repeated on different days. The data presented here are averages (± SD) of three to six experiments conducted on different days. The variation due to experimental treatments was analyzed for statistical significance using Student's *t*-test.

Chemicals

α-HPMS was from Aldrich Chemical Co, USA; acetyl-CoA, coenzyme A (sodium salt), carbonic anhydrase, Dowex-1 (acetate), Dowex-50 (H), DL-glyceraldehyde, glyceraldehyde-3-phosphate dehydrogenase, glycerate (monosodium salt), glycolate (potassium salt), glyoxylate (monosodium salt), hyamine hydroxide, β-hydroxypyruvate (lithium salt), lactate dehydrogenase, malate (monosodium salt), NAD-malate dehydrogenase, methionine sulfoximine, phospho*enol*pyruvate (monocyclohexylammonium salt), PEP carboxylase (from corn), phenylhydrazine hydrochloride, phosphoglyceric acid (trisodium salt), 3-PGA kinase, phosphoglycolate, protamine sulfate, pyridoxal 5-phosphate,

ribulose 1,5-bisphosphate (sodium salt), tetrahydrofolic acid, and tricine were from Sigma Chemical Co, USA; Dimedon from Fluka, Switzerland; 2-oxoglutarate (disodium salt) from Boehringer-Mannheim, Germany; Sephadex G-200 from Pharmacia Fine Chemicals, Uppsala, Sweden; INH from Citadel Fine Pharmaceuticals, Tamil Nadu; Seralose-6B from Sisco Research Laboratories, Bombay; and 2,7-dihydroxynaphthalene from Sisco-Chem Industries, Bombay; The enzymes for protoplast preparation, Cellulase 'Onozuka R-10' and Macerozyme R-10, were procured from Yakult Honsha Co. Ltd., Tokyo, Japan.

The radioactive chemicals namely [1- C]glycine, [1-^{14}C]glyoxylate, [1-^{14}C]serine, [U-^{14}C]serine and [^{14}C]$NaHCO_3$, were from Board of Radiation and Isotope Technology, Bombay, India.

DCDP and glycidate were gifts from Dr. C.L.D. Jenkins, CSIRO, Canberra, Australia and Prof. I. Zelitch, Connecticut Agricultural Experiment Station, Connecticut, USA, respectively.

All other chemicals were of analytical grade from either Sisco Research Laboratories or Spectrochem, Bombay, India.

Chapter 3

Chapter 3

LEAF CHARACTERISTICS AND ACTIVITY-LEVELS OF PHOTOSYNTHETIC AND PHOTORESPIRATORY ENZYMES

Introduction

C3-C4 intermediate species offer a good model to study the mechanism of photorespiration (Raghavendra 1980, Edwards and Ku 1987, Monson and Moore 1989, Rawsthorne 1992, Raghavendra and Das 1993). While some of these intermediates possess a partial or near complete C4 pathway, others do not show evidence of C4 cycle operation (Edwards and Ku 1987, Rawsthorne 1992, Raghavendra and Das 1993). Apparently photoresplration is reduced in these intermediates due to reasons other than C4 cycle, e.g. a decrease in enzymic capacity, or a different type of CO2 concentrating mechanism.

The levels of key C4 photosynthetic enzymes were 2-5 fold higher in several C3-C4 intermediates of *Flaveria* compared to C3 species (Ku et *al.* 1983, 1991, Hattersley and Stone 1986). The maximum catalytic activities of key photorespiratory enzymes were either comparable to C3 species or partially reduced in the leaves of C3-C« intermediates of *Flaveria* (Moore *et al.* 1988, Ku et *al.*

1991), *Mollugo* (Sayre and Kennedy 1977, Sayre *et al.* 1979) and *Moricandia* (Rawsthorne *et al.* 1988b, Kumar and Abrol 1989). Most of these studies involved a cursory examination of two or three of photosynthetic or photorespiratory enzymes in leaves of C3-C4 intermediates (Ku *et al.* 1983, 1991, Holaday and Chollet 1983, Hattersley and Stone 1986, Cheng *et al.* 1989). However, a detailed examination is yet to be made of photorespiratory enzyme complement in the intermediates of *Alternanthera* and *Parthenium*.

The aim of the present work is to assess the possibility of reduced photorespiration in C3-C4 intermediates of *Alternanthera* and *Parthenium* due to a restriction in enzymic capacity. We have chosen to assess the complement of photosynthetic and photorespiratory enzymes in leaf extracts of the three C3-C4 intermediate species, in comparison with those of the two C3 and the three C4 species. The activity-levels of several photosynthetic and photorespiratory enzymes were assayed in the three C3-C4 intermediate species of *Alternanthera* and *Parthenium* and were compared with C3 *{A. sessiles)* and C4 *(/. pungens)* species of *Alternanthera.* The enzyme complement of *Pisum sativum* (C3), Zea *mays* (NADP-ME type C4 monocot) and *Amranthus hypochondriacus* (NAD-ME type C4 dicot) were included as further references. The leaf anatomy was studied in order to establish the extent of development of Kranz anatomy in the intermediates in relation to other C3 or C4 species.

Results

Leaf anatomy

The cross-sections of leaves from *A. sessiles* showed normal C3 type leaf anatomy. The mesophyll was diffuse and composed of palisade and spongy parenchyma. There were no bundle sheath cells (Fig. 3.1A). The C4 species *Alternanthera pungens* showed typical **'Kranz'** anatomy, with two different photosynthetic cells, namely mesophyll and bundle sheath cells. The bundle sheath cells were distinct with numerous chloroplasts (Fig. 3.1B).

In contrast to C3 and C4 species, the three C3-C4 species (*A. ficoides*, *A. tenella* and *Parthenium hysterophorus)* showed an intermediate status ('partial Kranz' anatomy), with a chlorenchymatous bundle sheath layer, but these cells were not as distinct as in C4 plants. The chloroplasts in bundle sheath cells of these intermediates were arranged in a centripetal manner. The number of bundle sheath cells in each cross-sectional area of the leaf was considerably less in intermediates than in their C4 counterparts (Fig. 3.1 C to E).

Chlorophyll and protein content

The chlorophyll and protein (per unit fresh weight) content of leaves was similar among the plant species studied. However, the Chl *a/b* ratio increased progressively from C3 plants to C3-C4 intermediates and C4 species (Table 3.1). The C3 species had the lowest Chl a/b ratio of 2.75, while the C4 species had the highest ratio of nearly 3.5. The C3-C4 intermediate species had the

Fig. 3.1. Photomicrographs of cross-sections of leaves from A: *Alternanthera sessiles* (C3 plant), B: *Alternanthera pungens* (C4 species), C: *Alternanthera tenella,* D: *Alternanthera ficoides* and E: *Parthenium hysterophorus* (C to E are C_3-C_4 intermediates). Bundle sheath (BS) cells are indicated by arrows. The horizontal bar represents 50 μm.

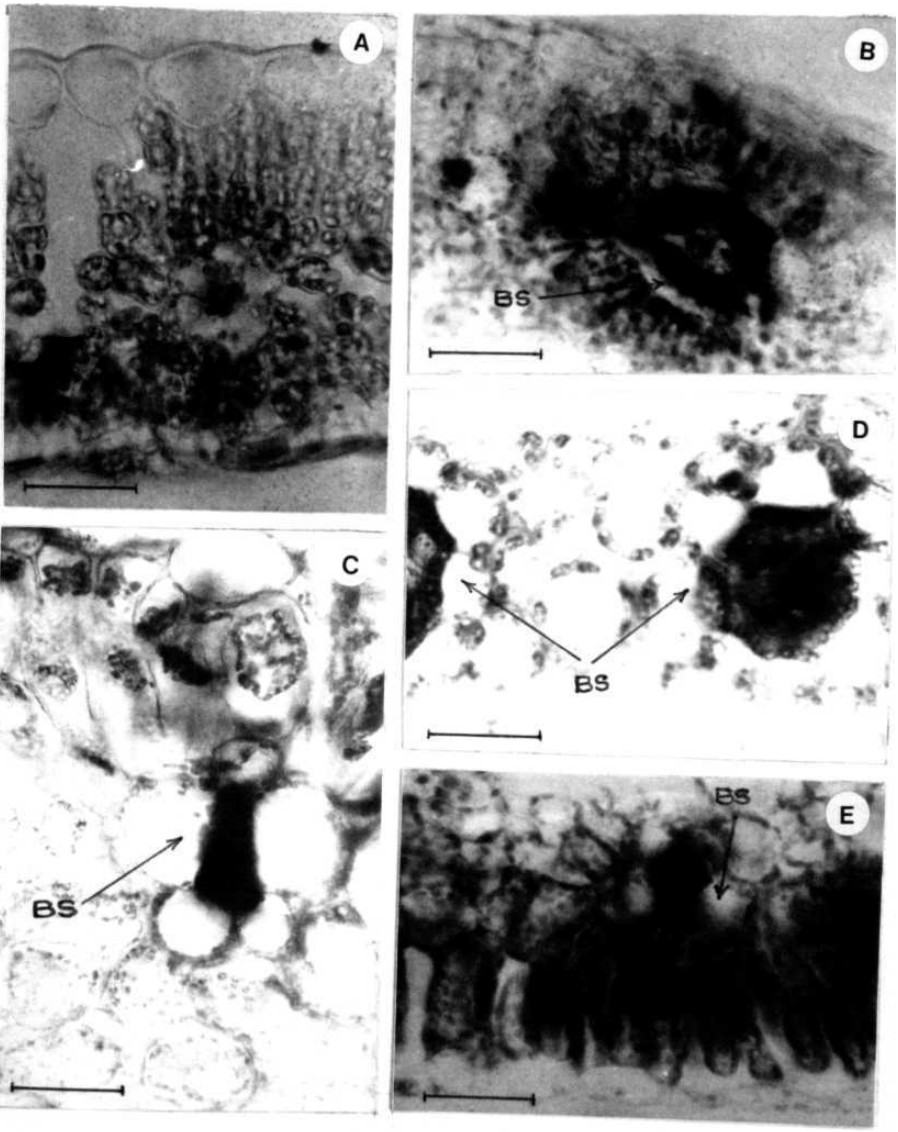

A
B
BS
D
BS
C
BS
BS
E

Table 3.1. Total chlorophyll, protein, Chl *a/b* and protein/ chlorophyll ratios in C_3, C3-C4 intermediate and C_4 species

Type/Species	Total Chlorophyll	Chl *a/b* ratio	Soluble protein	Protein/ Chlorophyll
	mg g F.wt		mg g F.wt	
C3 SPECIES				
Alternanthera sessiles	1.50	2.65	7.94	5.29
Pisum sativum	1.58	2.85	8.84	5.59
Average	1.54	2.75	8.12	5.44
C_3-C_4 INTERMEDIATES				
Alternanthera ficoides	1.46	2.94	8.11	5.55
Alternanthera tenella	1.54	3.06	8.69	5.65
Parthenium hysterophorus	1.50	3.00	7.92	5.55
Average	1.50	3.00	8.24	5.48
C4 SPECIES				
Alternanthera pungens	1.52	3.54	8.89	5.84
Zea *mays*	1.40	3.38	7.97	5.46
Amaranthus viridls	1.40	3.46	7.83	5.59
Average	1.44	3.46	8.23	5.63

intermediate value of 3.0. The protein/chlorophyll ratios were also similar in all the three types of species (Table 3.1).

Enzymes of photorespiration and photosynthesis

The activity-levels of GO, catalase, NADH-HPR and glycerate klnase in leaves of the intermediates were 28 to 35% less than those in C3 species (Table 3.2). The activities of photorespiratory enzymes in C« plants were only a fraction (<18%) of those in C_3 leaves.

The reduction of photorespiratory enzymes was not drastic, but the reduced levels were consistently recorded, during replicated enzyme assays made on different days. The reduction was not due to any endogenous inhibitors since either mixing of leaf extracts or cohomogenation of leaves of pea and the C_3-C_4 intermediates did not show any inhibition of expected activity (Table 3.3).

The activity-level of RuBPO was low in *Parthenium* but this may be due partly to an endogenous inhibitor as coextraction with *Pisum sativum* tissue recovered only 60% of the expected activity (Table 3.4).

The activities of C_4 photosynthetic enzymes were several fold higher in C« species compared to those in C3 species. For e.g., PPDK was very active in C_4 species, but was barely detectable in C3

Table 3.2. Activity-levels of key photorespiratory enzymes in C3, C3–C4 intermediate and C4 species

Type/Species	Glycolate oxidase	Catalase	NADH-hydroxy pyruvate reductase	Glycerate kinase
		μmol mg⁻ Chl h		
C3 SPECIES				
Alternanthera sessiles	126 ± 11	194 ± 18	747 ±71	97 ± 9
Pisum sativum	144 + 14	257 ± 25	710 ± 72	106 ± 10
Average	**135**	226	**729**	102
C3–C4 INTERMEDIATES				
Alternanthera ficoides	9 5 + 9	171 ± 18	507 +48	67 ± 6
Alternanthera tenella	110 + 10	182 + 17	499 ±43	6 9 + 6
Parthenium hysterophorus	80 ± 7	126 ± 11	560 ±53	61 ± 5
Average	95	**160**	522	66
C4 SPECIES				
Alternanthera pungens	12 ± 1	48 ± 4	459 ±41	19 ± 1
Zea mays	18 ± 1	27 + 2	396+38	16 + 1
Average	**15**	38	**408**	**18**

• , -1 .-1
mmol mg h .

Table 3.3. Glycolate oxidase and NADH-hydroxypyruvate reductase activities in co-homogenized leaf extracts or mixed leaf extracts of pea and C3-C4 intermediate species

Species combination	Glycolate oxidase		NADH-hydroxypyruvate reductase	
	Expected	Measured	Expected	Measured
		μmol mg^{-1} Chl h^{-1}		
Cohomogenation				
Pisum sativum + *Alternanthera tenella*	113	106 (94)	604	575 (95)
Pisum sativum + *A. ficoides*	108	104 (96)	608	566 (93)
Pisum sativum * *Parthenium hysterophorus*	118	115 (97)	635	610 (96)
Mixed leaf extracts				
Pisum sativum + *Alternanthera tenella*	127	115 (91)	605	571 (94)
Pisum sativum + *A. ficoides*	120	112 (93)	614	589 (96)
Pisum sativum + *Parthenium hysterophorus*	112	99 (88)	635	608 (96)

Values in parentheses indicate '/. control.

Table 3.4. Activity-level of RuBP oxygenase in co-homogenized leaf extracts or mixed leaf extracts of pea and *Parthenium hysterophorus*

	RuBPO activity (μmol mg^{-1} Chl h^{-1})	
	Expected	Measured
Pisum sativum	-	35.5
Parthenium hysterophorus	-	4.3
Pisum sativum * *Parthenium hysterophorus*		
(i) Co-homogenation	19.9	11.5 (58%)
(ii) Mixed after extraction	19.9	11.9 (60%)

Values in parentheses indicate *V.* of control (i.e. expected values).

species. The activity-levels of key C4 photosynthetic enzymes (PEPC, PPDK and NAD-ME) were 2 to 7 fold higher in the C3-C4 intermediate species than those in C3 but were not as pronounced as in C4 species. On the other hand, the level of carbonic anhydrase in all the plant species was similar (Table 3.5).

The activity-levels of photorespiratory aminotransferases, namely glutamate-glyoxylate aminotransferase and serine-glyoxylate aminotransf erase, were less (23 to 37%) in the intermediates than those in the C3 species. The activities of asp AT and ala AT were slightly increased (60 to 130% over C3 species) in C3-C4 species of *Alternanthera* and *Parthenium* (Table 3.6).

A histogram of the range of activity-levels of photorespiratory enzymes in the three photosynthetic groups is presented Fig. 3.2), to emphasize the reduction in some of these key enzymes. The actual values of these photorespiratory enzymes in each species are given in Table 3.2.

The ratio of PGA- to P-glycolate phosphatase confirmed the reduced photorespiratory enzymic capacity in the C3-C4 intermediate species. However, the ratios of PEPC/RuBPC and PEPC/RuBPO indicated that the intermediates were similar to the C3 species (Table 3.7).

Table 3.5. Activity-levels of key photosynthetic enzymes in C3, C3-C4 intermediate and C4 species

Type/Species	Carbonic anhydrase*	PEP carboxylase	PPDK	NAD-malic enzyme
		μmol mg^{-1} Chl h^{-1}		
C3 SPECIES				
Alternanthera sessiles	83 + 8	24 + 2	2 ± 0.2	54 + 5
Pisumsativum	98 + 9	29 + 3	3 ± 0.3	69 ± 6
Average	91	27	3	62
C3-C4 INTERMEDIATES				
Alternanthera ficoides	75 ± 7	71 + 7	19 ± 2	109 + 9
Alternanthera tenella	83 + 8	71 + 7	29 + 3	144 + 10
Parthenium hysterophorus	83 ± 8	55 + 5	13 ± 2	70 + 6
Average	80	66	20	**108**
C4 SPECIES				
Alternanthera pungens	86 ± 8	702 + 70	294 ± 29	293 + 17
Amaranthus hypochondriacus	85 + 8	908 + 81	285 ± 27	241 *t* 23
Zea *mays*	83 ± 8	348+34	**110** ± **11**	ND**
Average	**85**	653	**345**	**267**

* mmol mg^{-} Chl h^{-} .

ND: not determined.

Table 3.6. Activity-levels of aminotransferases in C3, C3-C4 intermediate and C4 species

Type/Species	GGAT	SGAT	Asp-AT	Ala-AT
		μmol mg Chl h		
C3 SPECIES				
Alternanthera sesslles	58 ± 5	64+6	170 ± 16	121 ± 12
Pisum sativum	40 ± 4	55 ± 5	164 ± 16	145 ± 14
Average	**49**	60	**167**	133
C3-C4 INTERMEDIATES				
Alternanthera ficoides	29 ± 2	47 ± 4	204 ± 19	314 ± 31
Alternanthera tenella	34 ± 3	41+4	238 ± 21	336 ± 32
Parthenium hysterophorus	31+3	49 ± 4	333 + 32	279 ± 25
Average	**31**	**46**	258	310
C4 SPECIES				
Alternanthera pungens	23+2	9 ± 0.6	1213 + 95	1012 ± 90
Amaranthus hypochondriacus	19 ± 1	7+0.5	1420 + 99	1160 ± 90
Average	**21**	**8**	**1317**	1086

GGAT - glutamate-glyoxylate aminotransferase; SGAT - serine-glyoxylate aminotransferase; Asp-AT - aspartate aminotransferase; Ala-AT - alanine aminotransferase.

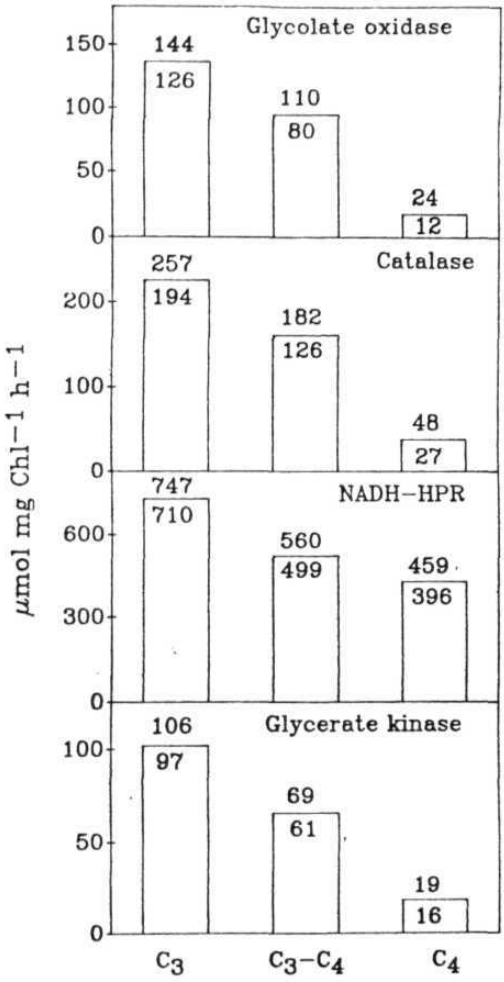


Fig. 3.2. The averaged activities of four photorespiratory enzymes, glycolate oxidase, catalase, NADH-hydroxypyruvate reductase and glycerate kinase in C3, C3-C4 intermediate and C_4 species. The species examined were two C3 (*Alternanthera sessiles, Pisum sativum*), three C3-C4 intermediates (*Alternanthera ficoides, A. tenella, Parthenium hysterophorus*) and three C_4 [*Alternanthera pungens, Amaranthus hypochondriacus, Zea mays*). The figures on each bar indicate the range of activities for each photosynthetic type. The activity of catalase is represented in mmol mg Chl h .

Table 3.7. Ratios of photosynthetic and photorespiratory enzymes in C3, C3-C4 intermediate and C4 species

Type/Species	PEPC/RuBPC*	PEPC/RuBPO	RuBPC/RuBPO	PGAP/PGP
C3 SPECIES				
Alternanthera sessiles	0.07	0.54	**11.2**	0.28
Pisum sativum	0.05	1.12	15.6	0.22
Average	0.06	0.83	**13.4**	0.25
C3-C4 INTERMEDIATES				
Alternanthera ficoides	0.20	3.0	15.7	0.62
Alternanthera tenella	0.18	3.1	15.5	0.58
Parthenium hysterophorus	0.15	3.2	15.9	**0.66**
Average	**0.18**	**3.1**	**15.7**	**0.62**
C4 SPECIES				
Alternanthera pungens	2.90	58.5	20.2	1.7
Zea mays	**4.24**	71.0	16.4	2.3
Average	**3.57**	**64.8**	**18.3**	2.0

*PGP - phosphoglycolate phosphatase; PGAP - phosphoglycerate phosphatase; PEPC - phospho*enol*pyuvate carboxylase; RuBPC - ribulose- 1,5-bisphosphate carboxylase; RuBPO - ribulose-1,5-bisphosphate oxygenase.

Discussion

This is the first study of a profile of several enzymes involved in photorespiratory carbon/amino acid metabolism in the three C3-C4 intermediate species of *Alternanthera* and *Parthenium*, which have been identified as C3-C4 intermediates based on their low Γ values and partial kranz anatomy (Rajendrudu and Das 1981, Rajendrudu et *al*. 1986, Moore *et al*, 1987a).

A partial 'Kranz' anatomy occured in the intermediates of *Alternanthera* (Rajendrudu *et al.* 1986 and Fig. 3.1 C,D) and *Parthenium* (Moore et *al.* 1987 and Fig. 3.1 E). Such partial differentiation of mesophyll and bundle sheath cells in intermediates may result in incomplete compartmentation of photosynthetic and photorespiratory enzymes (Edwards and Ku 1987, Brown and Hattersley 1989, Rawsthorne 1992). Interveinal distances and the number of plasmodesmata were also found to be intermediate in some C3-C4 intermediates (Kanai and Kashiwagi 1975, Morgan and Brown 1979, Brown et *al*. 1983a, Monson et *al.* 1984, Rawsthorne 1992). However, the leaf anatomy of the intermediates is not strictly correlated to their photosynthetic characteristics (Monson *et al.* 1986, Edwards and Ku 1987). The development of a partial Kranz anatomy in the intermediates may form only an initial stage during the evolution of C4 photosynthesis (Brown and Hattersley 1989, Monson and Moore 1989, Raghavendra and Das 1993).

The amount of chlorophyll and protein was similar in all the species, whereas the Chl *a/b* ratio increased progressively from C3 to C3-C4 and C4 species (Table 3.1). In C3 plants the light harvesting chlorophyll of PS I contains mostly Chl a and the light harvesting chlorophyll of PS II is expected to contain both Chl *a* and *b* (Ku *et al.* 1991). The higher ratios of Chl *a/b* reflects the higher energy requirements of C4 plants.

At least four enzymes of the photorespiratory metabolism, *viz.*, glycolate oxidase, catalase, NADH-hydroxypyruvate reductase, and glycerate kinase, were significantly reduced (28 to 35%) in the leaves of these intermediates (Table 3.2). The levels of two aminotransferases (GGAT and SGAT) related to photorespiration also were partly reduced in the intermediates (Table 3.6). Thus, our results strongly suggest that there is a partial reduction in photorespiratory enzymic capacity of the C3-C4 intermediate species of *Alternanthera* and *Parthenium*.

Another indication of decreased photorespiratory enzymic capacity is the ratio of PGA-/P-glycolate phosphatase activities. This ratio is about 2-4 in C4 plants, but is less than one in C3 species indicating that the metabolism of phosphoglycerate is far more active than that of P-glycolate in leaves of C4 plants (Randall *et al.* 1971). The ratios of PGA- to P-glycolate phosphatase (Table 3.7) suggest a decreased potential of metabolizing phosphoglycolate in C3-C4 intermediates. These observations appear to be similar to the limitation in

photorespiratory turnover of glycine was associated with C_3-C_4 intermediacy in *Moricandia arvensis* (Kumar and Abrol 1990).

The reduction observed in the activity-levels of photorespiratory enzymes (glycolate oxidase, hydroxypyruvate reductase) in these intermediate species was not due to any endogenous inhibitors since either coextraction or mixing of extracts had above 90% of the expected activity (Table 3.3). However the reduced activity-level of RuBPO observed for *Parthenium hysterophorus* could be due partly to some endogenous interference since these experiments recovered only 60% of the expected activity (Table 3.4).

Our data may not be able to explain readily the gas exchange phenotype of intermediates and their ability to grow in normal air. Mutants deficient in photorespiratory enzymes (Oliver and Kim 1990, Lea and Blackwell 1990, Somerville 1986) can not grow in air but survive in elevated CO_2. Therefore, the reduction in photorespiratory metabolism is normally detrimental to plants. The reasons for the ability of the intermediates of *Alternanthera* and *Parthenium* to survive in air are not clear, at present.

Our observation that the leaves of C3-C4 intermediates have slightly higher activities of at least three C4 enzymes: PEPC, PPDK and NAD-ME than C3 species (Table 3.5), is at variance with those of Rajendrudu *et al.* (1986) and Moore *et al.* (1987a), who have reported low activities of C_4 enzymes in *Alternanthera* and *Parthenium,* respectively. These enzyme activities could vary due

to several factors including growth conditions, but we are sure of the high levels of C4 cycle enzymes in these intermediates in view of our sufficiently replicated assays. Incidentally, no indication of variability is given in the earlier reports (Rajendrudu *et al.* 1986, Moore *et al.* 1987a). However, we doubt the operation of a typical C4 pathway in our intermediates, since the ratios of PEPC/RuBPC in leaves of *A. tenella*, *A. ficoides* and *P. hysterophorus* are close to those of C3 species (Table 3.7). Similarly, the leaves of *P. hysterophorus* did not incorporate much of C into C4 acids during short-term labelling experiments (Moore *et al.* 1987a).

In C4 plants, the Γ value does not change even at low light intensity, while a marked increase in Γ under low light intensities is expected in C3-C4 intermediate species having a light dependent C02 refixation mechanism (Monson *et al.* 1984). However, the decrease in Γ of *Alternanthera* intermediates under low light (Rajendrudu *et al.* 1986) was not as drastic as that of C3-C4 intermediates of *Panicum* (Brown and Morgan, 1980). We wish to cite this an additional evidence for non-significance of C4 pathway in our intermediates of *Alternanthera* and *Parthenium*.

The extent of increase in activity-levels of photosynthetic enzymes like PEPC, PPDK and NADP-ME over C3 plants varies among the intermediates. The enhanced activity levels of these photosynthetic enzymes correlate with the pattern of ^{14}C labelling during short-term $^{14}CO_2$ fixation (Monson *et al.* 1986, Moore *et al.* 1987b,

Chastin and Chollet 1989) and also conform to the quantitative increases in the PEPC protein levels (Ku *et al.* 1991). This increase in activity or protein levels of C_4 enzymes tends to correlate with the development of kranz anatomy (Ku *et al.* 1983, Holaday *et al.* 1984). The increased activity-levels of PEPC in these intermediates may help in the refixation of photorespired C02 (Bauwe and Chollet 1986, Holaday *et al.* 1981, Devi and Raghavendra 1992a, 1993a), without the involvement of typical C4 pathway.

In *Moricandia arvensis*, the reduced photorespiration is due to the predominant localization of glycine decarboxylase and serine hydroxymethyltransferase in bundle sheath cells, leading to an efficient recycling of photorespiratory CO_2 (Rawsthorne *et al.* 1988a). Our attempts to separate mesophyll and bundle sheath tissues from leaves of *A. ficoides* and *Parthenium hysterophorus* have not been successful. However, studies on mesophyll and bundle sheath tissues of *A. tenella* did not reveal any clear intercellular distribution of photosynthetic enzymes (Chapter 8 of this Thesis). Nevertheless, we wish to draw an attention to the significant reduction in photorespiratory enzymic capacity, along with a small increase in PEP carboxylase/NAD-ME system in the intermediates of *Alternanthera* and *Parthenium.*

Based on the differential localization of photorespiratory enzymes Rawsthorne (1992) proposed a model for photorespiratory mechanism in C3-C4 intermediates. According to this model, the mesophyll cells have the ability to produce glycine but lack the

capacity to decarboxylate it. Therefore glycine will be transported to the bundle sheath cells and further metabolism will occur in these cells. The CO_2 released during glycine decarboxylation will be refixed within the bundle sheath cells reducing the extent of photorespiratory CO2 loss. Serine or its product is transported back to mesophyll cells for further metabolism.

We do not know the exact mechanism which results in partial reduction of enzyme activities in leaves of C3-C4 intermediates. Recently a gradation in the level of NADP-ME transcripts has been noticed among different photosynthetic types (Rajeevan *et al.* 1991). The abundance of NADP-ME transcripts was less in C3 *Flaveria pringlei,* more in C4 *F. trinervia,* but intermediate in C3-C4 *F. linear is* (Rajeevan et *al*. 1991). The expression of NADP-ME in leaves could thus be regulated, at least in part, by the abundance of transcripts and the expression of genes rather than differences in primary structure or activation state of the enzyme. The partial reduction in photorespiratory enzymes in our intermediates can therefore be due partly to reduced abundance of enzyme transcripts or decreased gene expression. Further work is necessary to confirm the exact phenomenon.

The reduction in photorespiration in C3-C4 intermediates is proposed to be due to either an efficient refixation of photorespired CO_2, supported by either anatomical features or the operation of a limited (or near complete) C4 cycle (Edwards and Ku 1987, Monson 1989a, Monson and Moore 1989, Rawsthorne 1992,

Raghavendra and Das, 1993). Based on this criterion, the C3-C4 intermediates were classified into two categories: the first group with an operational C4 cycle (e.g. *Flaveria brownii*) and the second without a C_4 pathway (e.g. *Moricandia arvensis).* However, Rawsthorne (1992) proposed that the confinement of glycine decarboxylase and an enrichment of mitochondria in bundle sheath cells forms the physiological base of all C3-C4 intermediates. We suggest a partial reduction in enzymic capacity could be an additional factor for the reduction in the photorespiratory pathway at least in the intermediates of *Alternanthera* and *Parthenium*.

Chapter 4

Chapter 4

PHOTORESPIRATORY METABOLITES (AMMONIA, GLYCOLATE AND GLYOXYLATE) IN LEAF DISCS AND THEIR RESPONSE TO EXTERNAL BICARBONATE

Introduction

During photorespiration, ammonia is formed from glycine by decarboxylation in mitochondria (Oliver 1981, Oliver et *al.* 1990). Photorespiratory ammonia (PR-NH_3) is an important component during not only photosynthesis and photorespiration but also nitrogen metabolism of C3 plants (Keys *et al.* 1978, Oliver 1981, Miflin and Lea 1980, 1982, Singh *et al.* 1985, Givan *et al.* 1988, Lea *et al.* 1990, Lea and Blackwell 1992, Sechley *et al.* 1992).

PR-NH3 is reassimilated *in vivo* via mainly the GS-GOGAT pathway (Keys *et al.* 1978). Methionine sulfoximine (MSO) is an irreversible inhibitor of glutamine synthetase (EC 6.3.1.2), the enzyme that catalyzes the primary incorporation of NH3 into amino acids. Addition of MSO results in an increase in NH3 particularly in leaves of C3 plants, such accumulation being a reflection of PR-NH3 (Platt and Rand 1982, Berger and Fock 1983, Martin *et al.* 1983, Ikeda *et al* 1984, Rhodes *et al.* 1986, Magalhaes and Huber 1991, Sechley et *al.* 1992).

The extent of NH3 accumulation in the presence of MSO is two to three-fold higher in C3 than in C_4 leaves (Martin *et al.* 1983). Phosphinothricin (glufosinate). another irreversible inhibitor of GS, also causes the accumulation of ammonia in several C3 and C_4 plants (Lacuesta *et al.* 1989, Wendler et *al.* 1990, 1992, Shelp et *al* 1992).

Glycolate and glyoxylate are important intermediates of photorespiration (Zelltch 1979, Lorimer and Andrews 1981, Canvin 1990). Inhibitors of glycolate oxidase (EC 1.1.3.1) like α-hydroxypyridinemethane sulfonate (α-HPMS), butyl 2-hydroxy-3-butynoate (BHB) are used to measure the levels of photorespiratory glycolate (Salin and Homann 1973, Zelltch 1973, 1979, 1988, Servaites and Ogren 1977, Doravari and Canvin 1980, Cho *et al.* 1983). Glycidate is an inhibitor of glutamate-glyoxylate aminotransferase (EC 2.6.1.4) and can be used for determining PR-glyoxylate (Lawyer and Zelitch 1978, Zelitch 1978, 1979). Glycidate also increases the glutamate pool (Zelltch 1978).

Direct estimates of photorespiratory metabolites in C3-C4 intermediates are very few (cf. Holbrook *et al.* 1985, Ku *et al.* 1991), since most of the studies have been made on photosynthetic carbon metabolism. We examined the extent of the accumulation of photorespiratory ammonia, glycolate and glyoxylate in leaf discs of C_3-C_4 intermediates of *Alternanthera ficoides*, *Alternanthera tenella* and *Parthenium hysterophorus* and compared these results

with those from C3 *(A. sessiles, Pisum sativum)* and C_4 *(A. pungens, Amaranthus hypochondriacus, Zea mays)* species. Our findings indicate a reduction in the formation of photorespiratory ammonia and pools of glycolate and glyoxylate in leaves of C3-C4 intermediates of *Alternanthera* and *Parthenium*. We also report a marked modulation of these metabolites in leaf discs by external bicarbonate or glycine.

Results

Photorespiratory ammonia accumulation

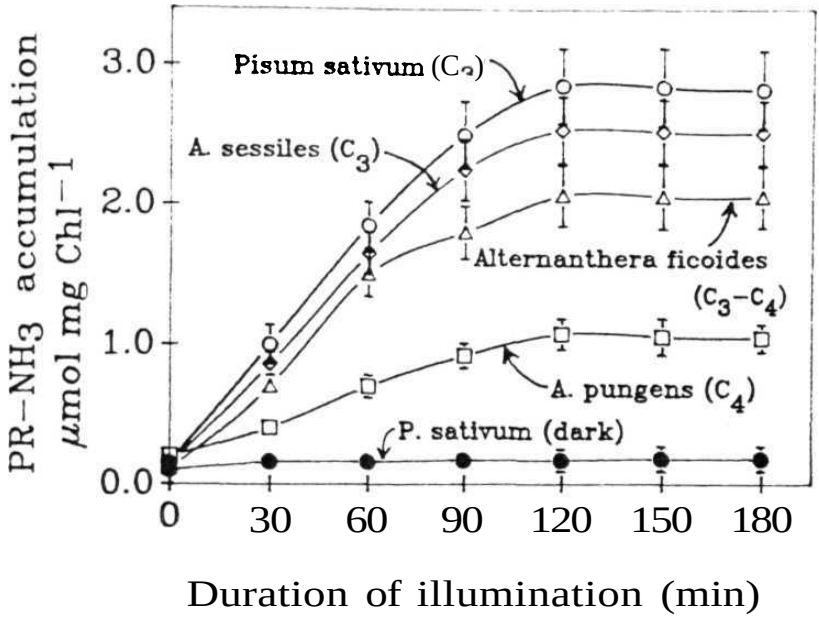
A steady increase in PR-NH3 occurred upon illumination of leaf discs for up to 90-120 min, but there was no such increase when leaves were kept in darkness (Fig. 4.1). The calculation of PR-NH3 is shown in Table 4.1. Accumulation of PR-NH3 was maximum in *Pisum sativum* and *Alternanthera sessiles* (C3), low in *A. pungens* (C4) and moderate in *A. ficoides* (C_3-C_4 intermediate). The average rates of PR-NH3 accumulation, estimated using MSO and either INH or α-HPMS, were reduced in the C3-C4 intermediates by 23-30% (Table 4.2 and 4.3), compared to C3 species, while PR-NH3 in C_4 plants was further lowered (≤ 42% of that in C3).

Effect of external bicarbonate and glycine on PR-ammonia

The presence of bicarbonate in the incubation medium reduced the formation of PR-NH3 in pea leaf discs. The inhibitiory effect of bicarbonate on PR-NH3 accumulation was saturated at 5 mM (Fig.

Table 4.1. An illustration of photorespiratory ammonia (PR-NH3) in leaf discs of *Pisum sativum* (C3 species) determined in presence of INH or α-HPMS

Parameter/ Test combination	Ammonia
	µmol mg Chl h"
1. Water	3.1
2. MSO (2.5 mM)	7.2
3. INH (35 mM)	2.3
4. a-HPMS (20 mM)	1.7
5. MSO + INH	4.6
6. MSO + a-HPMS	3.5
7. PR-NH3 (INH-dependent) (Row 2 - Row 5)	2.6
8. PR-NH3 (a-HPMS-dependent) (Row 2 - Row 6)	3.7



Tig. **4.1.** Photorespiratory ammonia (PR-NH_3) accumulation upon illumination (open symbols) in leaf discs of C3, C_4 and C_3-C_4 intermediate species. Ammonia accumulation in darkness (closed symbols) was negligible.

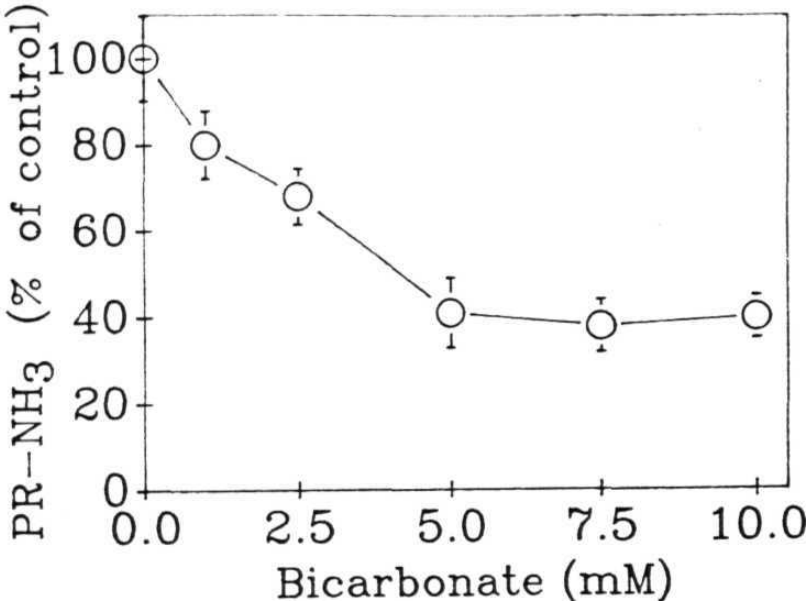


Fig. 4.2. Effect of bicarbonate on PR-NH3 accumulation in pea (*Pisum sativum*) leaf discs. The inclusion of 5 mM bicarbonate in the incubation medium caused a marked decrease in the levels of PR-NH3. The extent of PR-NH3 accumulated in control (water) was 2.36 ± 0.22 μmol mg Chl h .

Table 4.2. Effect of bicarbonate (5 mM) and glycine (15 mM) on accumulation of PR-NH_3 (measured in presence of 35 mM INH) in C3, C_4 and C3-C4 intermediate species. The calculations of PR-NH3 were done as per Table 4.1

Species	Photorespiratory ammonia		
	Water	Bicarbonate	Glycine
		µmol mg Chl h^{-1}	
C3 SPECIES			
Alternanthera sessiles	2.3 ± 0.1	1.0 ± 0.03 (44)*	4.0 *t* 0.4 (174)*
Pisum sativum	2.8 ± 0.2	**1.2** ± 0.05 (43)	4.2 ± 0.4 (150)
Average	2.6	**1.1** **(44)**	**4.1** **(162)**
C_3–C_4 INTERMEDIATES			
Alternanthera ficoides	2.0 + 0.1	1.2 ± **0.06** (60)	4.1 + 0.4 (205)
Alternanthera tenella	1.9 + 0.1	1.1 ± 0.05 (58)	4.2 + 0.3 (221)
Parthenium hysterophorus	2.0+0.1	1.3+0.07 (65)	3.9 ± 0.4 (195)
Average	2.0	**1.2** (61)	**4.1** **(207)**
C_4 SPECIES			
Alternanthera pungens	1.1 ± 0.1	1.0 ± 0.05 (91)	3.4+0.3 (309)
Amaranthus hypochondriacus	1.1+0.1	1.0 + 0.03 (91)	3.3+0.3 (300)
Zea *mays*	1.1+0.1	1.0 + 0.04 (91)	3.4 ± 0.4 (309)
Average	**1.1**	**1.0** **(91)**	**3.4** (306)

Values in parentheses indicate the effect of test compounds as '/. control (i.e. water taken as 100%).

Table 4.3. Effect of bicarbonate on α-HPMS (20 mM) dependent PR-NH3 accumulation in C3, C4 and C3-C4 intermediate species. The calculations of PR-NH3 were done as per Table 4.1

Species	Photorespiratory ammonia		inhibition by bicarbonate
	Water	Bicarbonate	
	µmol mg Chl h		'/.
C3 SPECIES			
Alternanthera sessiles	3.1 ± 0.3	1.6 ±0.1	48
Pisum sativum	3.4 + 0.2	1.8 ± 0.2	47
Average	3.3	1.7	48
C3-C4 INTERMEDIATES			
Alternanthera ficoides	2.3 ± 0.2	1.5+0.2	35
Alternanthera tenella	2.3+0.2	1.4 ± 0.1	39
Parthenium hysterophorus	2.2±0.2	1.5+0.2	32
Average	2.3	1.5	35
C4 SPECIES			
Alternanthera pungens	1.1+0.1	1.1 ± 0.1	0
Amaranthus hypochondriacus	1.1+0.1	1.1+0.1	0
Zea mays	1.2+0.1	1.2+0.1	0
Average	**1.1**	**1.1**	°

4.2). The level of PR-NH_3 accumulation (measured with MSO and either α-HPMS or INH) was markedly inhibited by 5 mM bicarbonate in not only C3 but also C3-C4 intermediate species (Tables 4.2 and 4.3). However, the magnitude of inhibition by bicarbonate of PR-NH_3 formation was less in C3-C4 intermediate species (about 40% decrease over control) than that in C3 species (nearly 55%). The effect of bicarbonate on PR-NH3 was almost negligible (<6% decrease) in C4 species.

Glycine enhanced ammonia accumulation in all the species examined (Table 4.2). Such stimulation by glyclne was maximum (>200%) in C4 species, low in C_3 plants (about 60%) and intermediate in C_3-C_4 intermediates (nearly 100%).

Photorespiratory glycolate and glyoxylate

The levels of photorespiratory glycolate increased steadily with illumination up to 60 min in C3 (*Pisum sativum*, *Alternanthera sessiles)* or C_3-C_4 intermediates *{Alternanthera ficoides*). Very little glycolate accumulated in leaf discs of *A. pungens*, a C4 species. In darkness the glycolate levels remained at a low level in *P. sativum,* a C3 species (Fig. 4.3).

The increase in glycolate due to a-HPMS is considered to determine the PR-glycolate (Table 4.4). The levels of PR-glycolate were very high in C_3 species, reduced by about 30% in C_3-C_4

Table 4.4. Determination of photorespiratory glycolate or glyoxylate in leaf discs of *Pisum sativum*, a C3 plant. The leaf discs were incubated in either water or 5 mM bicarbonate along with or without inhibitors (α-HPMS or glycidate)

Medium of incubation	glycolate/glyoxylate
	μmol mg Chl h^{-}
Experiment 1 :	
1. Water	6.9
2. a-HPMS (10 mM)	45.5
3. PR-glycolate (Row 2 - Row 1)	38.6
4. Bicarbonate (5 mM)	3.5
5. Bicarbonate (5 mM) + a-HPMS (10 mM)	18.7
6. PR-glycolate in presence of bicarbonate (Row **5** - Row **4)**	15.2
Experiment 2 :	
1. Water	7.9
2. Glycidate (20 **mM)**	11.8
3. PR-glyoxylate (Row **2** - Row **1)**	3.9
4. Bicarbonate (5 mM)	7.4
5. Bicarbonate (5 mM) + glycidate (20 **mM)**	9.5
6. PR-glyoxylate in presence of bicarbonate **(Row** 5 - **Row** 4)	2.1

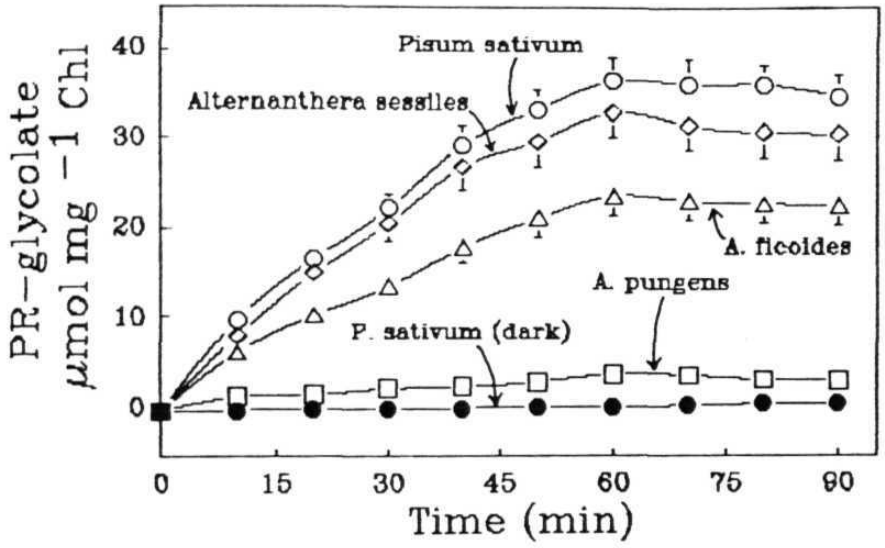


Fig. 4.3. Accumulation of photorespiratory glycolate upon illumination (open symbols) in leaf discs of C3, C4 and C3-C4 intermediate species. Glycolate accumulation in darkness (closed symbols) **was** negligible.

intermediates, and was very low in C_4 species (only about 10% of that in C3 species, Table 4.5).

Similarly PR-glyoxylate was determined in leaf discs by using glycidate in (Table 4.4). The levels of glyoxylate were only a fraction (about 10%) of glycolate. PR-glyoxylate levels in leaves of C3-C4 intermediates were markedly reduced (nearly 30% less than those in C3 plants). The glyoxylate content in leaves of C_4 species was very low and was less than 10% of that in C3 species (Table 4.6).

Effect of external bicarbonate on levels of glycolate and glyoxylate

Similar to PR-NH3, the levels of PR-glycolate and glyoxylate in C3 (*Pisum sativum*, *Alternanthera sessiles)* and C3-C4 intermediate (*A. ficoides*, *A. tenella* and *Parthenium hysterophorus*) species were decreased on incubation with 5 mM bicarbonate (Tables 4.5 and 4.6). However, no such inhibition was observed in C_4 species.

Glycine decarboxylation

The activity-levels of two (mitochondrial) enzymes involved in glycine decarboxylation, namely glycine decarboxylase (GDC) and serine hydroxymethyltransferase (SHMT), were partially reduced (75% of the levels in C3 plants) in C3-C4 intermediate species but were very low (<30% of C3) in C4 plants (Table 4.7).

Table 4.5. Glycolate accumulation in leaf discs of C3-, C4- and C3-C4 intermediates. The leaf discs were incubated in either water or 5 mM bicarbonate along with or without 10 mM α-HPMS. PR-glycolate was calculated as indicated in Table 4.4

Photosynthetic type/Species	PR-glycolate		Inhibition by bicarbonate
	Water	Bicarbonate	
	μmol mg Chl h		%
C3 SPECIES			
Alternanthera sessiles	33.1 +3.0	12.0 ±1.3	64
Pisum sativum	38.8 ± 3.4	15.1 ± 0.9	61
Average	36.0	13.5	63
C3-C4 INTERMEDIATES			
Alternanthera ficoides	23.8 ±2.1	14.1 ±1.1	41
Alternanthera tenella	26.5 ± 2.4	14.5 ± 1.2	45
Parthenium hysterophorus	27.6 ±2.1	13.7 ±1.1	50
Average	**26.0**	14.1	45
C4 SPECIES			
Alternanthera pungens	4.0 ±0.3	4.0 ±0.4	0
Amaranthus hypochondriacus	4.0 ±0.4	3.9 ±0.3	2
Average	4.0	4.0	1

Table 4.6. Glyoxylate accumulation in leaf discs of C3-, C4- and C3-C4 intermediates. The leaf discs were incubated either in water or 5 mM bicarbonate along with or without 20 mM glycidate. PR-glyxoylate was calculated as indicated in Table 4.4.

Photosynthetic type/Species	PR-glyoxylate		Inhibition by bicarbonate
	Water	Bicarbonate	
	µmol mg^{-} Chl h^{-}		%
C3 SPECIES			
Alternanthera sessiles	4.2 ±0.4	1.9 ±0.1	55
Pisum sativum	4.8 ± 0.5	1.9 i 0.2	60
Average	**4.5**	**1.9**	58
C3-C4 INTERMEDIATES			
Alternanthera ficoides	3.1 ±0.2	1.7 +0.1	45
Alternanthera tenella	3.6 t 0.3	1.8 ± 0.2	50
Parthenium hysterophorus	2.7 ±0.3	1.6 ±0.2	40
Average	3.1	**1.7**	**45**
C4 SPECIES			
Alternanthera pungens	0.36 ±0.04	**0.36** 0.03	0
Amaranthus hypochon-driacus	0.37 ±0.03	0.37 0.04	0
Average	0.37	**0.37**	**0**

Table 4.7. Activity-levels of mitochondrial glyclne decarboxylase and serine hydroxymethyl transferase in C3, C_4 and C_3-C_4 intermediate species

Species	Glycine decarboxylase	Serine hydroxy-methyltransferase
	µmol mg protein h"	
C3 SPECIES		
Alternanthera sessiles	3.74 ± 0.21	64 + 4.6
Pisum sativum	4.13 + 0.28	71 ± 6.7
Average	3.94	68
C3-C4 INTERMEDIATES		
Alternanthera tenella	2.78 ± 0.19	48 *t* 4.4
Alternanthera ficoides	2.91 ± 0.21	52 ± 4.6
Parthenium hysterophorus	3.05 ± 0.26	55 ± 4.9
Average	2.91	52
C4 SPECIES		
Alternanthera pungens	0.98 ± 0.05	19 ± 1.1
Amaranthus hypochondriacus	0.93+0.56	18 ± 1.2
Zea *mays*	0.99+0.06	24+2.1
Average	0.97	20

The rates of C02 evolution from [1- C]glycine in darkness were low in C4 leaf discs, intermediate in C3-C4 intermediate species and higher in C3 species (Table 4.8). However, the rates of glycine decarboxylation in the light by C4 species were only a fraction (s 2%) of those in C3 species, while the rates in C3-C4 intermediates were about 20% of those in C3 species. Evidently, the extent of refixation of CO_2 evolved from C-glycine in the light was very high in C4, low in C3 but quite considerable in C3-C4 intermediates.

Discussion

Photorespiration occurs only in the light when phosphoglycolate, the substrate for photorespiration, is formed (Lorimer and Andrews 1981, Ogren and Chollet 1982, Ogren 1984, Husic *et al.* 1987, Givan *et al.* 1988, Sharkey 1988). Accordingly, PR-NH_3 and glycolate accumulated during illumination of leaf discs (Fig. 4.1 and 4.3), demonstrating the dependence of photorespiratory NH3 and glycolate on photosynthetic metabolism. On the other hand accumulation of PR-ammonia and glycolate in the dark was essentially negligible due to the absence of photorespiration. The lowered levels of PR-NH3 accumulation in the C3-C4 intermediates (Tables 4.2 and 4.3) suggest that photorespiratory metabolism is significantly reduced in these species. Earlier reports suggested a reduction in PR-NH3 metabolism in C3-C4 intermediates of *Moricandia arvensis* and *Parthenium hysterophorus* (Kumar and Abrol 1989, 1990).

Table 4.8. Decarboxylation of [1- C]glycine in dark or light and apparent refixation by leaf discs of C3, C4 and C3-C4 intermediate species

Species	Glyclne decarboxylation			Apparent refixation
	Dark	Light	Dark/ Light	
	μmol $^{14}CO_2$ mg^{-1} Chl h^{-1}			(%)
C3 SPECIES				
Alternanthera sessiles	5.88 ± 0.46	2.93 ± 0.18	2.0	50
Pisum sativum	4.97+0.43	2.62+0.16	1.9	47
Average			2.0	49
C3-C4 INTERMEDIATES				
Alternanthera tenella	4.12 ± 0.26	0.65 + 0.02	6.3	84
Alternanthera ficoides	4.41 ± 0.27	0.49 ± 0.03	9.0	89
Parthenium hysterophorus	4.01 + 0.19	0.55 + 0.02	7.3	86
Average			7.5	86
C4 SPECIES				
Alternanthera pungens	2.78 + 0.22	0.05 ± 0.01	56	98
Amaranthus hypochondriacus	2.64+0.25	0.04+0.01	66	99
Zea *mays*	2.54 + 0.18	0.04 + 0.01	64	98
Average			62	98

'/. of photoresplratory C02 refixed in light.

The decrease in the formation of photorespiratory ammonia, glycolate and glyoxylate on incubation of leaf discs in 5 mM bicarbonate (Tables 4.2, 4.3, 4.5 and 4.6) reflects the inhibitory effect of C02 on photorespiration. The primary reaction of photorespiration is the oxygenase activity of Rubisco, which is modulated by the availability of CO2 versus 02 (Lorimer 1981, Ogren 1984, Woodrow and Berry 1988). An increase in the CO2 concentration is expected to increase carboxylase activity, while reducing the oxygenation and thereby photorespiratory metabolism (Reviews: Bowes 1991, 1993, Long 1991). However exogenous bicarbonate did not completely inhibit photorespiratory ammonia accumulation, even in C3 plants. The reason for this is not clear, but could be due partly to photosynthetic 02 evolution in light. The reduced sensitivity of photorespiratory metabolites to bicarbonate is an indication of efficient CO_2 recycling/concentration in the C_3-C_4 intermediates. Bicarbonate had no effect on the accumulation of photorespiratory metabolites in C_4 plants (Tables 4.1, 4.2, 4.5, and 4.6), presumably due to their highly efficient CO_2 concentrating mechanism (Hatch 1987).

Photorespiratory carbon metabolism in leaves of higher plants involves the coordinated functioning of three organelles (viz., chloroplast, peroxisome and mitochondrion) and is closely related to the C3 photosynthetic carbon reduction cycle (Schnarrenberger and Fock 1976, Zelitch 1979, Ogren and Chollet 1982, Ogren 1984, Sharkey 1988, Canvin 1990, Sechley *et al*. 1992).

Therefore, the reduction in photorespiration of C3-C4 intermediates could be due to a limitation in any of the steps/reactions. Data presented in Chapter 3 (of this Thesis) indicated a partial reduction in levels of key photorespiratory enzymes in intermediates of *Alternanthera* and *Parthenium* (Devi and Raghavendra 1993a). The present results reveal that the C3-C4 intermediates have a reduced capacity of not only production but also decarboxylation of glycine (Table 4.8). This may be due in part to the reduction in related enzymes (Table 4.7). The studies of Kumar and Abrol (1990) and Ku et *al.* (1991) also indicated a reduction in the activity-levels of GDC and SHMT in C3-C4 intermediates of *Moricandia* and *Flaveria,* respectively.

The decarboxylation of glycine constitutes a major source of NH3 during photorespiration in soybean (Oliver 1981), barley (Lea *et al.* 1990) and maize leaves (Berger and Fock 1983). The enhancement of NH3 accumulation by exogenous glycine was earlier observed in C3 (mungbean, Kumar *et al.* 1984) and C_4 species (maize, Berger and Fock 1983; *Amaranthus*, Kumar *et al.* 1984). The enhancement of ammonia accumulation by glycine in not only C4 species but also in the intermediates (Table 4.3) suggests that another reason for reduced photorespiration in these plants is a marked limitation in glycine production.

The reduced apparent photorespiration in *Moricandia arvensis,* a C3-C4 intermediate, has been attributed to mostly the

exclusive confinement of GDC in bundle sheath cells (Rawsthorne *et al.* 1988a, Rawsthorne 1992, Morgan *et al.* 1992, 1993). Nevertheless, there are reports of reduced turnover (synthesis and metabolism) of glycine in *Parthenium hysterophorus* and *Moricandia arvensis* (Kumar and Abrol 1989, 1990).

To our knowledge this is the first report on the estimation of glycolate and glyoxylate in leaves of C_3-C_4 intermediates and their modulation by external bicarbonate. Glycolate and glyoxylate measurements were occasionally made in leaves of C_3 plants (Salin and Homann 1973, Zelitch 1973, 1978, 1979, Lawyer and Zelitch 1978, Creach and Stewart 1982) and such attempts in C_4 leaves are very few (Zelitch 1973).

Low levels of photorespiratory metabolites and reduced response to α-HPMS and glycidate reflect the reduced photorespiration in C_3-C_4 intermediates. Variation in glycolate and glyoxylate accumulation correlate with the variation in the extent of photorespiration in these three photosynthetic types. Glycolate levels are reported to be high in C_3 species such as tobacco but are very low in C4 species like maize (Zelitch 1973). Reduced incorporation of CO_2 into glycolate, compared to C3 species, has been noticed in C_3-C_4 intermediates of *Panicum milioides* (Servaites *et al.* 1978) and *Parthenium hysterophorus* (Moore *et al.* 1987a). Our estimates of glycolate in C_3 and C_4 species are similar to the earlier reports (Salin and Homann 1973, Zelitch 1973).

The decrease in C02 evolution from exogenous glycine in the light is interpreted to be due to the internal refixation of released CO2 by photosynthesis. Therefore, the ratio of glycine decarboxylation in dark to light provides an apparent measure of the extent of recycling of photorespiratory CO_2 (Holbrook *et al.* 1985, Kumar and Abrol 1990). This ratio in C3-C4 intermediates is nearly four times greater than that in C3 species, indicating a significant refixation of CO_2 in *vivo.* In C4 species, the ratio is very high (Table 4.8) due to the efficient carbon fixation mechanism of C_4 photosynthesis.

We suggest that the reduced photorespiration in C3-C4 intermediates of *Alternanthera* and *Parthenium* is due both to a decrease in glycine formation/decarboxylation and an increase in the capacity for internal refixation of photorespired CO2 in the light. Significant levels of PEP carboxylase in leaves of these intermediates (Devi and Raghavendra 1993a) may lead to enhanced CO_2 refixation and subsequent recycling through NAD-ME and Rubisco. The reduced sensitivity of PR metabolites to exogenous bicarbonate is an indication of an efficient CO2 recycling mechanism in these C3-C4 intermediates.

Chapter 5

Chapter 5

CHARACTERISTICS OF PHOTOSYNTHESIS AND RESPIRATION

PART I

STANDARDIZATION OF MEASURING O_2 EXCHANGE BY LEAF DISCS

Introduction

In the present study we used a leaf disc oxygen electrode for monitoring photosynthetic and respiratory O_2 exchange in leaf discs (20 mm area) of our experimental plants. The detailed description of the apparatus "Leaf Disc Electrode" and its functioning are given elsewhere (Delieu and Walker 1981, Walker and Osmond 1986, Walker 1988, 1990).

Initially we standardized the use of leaf disc electrode in order to optimize the conditions to measure photosynthetic O2 evolution rates by small leaf discs. This was necessary as the leaves of some of our test plants (e.g. *Alternanthera pungens)* were too small to allow the use of a single large disc (to occupy the entire chamber). Experiments were therefore designed to assess the number and orientation of leaf discs to be used on the capillary **mat,** buffering capacity of bicarbonate buffer, necessity of carbonic anhydrase and the effect of infiltration of leaf discs.

Results

The rate of 02 evolution was dependent on the arrangement of leaf discs on the capillary mat. Maximum rate of 02 evolution was achieved when leaf discs were arranged in two concentric circles of 6 (inner circle) and 12 (outer circle). The rate of photosynthetic 02 evolution was more when small circular discs were used than that by irregularly sized pieces covering the whole area (Table 5.1).

The quantity of bicarbonate buffer used for wetting the capillary mats was also crucial. An amount of 100 µl buffer was optimal for achieving maximal photosynthetic rates (Fig. 5.1). Any variation (from 100 µl) in the amount of bicarbonate used for wetting the capillary mats lead to a decrease in the rates of photosynthetic 02 evolution.

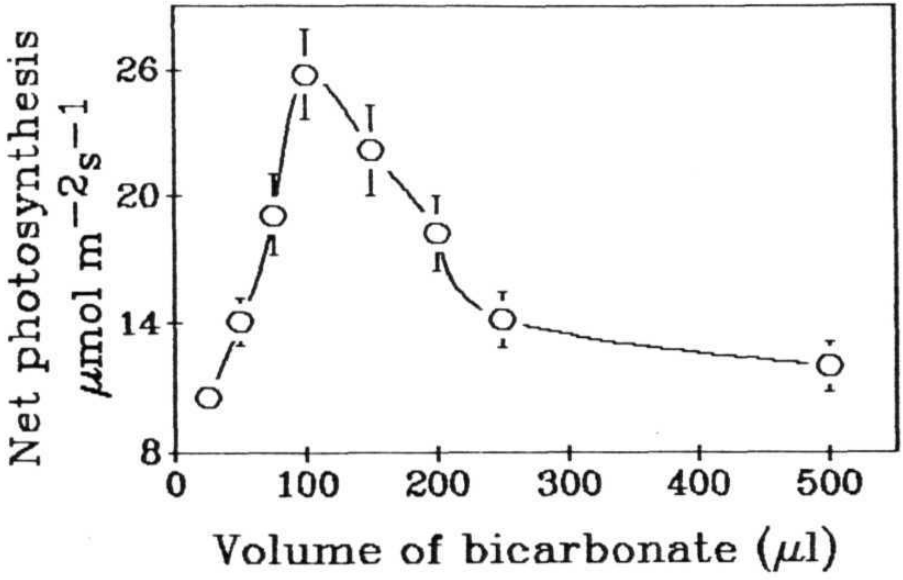
Normally bicarbonate buffer alone (NaHC03 + Na2C03; pH 8.5) was used to wet the capillary mats during measurement of photosynthetic 02 evolution. To check the buffering capacity we supplemented different concentrations of bicarbonate solutions with 25 mM Ches buffer (pH 8.5) in both C3 (*Pisum sativum*) and C4 (*Amaranthus viridis*) species. The Ches buffer had no influence on the photosynthetic oxygen evolution rate in a wide range of bicarbonate concentration tested on these two plants (Table 5.2).

Table 5.1. Rate of photosynthetic 02 evolution (measured at 100 mM bicarbonate) with respect to the orientation and number of leaf discs in *Pisum sativum*. The leaf discs are arranged symmetrically in circles

Leaf arrangement	O_2 evolution	Quantum Requirement
	μmol 02 m s	Quanta mol^{-} 02
1. Whole area (10 cm^2) (leaf pieces)	25.8 + 2.5	10.8 ± 1.0
2. Leaf discs (each of 0.2 cm^2) arranged in circles:		
(a). Three circles (6+12+18)	29.7 + 2.6	10.0 *i* 1.0
(b). Two circles (6+12)	33.7 + 3.3	9.8 + 0.9
(c). Two circles (6+6)	31.6 ± 3.0	10.0 ± 0.9
(d). Single circle (6)	30.1 + 3.0	10.3 ± 1.0

The means of each treatment are significantly different ($P < 0.01$).

No. of leaf discs, in the innermost, middle and outer circles, respectively.



0 100 200 300 400 500
Volume of bicarbonate (μl)

Fig. 5.1. Effect of volume of bicarbonate buffer (used to moisten **the** capillary mats) on net photosynthetlc 02 evolution **in leaf** discs of *Pisum sativum* (a C3 species).

Table 5.2. Effect of supplementing bicarbonate solution **with** 25 **mM** Ches buffer (pH 8.5) on net photosynthesis in leaf discs of *Pisum sativum* (C3) and *Amaranthus viridis* (C_4) at various bicarbonate concentrations

Bicarbonate (mM)	*Pisum sativum*		*Amaranthus viridis*	
	water (control)	buffer	water (control)	buffer
	μmol O_2 evolved m s			
0.01	9.9	10.4 (105)*	21.3	21.5 (101)
0.1	13.2	13.6 (103)	22.1	22.2 (101)
1.0	17.2	17.6 (103)	23.3	23.4 (101)
10	21.2	21.1 (99)	25.1	25.2 (100)
50	23.3	23.5 (101)	25.4	25.2 (99)
100	**25.5**	25.1 (98)	25.7	25.5 (99)

Values in parentheses indicate ⁄. control.

In order to check the effect of carbonic anhydrase on the photosynthetic O_2 evolution rates, experiments were run with different concentrations of carbonic anhydrase (ranging from 3,000 to 10,000 units ml^{-1}) in presence of 0.1 M bicarbonate buffer using *Pisum sativum.* The 02 evolution rate remained constant irrespective of the presence of CA (Table 5.3).

The effect of CA (3,000 units ml^{-1}) on photosynthetic 02 evolution in both and C3 and C_4 plants was studied also at different concentrations of bicarbonate. The photosynthetic rates were insensitive to the presence of CA in both *Pisum sativum* (a C3 species) and *Amaranthus viridis* (a C4 plant) (Table 5.4).

When leaf discs were infiltrated with water (for 5 min) their photosynthetic rates were decreased (at all concentrations of bicarbonate) in both C3 and C_4 species (Tables 5.5 and 5.6). The rate of respiration was unaltered as a result of infiltration of leaf discs (Tables 5.5 and 5.6).

Discussion

Photosynthetic studies were successfully made with leaf segments/pieces (Jones and Osmond 1973, Ishii *et al.* 1977, Imaizumi *et al.* 1990). We used leaf discs in our experiments for photosynthetic measurements. Previous studies with leaf slices and leaf discs of *Lolium* showed similar quantum yields (Wilson *et al.* 1969).

Table 5.3. Effect of different concentrations of carbonic anhydrase on photosynthesis in leaf discs of *Pisum sativum*, using 0.1 M bicarbonate buffer

Addition of carbonic anhydrase	Photosynthetic 02 evolution
	μmol m^{-2} s^{-1}
None (control)	32.1 ± 2.7 (100)"
3,000 units ml"[1]	27.2 ± 2.3 (85)
5,000 units ml"[1]	**28.1 ± 2.1** (88)
8,000 units ml"[1]	27.9 ±2.1 (87)
10,000 units ml"[1]	28.2 + 2.5 (88)

Values in parentheses indicate '/. control.

Table 5.4. Effect of carbonic anhydrase (CA) (3000 units ml"[1]) on photosynthetic 02 evolution in C3 *[Pisum sativum)* and **C4** *{Amaranthus viridis)* species

Bicarbonate (mM)	*Pisum sativum*		*Amaranthus viridis*	
	-CA	+CA	-CA	+CA
	μmol O_2 evolved m s			
0.1	23.8	21.9 (92)*	28.2	26.8 (95
1.0	25.7	22.6 (88)	28.0	26.1 (93
10	27.3	24.8 (91)	29.7	27.0 (91
100	32.1	28.2 (88)	29.8	27.0 (91
1000	32.1	28.2 (88)	29.9	27.1 (91

Values in parentheses indicate '/. control.

Table 5.5. Effect of vacuum infiltration on net photosynthesis and respiration in leaf discs of *Pisum sativum* (C3)

Bicarbonate (mM)	Photosynthesis		Respiration		
	Control	Infiltrated	Control	Infiltrated	
	μmol O_2 evolved/consumed m s				
0.01	9.9 + 0.7	5.2 ± 0.3 (53)*	4.1 ± 0.3	4.0 ± 0.2	(98)
0.1	13.2 ± 0.8	6.3 i 0.5 (48)	3.9 + 0.3	3.7 ± 0.3	(95)
1.0	17.2 + 0.9	7.2 i 0.6 (42)	3.4 ± 0.2	3.3 ± 0.3	(97)
10	21.2 + 1.3	8.3 ± 0.6 (39)	3.3 ± 0.2	3.2 ± 0.3	(97)
100	25.5 + 1.9	8.7 ± 0.6 (34)	3.0 + 0.3	2.8 ± 0.2	(93)
1000	25.8+2.1	5.7+0.3 (22)	2.0+0.1	1.9+0.1	(95)

Values in parentheses indicate *V.* control.

Table 5.6. Effect of vacuum infiltration on photosynthesis and respiration in leaf discs of *Amaranthus viridis* (C_4)

Bicarbonate (mM)	Photosynthesis		Respiration	
	Control	Infiltrated	Control	Infiltrated
	μmol O_2 evolved/consumed m s			
0.01	21.3 ± 1.95	9.3 ± 0.71 (44)*	3.0 ± 0.12	3.0 ± 0.71 (100)
0.1	22.1 ± 2.01	9.9 ± 0.75 (45)	2.9 *t* 0.13	2.8 ± 0.17 (97)
1.0	23.3 + 2.01	10.2 1 0.93 (44)	2.8 ± 0.19	2.9 ± 0.16 **(104)**
10	25.1 ± 2.31	11.6 ± 1.01 (46)	2.7 ± 0.15	2.8 + 0.19 **(104)**
100	25.5 ± 2.36	11.6 ± 1.06 (48)	2.6 ± 0.16	2.4 ± 0.14 (92)
1000	25.8 ± 2.41	12.5 ± 1.03 (48)	2.1 ± 0.14	1.9 ± 0.12 (91)

• Values in parentheses indicate % control.

In our experiments we could not use a single large leaf disc (10 cm area), as was originally suggested (Delieu and Walker 1981), because the leaves of some of the species (for example, *Alternanthera pungens*) were too small to occupy the entire area of the leaf chamber. The orientation of leaf discs was very important, since a uniform illumination of all the leaf discs is needed to get good O_2 exchange measurements (our results; DA Walker, personal communication). Our studies showed that 18 leaf discs (each 20 mm) arranged in two concentric circles of 6 (inner circle) and 12 (outer circle) could accomplish maximum photosynthetic rates (Table 5.1).

It is known that carbonic anhydrase (CA, EC 4.2.1.1) is needed for the dissociation of bicarbonate and to provide free CO2 (Sültemeyer *et al*. 1993), as shown below:

$$C02 + H_2O \xleftrightarrow{\text{Carbonic anhydrase}} HCO_3^- + H^+$$

The gaseous CO2 from this reaction can be used for photosynthesis. The presence of CA is therefore expected to optimize photosynthesis at low or limiting bicarbonate. In the present study there was no effect of CA on photosynthesis in either C3 or C4 species, when different bicarbonate concentrations were tested (Tables 5.3 and 5.4). Similar results were shown with leaf tissues of wheat, barley, *Lemna* and bean (MacDonald 1975). Therefore, we have omitted CA in our further experiments for measuring photosynthesis.

Infiltration of leaf discs with water or bicarbonate buffer decreased photosynthetic O_2 evolution rates in both C3 and C_4 species (Tables 5.5 and 5.6). In tobacco leaf discs about 50% of the photosynthetic 02 evolution was inhibited when leaf discs were floated on water (Glinka and Meidner 1968). This could be due to the decreased diffusion of CO2 into the intercellular spaces of the leaf (Jones and Slatyer 1972). The diffusion coefficient of CO2 in the liquid phases is 10 lower than that in the gaseous phase and results in a severe decrease in the CO_2 supply to the chloroplasts (Jarvis 1971). The impediment to the diffusion of gases through liquid phases may be the reason for also the decrease in photosynthetic rates when the volume of bicarbonate buffer used to moisten the capillary mat was increased above 100 µl.

Respiration was not affected as a result of infiltration of leaf discs with water (Table 5.5 and 5.6). This can be due to the following reasons: (i) the K_M value for 02 uptake permits normal respiration at low O_2; (KM(02) for leaf respiration is 2.5 µM, where as the $K_M(CO_2)$ for photosynthesis was 30 (iM); (ii) water has dissolved $[O_2]$ of 250 µM when it is in equilibrium with water, whereas the $[CO_2]$ was 9 µM; and (iii) the tissue requirement of 02 to maintain its normal respiration is a mere 5%. or less of its normal photosynthetic requirement (MacDonald 1975). The impediment to the diffusion of gases through liquid phases may be the reason for also the decrease in photosynthetic rates, when the volume of

bicarbonate buffer used to moisten the capillary mat was increased above 100 µl.

In addition to these factors stomatal closure may aggravate the difficulty of gas diffusion (as cuticle is impervious to the diffusion of CO_2 and O_2) and result in the inhibition of photosynthesis (MacDonald and Macklan 1975). Therefore infiltration seems to be inappropriate for photosynthetic studies either with leaf segments or leaf discs.

Chapter 5

Part II

REDUCED SENSITIVITY TO CO_2 OF PHOTOINHIBITION AND RESPIRATION IN C_3-C_4 INTERMEDIATES COMPARED TO C_3 SPECIES

Introduction

The C_3-C_4 intermediates of *Alternanthera* and *Parthenium* appear to have no functional C_4 cycle (See Chapter 3 of this Thesis; Rajendrudu *et al.* 1986, Moore *et al.* 1987a, Devi and Raghavendra 1992a, 1993a). The information on the photosynthetic features of these intermediates of *Alternanthera* and *Parthenium* is quite limited (Rajendrudu *et al.* 1986, Moore *et al.* 1987a). The photosynthetic and photoresplratory characteristics of the intermediates of *Flaveria, Panicum, Moricandia* and *Neurachne* are studied in detail (Brown and Brown 1975, Holaday *et al.* 1984, Bauwe 1984, Hattersley *et al.* 1986, Bauwe *et al* 1987, Apel *et al.* 1988, Huber *et al.* 1989, Brown et *al.* 1991, Ku et *al.* 1991). The photosynthetic performance of C3-C4 intermediates of *Flaveria* becomes more efficient than that of C3 plants at low CO2 levels (Chastin and Chollet, 1989). It is not known if such high photosynthetic efficiency at low CO2 occurs in the intermediates of *Alternanthera* and *Parthenium.*

The present study evaluates the characteristics of photosynthesis and dark respiration in leaf discs of the C3-C4 intermediates of *Alternanthera ficoides*, *A. tenella* and *Parthenium hysterophorus* at different CO2 concentrations. While demonstrating that the C3-C4 intermediates are less photoinhibited than the C3 plants at limiting CO_2 (<0.3 mmol mol^{-1}), this chapter describes marked differences between C3, C_4 and the intermediate species in their response of dark respiration to CO2.

Results

Photosynthetic rates in both C3 and C3-C4 intermediate species increased as CO2 levels were raised, and the saturation was reached at 3.6 mmol mol^{-1} CO_2 (Fig. 5.2 A,B). In C4 species photosynthetic rates were already saturated at 0.3 mmol mol CO2 (Fig. 5.2 C). Maximal rates of photosynthesis at high CO2 were similar for all the species.

The rate of photosynthetic O2 evolution by the leaf discs increased along with light intensity in C3 plants (Fig. 5.3 A) and C3-C4 intermediates (Fig. 5.3 B) both at limiting (0.3 mmol mol^{-1}) and at saturating C02 (3.6 mmol mol^{-1}). However, the saturation of photosynthetic capacity occurred at 300 $\mu mol\ m^{-}$ s under limiting CO_2 and at 500 $\mu mol\ m^{-2}\ s^{-1}$ in 3.6 mmol mol CO2. A decrease in photosynthesis occurred on exposure to red light intensities exceeding 500 $\mu mol\ m^{-2}\ s^{-1}$ but only at low CO_2, presumably due to

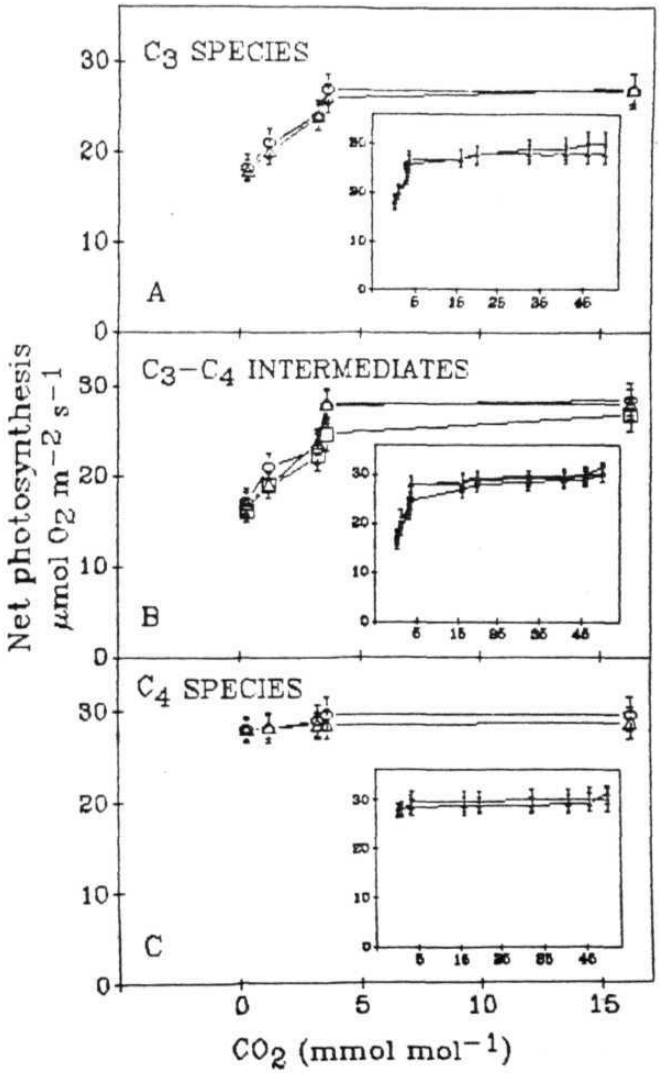


Fig. 5.2. Photosynthetic 02 evolution in response to varying concentrations of CO_2 in C3 (*Pisum sativum*, *Alternanthera sessiles*), **C3-C4** intermediate *[A. ficoides*, *A. tenella*, *Parthenium hysterophorus*) and C_4 (*A. pungens*, *Amaranthus viridis)* species.

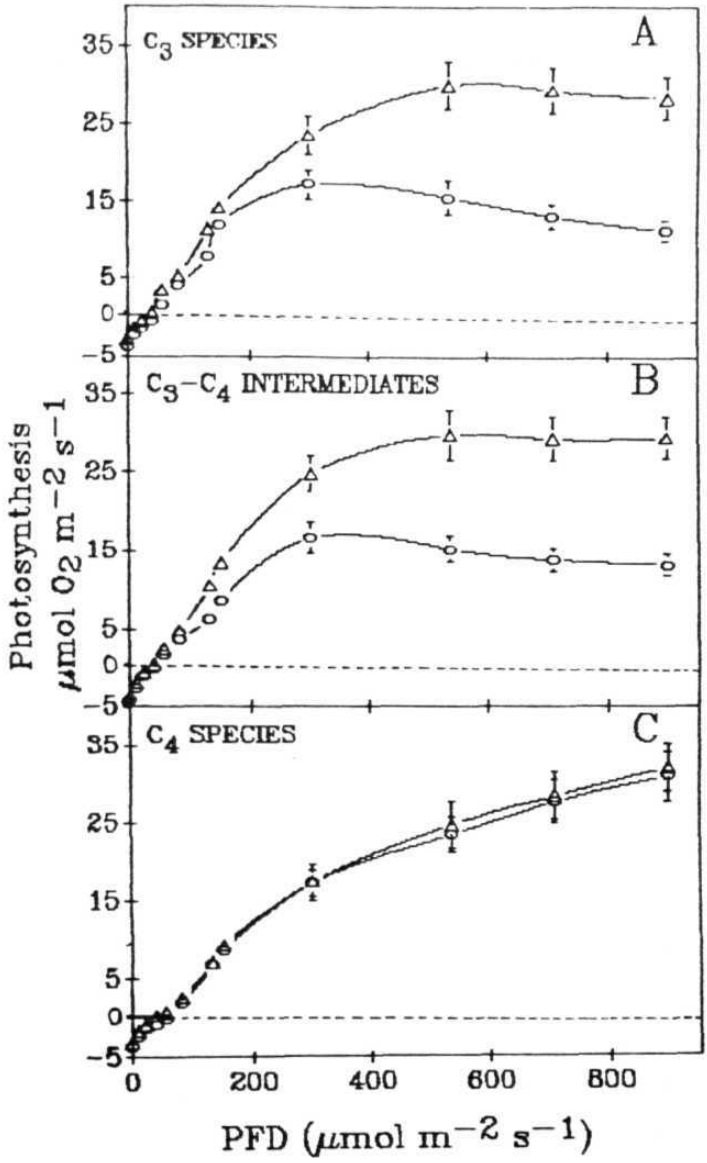


Fig. 5.3. **Photosynthetic** rates as a function of light intensity **In leaf discs of** C_3, C_3-C_4 and C_4 species, at low (0.3 mmol mol , **o-o) or saturating** (3.6 mmol mol^{-1}, Δ-Δ) CO_2 concentration. The **curves are drawn** from **the** averages of data obtained **with two C3 species, three C3-C4 intermediate** species and two C_4 species. **The species are as indicated in Fig. 5.2.**

photoinhibition. In C4 plants photosynthesis was not saturated at 900 μmol m s" at either CO_2 concentration and photoinhibition was not noticed (Fig. 5.3 C).

Increase in CO2 concentration relieved photoinhibition, in both C3 and C3-C4 intermediate species (Figs. 5.3 A,B, 5.4). The extent of photoinhibition and its relief by CO2 were less in the intermediates than those in C3 species. The differences in the extent of photoinhibition in C3 plants and C3-C4 intermediates were statistically significant ($P < 0.001$) at CO2 concentrations in the range of 0.3 mmol mol to 3.6 mmol mol^{-} . The quantum yields of C3-C4 intermediates (0.093 to 0.099 μmol 02 mol"^{1} quanta) were slightly less than those of C3 species (0.093 to 0.1 μmol 02 mol"^{1} quanta), but the differences were not statistically significant (Fig. 5.5).

There was a small (but significant) difference in the rate of dark respiration (Fig. 5.6) at 0.3 mmol mol"^{1} C02 of C3, C4 and C3-C4 intermediate species. As the CO2 level was increased, dark respiration and LCP decreased in all the three types of species. Such inhibition of dark respiration was nearly 40% in C3, 30% in C3-C4 intermediates and only 20% in C4 plants (Fig. 5.6). The differences between these plants in the extent of inhibition by CO2 were highly significant at all CO2 concentrations above 0.3 mmol mol"^{1} ($P < 0.005$). Similarly the shift in LCP due to elevated CO2 was largest in C3 species, intermediate in C3-C4 species and least

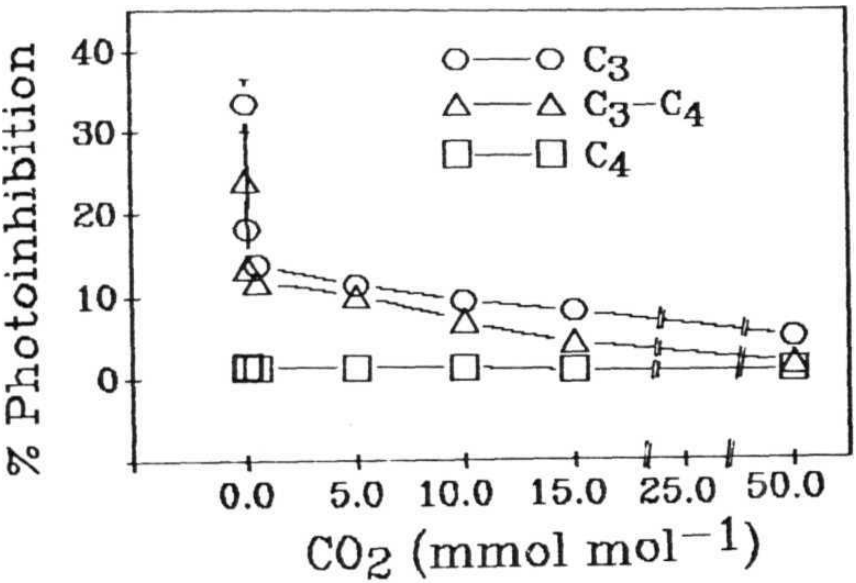


Fig. 5.4. Relief of photoinhibition of photosynthesis at high CO_2 **concentrations in leaf** discs of C3 or C3-C4 intermediate species. Photoinhibition is calculated in relation to maximal photosynthetic **rate of same leaf discs at optimal light intensities. No inhibition of photosynthesis was observed in C4 species.**

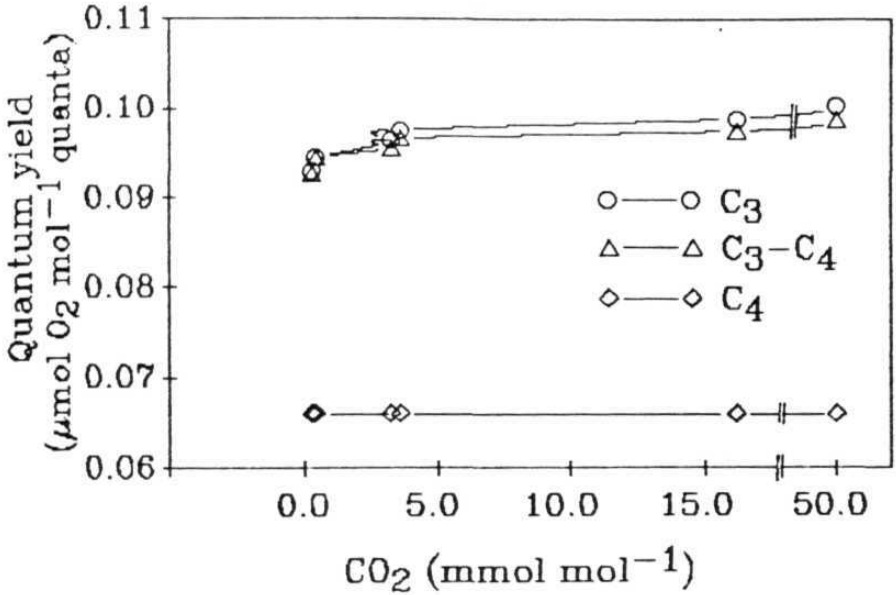


Fig. 5.5. Effect of CO2 on quantum yields of photosynthesis in C_3, C3-C4 and C4 species. The species included in the study are indicated in Fig. 5.2. Other details are as in Fig. 5.3.

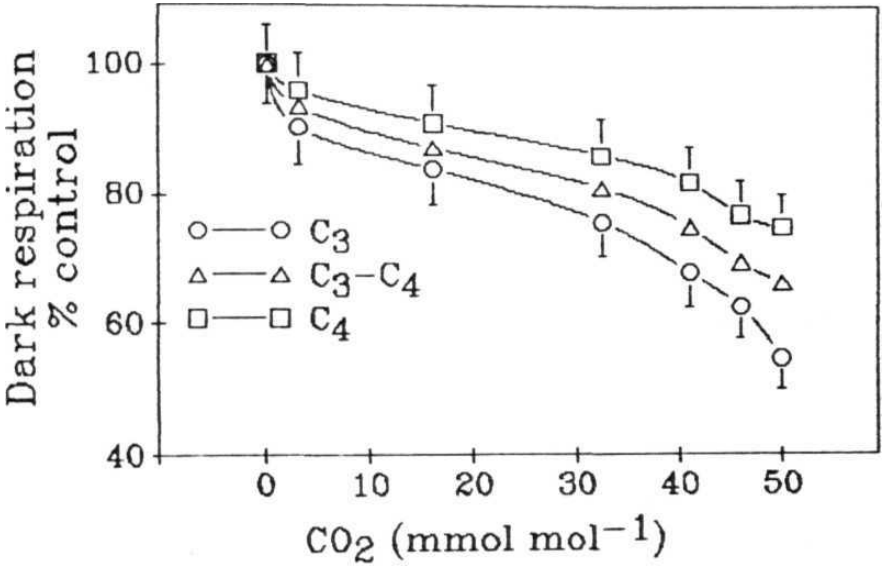


Fig. 5.6. Effect of CO_2 on dark respiration in leaf discs of C3, C3-C4 intermediate and C4 species. Other details are indicated in Figs. 5.2 and 5.3.

in C_4 plants (Fig. 5.7). The differences (in the responses of LCP to CO_2) between different photosynthetlc types also were statistically significant (P <0.005).

The relative predominance of PEP carboxylase in C3-C4 intermediates during photosynthesis at suboptimal or saturating CO_2 concentrations was assessed by using DCDP (an inhibitor of PEP carboxylase). At 2 mM DCDP concentration maximal inhibition of photosynthesis occurred in all the three types of species (Fig 5.8). Therefore 2 mM DCDP was used in further experiments.

Incubation of leaf discs with 2 mM DCDP (1 h) resulted in an inhibition of photosynthesis. The extent of such inhibition of photosynthesis with DCDP was more pronounced in C4 species (89% inhibition) (Table 5.7). In C3-C4 intermediates of *Alternanthera* and *Parthenium,* the extent of inhibition of photosynthesis by DCDP was slightly higher than that in C3 species but much less than that in C4 plants. The differences in inhibitor (DCDP) sensitivity between the C3 plants and the C3-C4 intermediates was significant (P <0.001) only at limiting CO_2 (0.3 mmol mol^{-1}) but not at saturating CO_2 (3.6 mmol mol"). The inhibition with DL-glyceraldehyde was more in C3 species, low in C_4 species and C_3-like in the intermediates (Table 5.8). However, the effect of DL-glyceraldehyde was not statistically different between C3 and C3-C4 species.

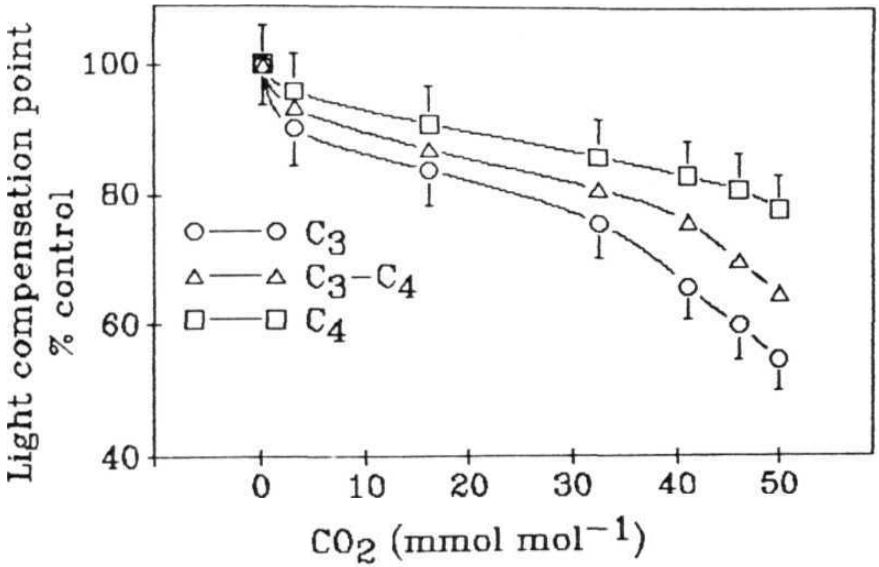


Fig. 5.7. Effect of C02 on light compensation point of **photosynthesis in leaf** discs of C3, C3-C4 intermediate and C_4 **species. Other details** are as indicated in Figs. 5.2 and 5.3.

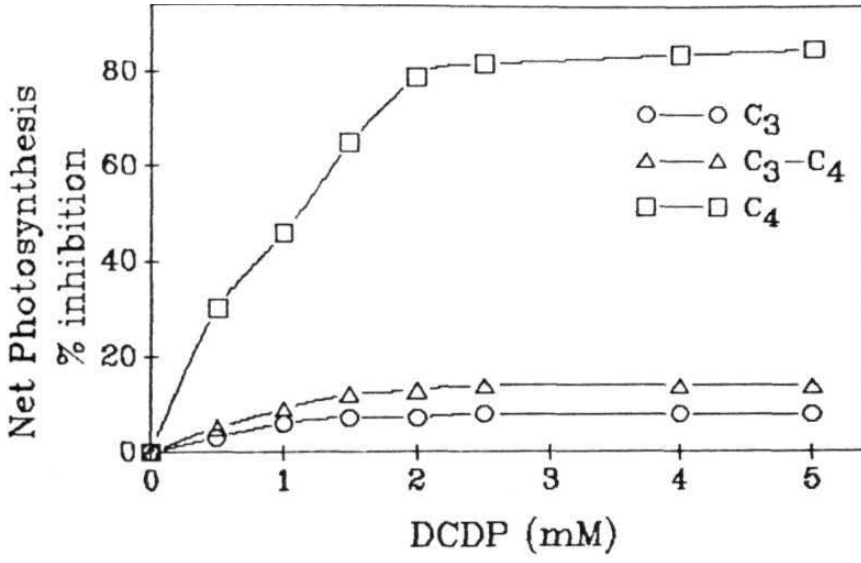


Fig. **5.8. Effect of** DCDP on photosynthesis in leaf discs of C3 (*Pisum* ***sativum),*** **C3-C4 intermediate** (*Alternanthera ficoides)* and **C4** (*Alternanthera* ***pungens)*** **species. The experiments were** done **at low (0.3 mmol** mol^{-1}**) CO2.**

Table 5.7. Effect of DCDP (2 mM) on net photosynthesis in **low** (0.3 mmol mol) and saturated (3.6 mmol mol^{-1}) C02 of C_3, C4 and C_3-C_4 intermediate species

Species	Net Photosynthesis (µmol O2 evolved m~ s)					
	Low CO_2			Saturated CO_2*		
	Control	DCDP	Inhibition	Control	DCDP	Inhibition
			%			'/.
C3 SPECIES						
Alternanthera sessiles	14.0 ±1.1	12.7 ±1.2	9	25.5 ±2.6	23.0 ±2.1	10
Pisum sativum	14.6 ± 1.3	13.6 *t* 1.2	7	27.0 ± 2.5	25.5 ± 2.3	6
C3-C4 INTERMEDIATES						
Alternanthera ficoides	14.5 ±1.4	12.3 ±1.0	15	23.1 ±2.0	21.2 ±1.7	8
Alternanthera tenella	14.9 ±1.4	13.0 ±1.2	13	23.9 ±2.3	21.4 ±2.1	11
Parthenium hysterophorus	13.8 ±1.2	12.1 ±1.1	12	23.1 ±2.0	21.2 ±2.1	8
C4 SPECIES						
Alternanthera pungens	23.8 ±2.2	2.6 ±0.3	89	27.0 ±2.6	4.0 ±0.4	85
Amaranthus hypochondriacus	22.6 ±2.3	2.5 ±0.2	89	26.5 ±2.4	4.6 ±0.5	83
Amaranthus viridis	21.3 ± 2.1	2.6 ± 0.3	88	26.6 ± 2.6	3.9 ± 0.4	85

*The differences in effect of DCDP on photosynthesis in C3-C4 intermediate species were significantly different ($P < 0.05$) from those of C3 species at low (0.3 mmol mol") CO2 but not at high (3.6 mmol mol^{-1}) CO2.

Table 5.8. Effect of DL-glyceraldehyde [100 mM) on net photosynthesis in low (0.3 mmol mol") and saturated (3.6 mmol mol"") C02 of C3, C4 and C3-C4 intermediate species

Species	Net Photosynthesis (μmol 02 evolved m^{-2} s^{-1})					
	Low CO2			Saturated CO2		
	Control	Glyceraldehyde	Inhibition	Control	Glyceraldehyde	Inhibition
			%			%
C3 SPECIES						
Alternanthera sessiles	14.0 ±1.1	4.2 ±0.4	70	25.5 ±2.6	5.4 ±0.4	79
Pisum sativum	14.6 ± 1.3	3.8 ± 0.3	74	27.0 ± 2.5	6.5 ± 0.6	76
C3-C4 INTERMEDIATES						
Alternanthera ficoides	14.5 ±1.4	3.8 ±0.3	71	23.1 ±2.0	6.5 ±0.6	74
Alternanthera tenella	14.9 ± 1.4	5.2 ± 0.5	65	23.9 ± 2.3	5.6 ± 0.6	75
Parthenium hysterophorus	13.8 ±1.2	4.9 ±0.5	63	23.1 ±2.0	7.9 ±0.7	66
C4 SPECIES						
Alternanthera pungens	23.8 ±2.2	12.1 ±0.3	49	27.0 ±2.6	13.2 ±0.4	51
Amaranthus hypochondriacus	22.6 ± 2.3	13.1 ± 0.2	42	26.5 ± 2.4	15.1 + 0.5	43
Amaranthus viridis	21.3 ± 2.1	11.9 ± 0.3	44	26.6 ± 2.6	14.3 ± 0.4	46

Discussion

We report for the first time a marked variation in the extent of inhibition by carbon dioxide (3.6 mmol mol^{-1} or above) of dark respiration in C3-, C4- and C3-C4 intermediate species. Dark respiration was markedly inhibited by the increase in ambient CO2 in all the species. Nevertheless, the extent of such inhibition was more pronounced in C3 than in the C4 species (Fig. 5.6). Although the standard errors were high, the statistical analysis indicated that differences in the inhibition of dark respiration between not only C3 and *Ci* plants, but also those between C3 plants and C3-C4 intermediates were statistically significant *[P* <0.005).

The effects of CO2 on plant respiration has not attracted as much attention as that of photosynthesis. There is a considerable disagreement in the available literature on this aspect (Reviews: Bunce 1990, Amthor, 1991, Eamus, 1991). Elevated CO2 during growth has been reported to either decrease (Gifford *et al.* 1985, Reuveni and Gale, 1985) or increase (Hrubec *et al.* 1985) or has no effect (Poorter *et al.* 1988) on dark respiration. However, most of these reports deal with long-term responses and some with tissues other than leaves.

Studies on short-term responses of respiration to CO_2, particularly in green tissues are extremely limited and these also are contradictory. Some authors have reported an inhibition by

high CO_2 of dark respiration in leaf epidermal strips (Shaish et al. 1989) and alfalfa leaves (Reuveni and Gale, 1985). On the other hand, high CO2 stimulated respiration in peanut leaves (Willmer *et al.* 1983). The studies of Palet *et al.* (1991) revealed that the short-term exposure to C02/bicarbonate resulted in partial inhibition of the cytochrome pathway and elicited a transient engagement of the alternate pathway in *Dianthus caryophyllata* callus and *Elodea canadensis* leaves. There are also reports where the decrease in CO2 concentration increases respiration in leaves of *Rumex crispus* (Amthor *et al.* 1992).

The light compensation point (LCP) is high in C_4 plants compared to that of C3 species (Berry and Downton 1982, Edwards and Walker 1983, Hatch 1987). LCP is known to decrease with increase in external CO2 concentration (Heath and Meidner 1967). The extent of decrease in LCP (at 50 mmol mol CO2) of intermediate species (35%) was intermediate between those of C3 (48%) and C_4 (29%) species (Fig. 5.7). LCP depends on both dark and photorespiration (Heath and Meidner, 1967). We emphasize that there are marked variations in the characteristics of not only photorespiration but also dark respiration between C3 ad C3-C4 intermediate species. The decrease in LCP with an increase in external CO2 is comparable to the decrease in photorespiration.

A consistent feature of the C3-C4 intermediates is their relatively low sensitivity to external CO_2 when compared to C3

plants. The relief of photoinhibition or inhibition of dark respiration or shift in LCP, due to high CO_2 was less in the C_3-C_4 intermediates than that in the C3 species. These differences were statistically significant ($P < 0.005$). Further the quantum yields of intermediates were not as much affected by CO2 as in C3 species. We suggest that the internal pool of CO2 in the intermediates could be larger than that in C3 species. Our earlier studies have already demonstrated the reduced sensitivity of photorespiratory metabolites in leaf discs to external bicarbonate (Chapter 4 of this Thesis).

DCDP, an analog of phosphoenoipyruvate (Jenkins *et al.* 1987, Jenkins 1989), was used to assess the degree of C4 cycle operation in C_3-C_4 intermediate species of *Flaveria, Panicum, Moricandia* and *Neurachne* (Brown *et al.* 1991) and also various *Flaveria* hybrids (Brown *et al.* 1992). The increased sensitivity of photosynthesis to DCDP (Table 5.7) suggests that PEP carboxylase may play an important role in either net carbon fixation or increasing the internal CO2 pool in the intermediates. However, the high quantum yields and the pattern of light response curves of photosynthesis in the intermediates (similar to C3 species) indicate the insignificance of C_4 cycle operation, confirming our earlier suggestion based on the activity of C_4 pathway enzymes (Chapter 3 of this Thesis).

Glycine decarboxylase, a mitochondrial enzyme is shown to be localized predominantly in the bundle sheath cells of intermediate species of *Moricandia*, *Panicum*, *Mollugo* and *Flaveria* (Hylton et al. 1988, Rawsthorne et *al.* 1988a, Morgan *et al.* 1992, 1993, Rawsthorne 1992). Further, the intercellular distribution of several respiratory enzymes indicated a marked mitochondrial concentration in bundle sheath cells of *A. tenella,* a C3-C4 intermediate (Chapter 8 of this Thesis). The present finding of highly significant variation in the response of dark respiration to CO_2 between not only C3 and C_4 plants but also between C3 and C_3-C_4 intermediates is a further indication that the mitochondrial metabolism in the C_3-C_4 intermediates is an important feature.

Chapter 6

Chapter 6

COMPARATIVE CHARACTERISTICS OF GLYCOLATE OXIDASE FROM C_3, C_3-C_4 AND C_4 PLANT SPECIES

Introduction

Glycolate oxidase (GO, Glycollate:oxygen oxidoreductase, EC 1.1.3.1) is a flavoprotein catalyzing the oxidation of glycolate to glyoxylate using 02 (Clagett *et al.* 1949, Zelitch and Ochoa 1953, Kerr and Groves 1975, Tolbert 1981). It is an ubiquitous enzyme, being present in algae, higher plants and animals (Frigerio and Harbury 1958, Dickinson and Massey 1963, Tolbert *et al.* 1968, Stabenau and Saftel 1982, Huang *et al.* 1983). The enzyme is located in peroxisomes of higher plants and plays an important role in photorespiration (Tolbert *et al.* 1969, Zelitch 1979, Tolbert 1981, Ogren 1984, Canvin 1990, Sechley *et al.* 1992).

The enzyme can also oxidize L-lactate to pyruvate with the production of H_2O_2 (Tolbert *et al.* 1949, Tolbert and Burris 1950, Noll and Burris 1954), but glycolate is the most preferred substrate.

Glycolate oxidase was purified from leaves of mostly C3 plants (Tolbert *et al.* 1949, Kerr and Groves 1975, Lindquist and

Branden 1979, Behrends et *al*. 1982, Nishimura *et al*. 1983, Davies and Asker 1983, Ernes and Erismann 1984), algae (Stabenau and Saftel 1982, Gross and Beevers 1989, Betsche *et al.* 1992, Iwamoto and Ikawa 1992), and nongreen cotyledons of cucumber seedlings (Kindl 1982). The enzyme was crystallized from spinach (Frigerio and Harbury 1958, Lindquist and Branden 1979, 1980, 1985) and pumpkin cotyledons (Nishimura *et al.* 1983). However, the enzyme has so far not been purified from any of the C_3-C_4 intermediate and C_4 species.

The exact mechanism by which photorespiration is reduced in the C_3-C_4 intermediates of *Alternanthera ficoides, A. tenella,* and *Partheniumhysterophorus* is yet to be established. Our earlier results suggested that the activity-levels of key photorespiratory enzymes (Chapter 3 of this Thesis) and the levels of photorespiratory metabolite pools (Chapter 4 of this Thesis) were partially reduced in these intermediate species. GO is one of the important photorespiratory enzymes which is reduced. It is possible that the reduced activity of this enzyme is a consequence of change in the kinetic/regulatory properties of enzyme. The differences in the kinetic and regulatory properties of GO from C3 and C_4 species are not known and a comparative study of these properties from the three different photosynthetic types will be useful in understanding the mechanism of reduced photorespiration in the intermediates.

The present study describes our efforts to purify glycolate oxidase from leaves of a C_3-C_4 intermediate species *(Parthenium hysterophorus)* and compare the properties of enzyme with those of C3 *(Pisum sativum)* and C_4 species (*Amaranthus hypochondriacus*). An attempt is made to assess if there are any differences in the characteristics of this important photorespiratory enzyme among plant species belonging to three different photosynthetic types.

Results

Glycolate oxidase was partially purified from the three species (*Pisum sativum, Parthenium hysterophorus* and *Amaranthus hypochondriacus*) (Tables 6.1 to 6.3). Precipitation by ammonium sulfate and fractionation on Seralose were highly effective in purifying the enzyme. The specific activity of the partially purified enzyme from *Pisum sativum* and *Parthenium hysterophorus* was 26.7 and 25.0 units mg^{-} protein, respectively, whereas it was only 3.3 units mg protein in *Amaranthus hypochondriacus* (Tables 6.1 to 6.3).

The absorption spectra of purified enzyme from the three species were similar with a major peak at 278 nm, and two minor peaks at 340 and 440 nm (Fig. 6.1 A,B,C). These peaks at 340 and 440 nm are believed to correspond to the flavin moiety of the enzyme. The E280/E450 for the enzyme varied from 6.7 *(Parthenium)* through 7.0 *(Pisum sativum)* to 7.4 *(Amaranthus)* (Table 6.4).

Table 6.1. Purification of glycolate oxidase from *Pisum sativum* (C3 species)

Purification Step	Total protein	Total activity	Specific activity	Purification fold
	mg	Units	U mg^{-1} protein	Fold
1. Crude extract	155.7	14.01	0.09	
2. Acetic acid (pH 5.3)	78.9	12.53	0.16	2
3. 25% $(NH_4)_2SO_4$ supernatant	17.1	6.11	0.36	4
4. 45% $(NH_4)_2SO_4$ pellet	1.14	1.16	1.40	16
5. 0.2% (w/v) Protamine sulphate	1.01	1.52	1.51	17
6. Seralose 6B	0.15	0.77	5.31	59
7. 65% $(NH_4)_2SO_4$ pellet	0.05	0.64	13.06	145
8. Sephadex G-200	0.01	0.24	26.67	296

Table 6.2. Purification of glycolate oxidase from *Parthenium hysterophorus* (C3-C4 intermediate species)

Purification Step	Total protein	Total activity	Specific activity	Purification fold
	mg	Units	U mg^{-1} protein	Fold
1. Crude extract	68.3	6.06	0.09	
2. Acetic acid (pH 5.3)	34.1	5.87	0.17	2
3. 25% $(NH_4)_2SO_4$ supernatant	6.9	4.29	0.62	7
4. 45% $(NH_4)_2SO_4$ pellet	0.4	1.42	3.55	40
5. 0.2% (w/v) Protamine sulphate	0.36	1.36	3.78	43
6. Seralose 6B	0.51	0.54	10.59	119
7. 65% $(NH_4)_2SO_4$ pellet	0.014	0.24	17.14	193
8. Sephadex G-200	0.004	0.10	25.00	281

Table 6.3. Purification of glycolate oxidase from *Amaranthus hypochondriacus* (C4 species)

Purification Step	Total protein	Total activity	Specific activity	Purification fold
	mg	Units	U mg^{-1} protein	Fold
1. Crude extract	40.3	0.48	0.01	
2. Acetic acid (pH 5.3)	20.1	0.42	0.02	2
3. 25% $(NH_4)_2SO_4$ supernatant	1.17	0.21	0.18	15
4. 45% $(NH_4)_2SO_4$ pellet	0.13	0.05	0.39	33
5. 0.2% (w/v) Protamine sulphate	0.12	0.05	0.42	35
6. Seralose 6B	0.014	0.03	1.72	149
7. 65% $(NH_4)_2SO_4$ pellet	0.01	0.02	2.22	185
8. Sephadex G-200	0.003	0.01	3.33	278

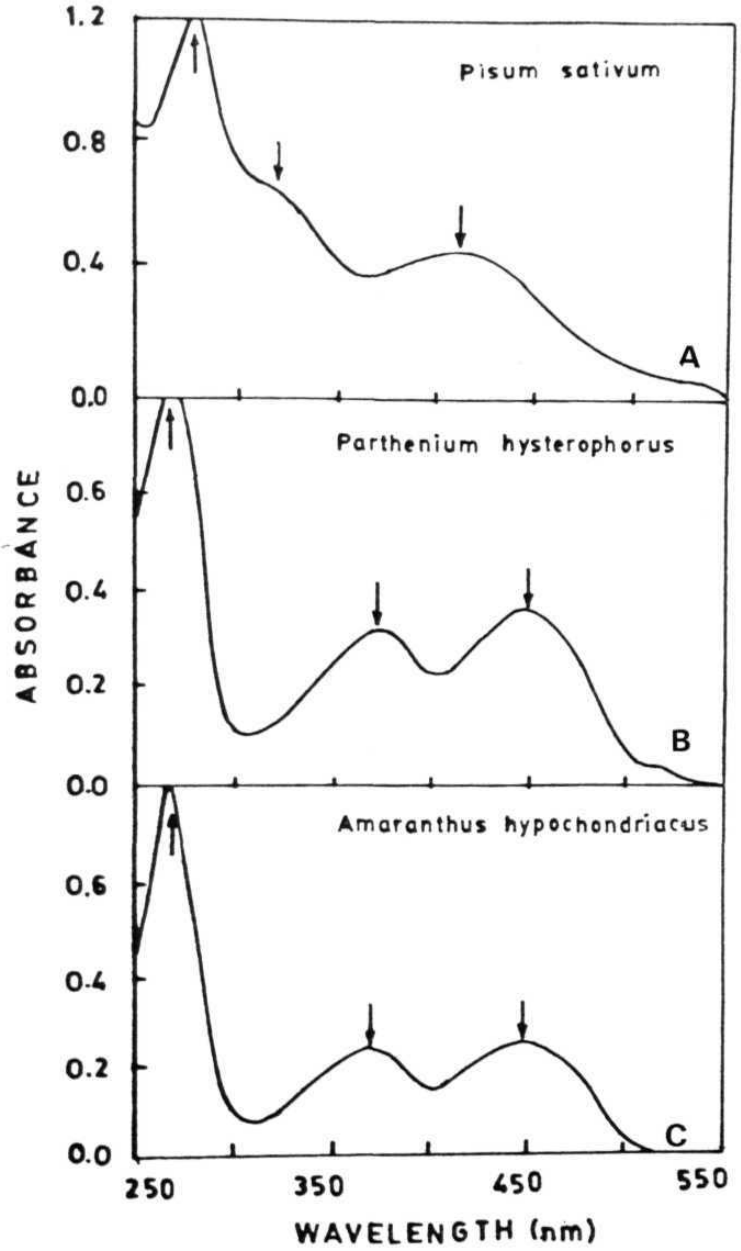


Fig. 6.1. Spectra of purified glycolate oxidase from leaves of *Pisum sativum* (C3 species). *Parthenium hysterophorus* (C3-C4 intermediate) and *Amaranthus hypochondriacus* (C4 plant).

Table 6.4. Comparative characteristics of glycolate oxidase from *Pisum sativum* (C_3), *Parthenium hysterophorus* (C3-C4 intermediate) and *Amaranthus hypochondriacus* (C_4)

Character	Enzyme source		
	Pisum sativum	*Parthenium hysterophorus*	*Amaranthus hypochondriacus*
1. Vmax (μmol mg^{-1} protein h^{-1})	1413 ± 101	1301 +89	125 +12
2. KM (glycolate) mM	0.27	0.21	0.07
3. K_I (α-HPMS) mM	0.8	1.6	5.5
4. E_{280}/E_{450}	7.0	6.7	7.4
5. Inhibition due to 3 mM a-HPMS (%)	78	75	59
6. Optimum pH	8.3	8.3	7.5
7. Activity with substrate (10 mM) (μmol mg protein h)			
Glycolate	1402 (100)	1134 (100)	158 (100)
Glyoxylate	451 (32)	250 (22)	6.0 (4)
β-hydroxypyruvate	55 (4)	45 (4)	0.0 (0)
L-lactate	885 (63)	645 (57)	78 (49)

Values in parentheses indicate % control.

The kinetic and regulatory properties of GO from leaves of C3 species were quite different from those of C4 plant (Table 6.4). The K_M (glycolate) of the enzyme from *Pisum sativum* was nearly four fold higher than that of the enzyme from *A. hypochondriacus*. On the other hand, the K_I (α-HPMS) was very higher for the enzyme from *A. hypochondriacus* than the one from pea. The enzyme from pea and *Parthenium hysterophorus* was severely inhibited by a-HPMS (78 and 75% respectively), compared to the moderate inhibition of enzyme from *A. hypochondriacus* (59% inhibition). The properties of GO from *Parthenium hysterophorus* (C_3-C_4 intermediate) were similar to those of pea (C_3) enzyme (Table 6.4).

The optimum pH of the enzyme from C_4 species was different from that of C_3 ones. While the enzyme from *Pisum sativum* and *Parthenium hysterophorus* had an optimum pH of 8.3, the enzyme from *A. hypochondriacus* showed an optimum of 7.5 (Fig. 6.2 A,B,C and Table 6.4).

The ability of GO to oxidize other substrates such as L-lactate, glyoxylate and hydroxypyruvate varied between C_3 and C_4 species. However, the enzyme from *Parthenium hysterophorus* was similar to that from *Pisum sativum* in its ability to oxidize different substrates (Table 6.4). The enzyme from C_3 and C_3-C_4 species showed maximum stimulation by FMN at 60-70 μM, above which the activity was inhibited. However, the enzyme from *Amaranthus*

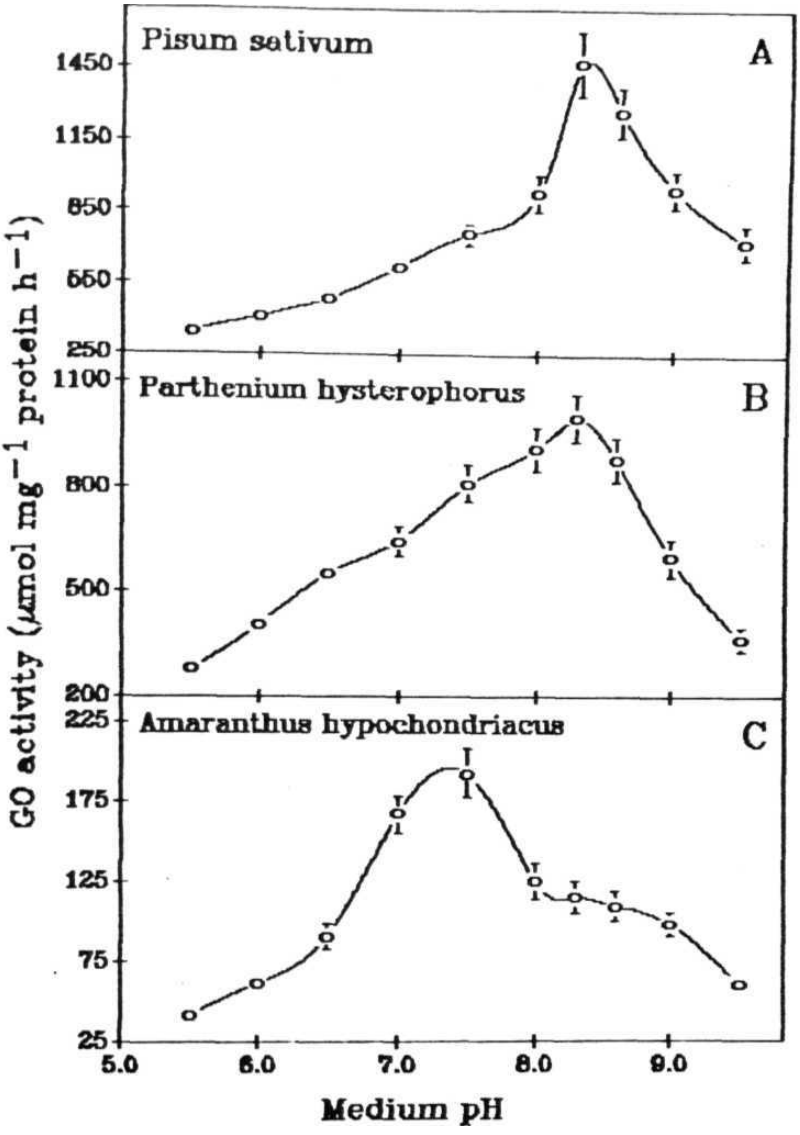


Fig. 6.2. The effect of assay pH on the activity of purified glycolate oxidase activity in C3 (*Pisum sativum*), C3-C4 Intermediate *(Parthenium hysterophorus)* and C4 (*Amaranthus hypochondriacus*) plants.

hypochondriacus was not saturated with external FMN concentration of even 100 μM (Fig. 6.3 A,B,C).

Discussion

To our knowledge this is the first report on the partial purification and characterization of the enzyme from not only a C_3-C_4 intermediate but also C_4 species. The kinetic and regulatory properties of GO from C3 plant were similar to those from C_3-C_4 species (Table 6.4), but the enzyme from *A. hypochondriacus* was quite distinct (Table 6.4).

The V_{max} of the enzyme from *A. hypochondriacus* was only one-fifth of that of *Pisum* or *Parthenium.* Similarly, the K_M(glycolate) and K_I(HPMS) also varied for the enzyme from these three species (Table 6.4). The observed K_M(glycolate) for pea (267 μM) correlated well with the previous reports for pea (262 μM; Corbett and Wright 1971, 250 μM; Kerr and Groves 1975, 220 μM; Betsche *et al*. 1992) and spinach (380 μM; Zelitch and Ochoa 1953).

The E_{280}/E_{450} for glycolate oxidase was reported to be between 6.0 and 6.3 (Davies and Asker 1983, Nishimura et *al*. 1983). In the present study, it varied between 6.7 and 7.4 (Table 6.4). The E_{280}/E_{450} ratio indicates the presence of the prosthetic group (FMN) and the relative purity of the enzyme.

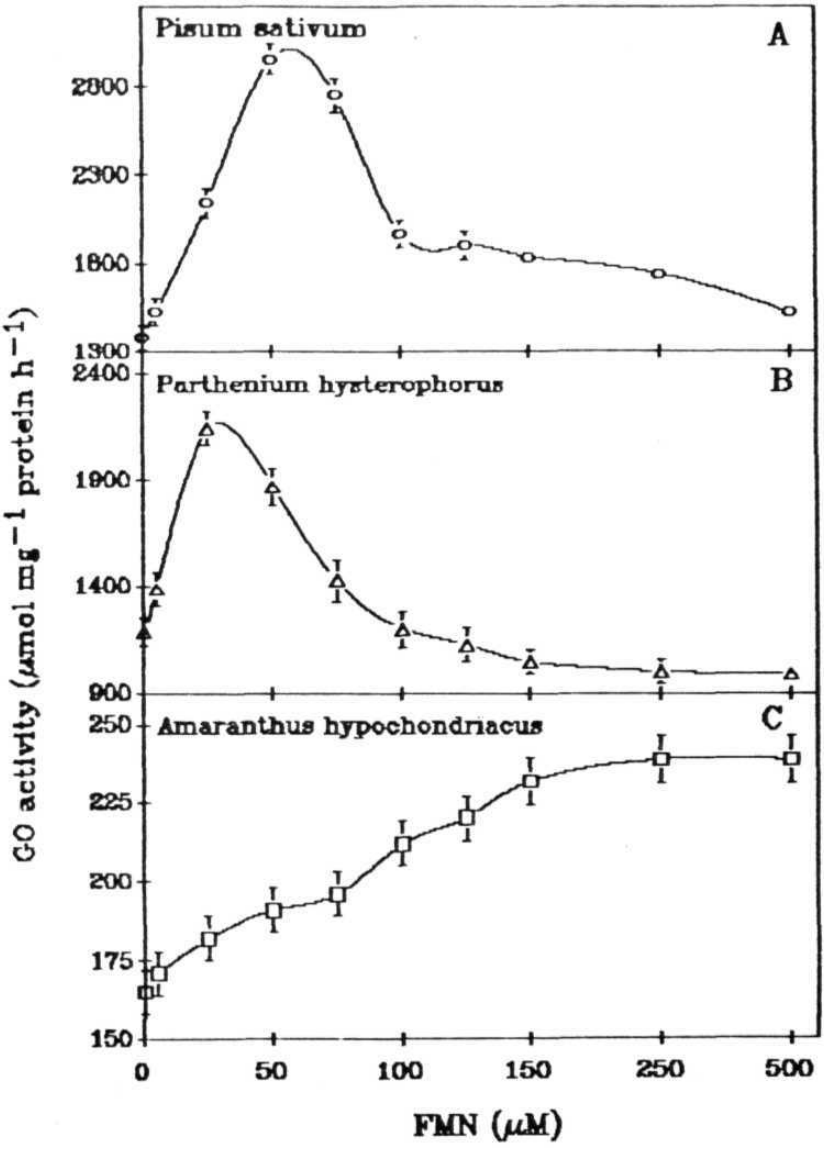


Fig. 6.3. The effect of different concentrations of flavin mononucleotide (FMN) on the activity of glycolate oxidase purified from C_3 (*Pisum sativum*), C3-C4 intermediate (*Parthenium hysterophorus*) and C_4 (*Amaranthus hypochondriacus*) species.

The optimal pH for glycolate oxidase in C3 and C_4 plants was different. However, the observed optimal pH of GO for pea and *Parthenium* (8.3) were similar to the previous reports for the enzyme from pea (8.3, Betsche *et al.* 1992), lettuce (8.5, Davies and Asker 1983), *Spathoglossum pacificum* (8.3, Iwamoto and Ikawa 1992), pumpkin cotyledons (8.0, Nishimura *et al.* 1983), *Lemna minor* (8.3, Ernes and Erismann 1984) and wheat (8.3, Baker and Tolbert 1965).

GO is a highly basic (pI 8.0) flavoprotein (Kerr and Groves 1975, Behrends *et al.* 1982, Ernes and Erismann 1982, 1984, Davies and Askar 1983) and uses a wide range of α-hydroxyacids as substrates with glycolate being the most preferred form (Zelitch and Ochoa 1953, Betsche *et al.* 1992, Davies and Asker 1983, Stabenau and Saftel 1982, Gross and Beevers 1989, Ernes and Erismann 1984). The present study also confirms that glycolate is the most preferred substrate than other a-hydroxyacids (Table 6.4).

Generally GO is associated with flavin prosthetic group, however, the presence of a non-flavin glycolate oxidase in etiolated wheat leaves and green tobacco leaves in addition to the flavoprotein was reported (Kuczmac and Tolbert 1962, Baker and Tolbert 1965, 1967). FMN is the prosthetic group of spinach GO as indicated by several lines of evidence (Zelitch and Ochoa 1953). The absorption peaks of FMN are at 266, 375, and 445 nm which is typical of flavoproteins (Blanchard *et al.* 1945, Horecker 1950).

The spectral properties of GO from C3, C3-C4 intermediate and C4 species (Fig. 6.1 A,B,C) indicated the presence of a flavin prosthetic group since two important peaks at 345 and 440 nm were observed for all the three enzymes. This also demonstrates that the flavin cofactor was not completely lost during the isolation of the the enzyme. The spectra of GO from these species is highly similar to that of *Lemna minor* (Ernes and Erismann 1984), spinach (Zelitch and Ochoa 1953), cucumber cotyledons (Behrends *et al.* 1982) and pumpkin cotyledons (Nishimura et *al.* 1983).

The activity of GO from wheat enzyme was only slightly stimulated by the addition of FMN (Baker and Tolbert 1969). In the present study, the activity of GO was stimulated by FMN up to 70 μM FMN, both in C_3 and C_4 plants, whereas in C_4 plant the stimulation required 100 μM FMN (Fig. 3 A,B,C). In pumpkin cotyledons the addition of either FMN (prosthetic group) or NaN3 (catalase inhibitor) did not alter the specific activity of the purified enzyme indicating that FMN was not depleted completely during purification and that catalase was not a contaminant in the final enzyme preparation (Nishimura et *al.* 1983).

GO is regulated by several factors. It is induced by light which is independent of chlorophyll formation (Tolbert and Burris 1950, Behrends et *al.* 1982) or glycolate (Tolbert and Cohan 1953). Its activity is also reported to be regulated by blue light (Schmid 1969), phytochrome (Roth-Bejerano and Lips 1978), phenolic acids

(De Jong 1973, Galzin and Monties 1979) and the form of nitrate supply (Vaklinova and Moskova 1973, Ernes and Erismann 1982).

Sulfhydryl inhibitors indicate that GO requires -SH groups for enzyme activity (Noll and Burris 1954). Potent inhibitors of GO are: α-hydrosulphonates, isonicotinyl hydrazide, and 2-hydroxy-3-butynoate (Zelitch 1971, 1973, 1979, Salin and Homann 1973, Doravari and Canvin 1980, Huang *et al*. 1983, Baumann and Przybilski 1990). Iodoacetate, iodoacetamide, phenylmercuric nitrate and p-chloro- mercuribenzoate are known to inhibit the crude enzyme activity from barley and purified spinach enzyme (Barron 1951, Noll and Burris 1954, Frigerio and Harbury 1958).

There were clear differences in the V_{max}, K_M(glycolate), K_I(HPMS), pH and substrate specificities of GO from the C_3 and C_4 plants, whicn suggest a marked alteration in the kinetic properties of the enzyme in C_4 plants. The kinetic and regulatory properties of GO from the C_3-C_4 intermediate *Parthenium hysterophorus* suggest that the enzyme is similar to that of a typical C_3 plant, *Pisum sativum*, since there were no conspicuous differences between the properties of GO from C_3 plant and C_3-C_4 intermediate.

These observations suggest that the reduced photorespiration in *Parthenium hysterophorus* is not the result of an alteration in molecular or kinetics properties of GO. On the other hand the enzyme from C_4 species (*Amaranthus hypochondriacus)* is quite

distinct from that in C3 species. Further experiments are needed to confirm this phenomenon. There is already enough evidence to indicate distinct isoforms of C_4 cycle enzymes in C4 species (e.g. Hermans and Westhoff 1992, Nelson and Langdale 1992). But this is the first report of a similar instance in case of a key photorespiratory enzyme.

Chapter 7

Chapter 7

COMPARATIVE CHARACTERISTICS OF PEP CARBOXYLASE FROM LEAVES AND LIGHT ACTIVATION OF ENZYME IN MAIZE MESOPHYLL PROTOPLASTS

Introduction

Phosphoenoipyruvate carboxylase (PEPC, Orthophosphate: oxaloacetate carboxylase (phosphorylating), EC 4.1.1.31) catalyzes the β-carboxylation of PEP to yield OAA and Pi (Reviews: O'Leary 1982, 1983, Andreo *et al.* 1987, Devi *et al* 1992a, Rajagopalan *et al*. 1993b). PEPC is a key enzyme involved in primary C02 fixation by C4 and CAM plants (Andreo *et al.* 1987). The enzyme is also present in C3 plants and algae (O'Leary 1982, Latzko and Kelly 1983, Rajagopalan *et al.* 1993b). Apart from photosynthetic carbon assimilation, other functions of PEPC include replenishment of tricarboxylic acid cycle intermediates, generation of NAD(P)H, refixation of respired CO_2, nitrogen assimilation, amino acid synthesis and maintenance of pH/electroneutrality (Review: Latzko and Kelly 1983). PEPC is a cytosolic enzyme and occurs in leaves as well as non-photosynthetic organs. In C4 plants, PEPC is localized primarily in mesophyll cytosol (Perrot-Rechenmann *et al.* 1982, 1984).

The kinetic and regulatory properties of PEPC undergo marked changes during light/dark transitions, particularly in C4 and CAM

plants (Huber and Sugiyama 1986, Doncaster and Leegood 1987, Nimmo *et a.1.* 1987, Jiao and Chollet 1988, 1991, Nimmo 1993). The enzyme extracted from preilluminated C_4 leaf tissue exhibits two to three fold more activity than the dark form when assayed under suboptimal (but physiological) assay conditions. In parallel with these light-induced changes in catalytic activity, PEPC extracted from illuminated leaves exhibits less sensitivity to malate inhibition than that from darkened ones (Karabourniotis et *al.* 1983, 1985, Huber and Sugiyama 1986, Doncaster and Leegood 1987, Jiao and Chollet 1988). The effect of light on PEPC is hardly discernible in leaves of C3 plants (Chastin and Chollet 1989).

Significant variations in the kinetic/regulatory properties (including light activation pattern) were reported between the PEPC of C3 and C_4 plants (Holaday and Black 1981, Bauwe and Chollet 1986, Chastin and Chollet 1989). We therefore examined the properties of PEPC in order to establish whether the enzyme from C3-C4 intermediates had kinetic and regulatory properties different from those of the enzymes from C3 and C_4 species. These studies could help us in understanding the possible evolutionary tendencies of PEPC, along with variation in photosynthesis/photorespiration.

Attempts were made to examine the light activation pattern of PEPC at three levels of organization: leaf discs, leaf homogenates and mesophyll protoplasts. The response to light of PEPC was maximum in leaf discs of particularly C_4 plants. The

extent of such light activation in leaf discs of C3-C4 species was quite limited as in C3 species. Therefore, the kinetic and regulatory properties of dark-form PEPC were studied in C3, C4 and C3-C4 intermediate species.

During our experiments with maize mesophyll protoplasts, very interesting observations were made on light activation of PEPC. It is for the first time such (even limited) light activation of PEPC could be demonstrated in isolated C4 mesophyll protoplasts. Being a novel finding, these results are also incorporated into this thesis (although not directly related to C3-C4 intermediates). The studies with maize mesophyll demonstrate that pH could be an important factor in modulating light activation of PEPC in C4 mesophyll cells.

Results

When the predarkened leaf discs of maize were illuminated, the activity of PEPC increased. Light activation of PEPC occurred in also mesophyll protoplasts and leaf homogenates of maize. However, the light/dark ratio was more in leaf discs than that in leaf homogenates or mesophyll protoplasts (Fig. 7.1).

The kinetic and regulatory properties of PEPC isolated from illuminated or darkened samples were different in the three systems (mesophyll protoplasts, leaf homogenates and leaf discs) (Table 7.1). Both the V_{max} and the affinity for PEP of the enzyme

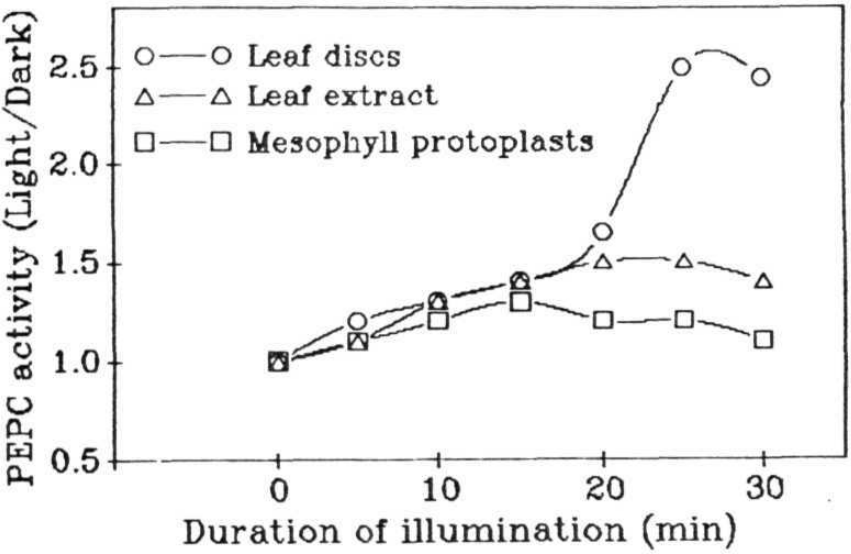


Fig. 7.1. The activation of PEP carboxylase in leaf discs or leaf extracts or mesophyll protoplasts of *Zea mays* in relation to the duaration of illumination. Maximal activation of PEPC required 15 (mesophyll protoplasts) to 25 min (leaf discs) of illumination. PEPC extracted from preilluminated leaf discs showed maximum activation than that from either illuminated leaf extracts or mesophyll protoplasts.

Table 7.1. The characteristics of dark- and light-forms of PEP carboxylase from maize mesophyll protoplasts or leaf homogenates or **leaf** discs

Parameter	Mesophyll protoplasts		Leaf homogenate		Leaf discs	
	Dark	Light	Dark	Light	Dark	Light
Kinetic properties						
Vmax (μmol mg Chl h)	195 ± 2 1	213 + 2 2	356 ±27	568 ±45	348 ± 26	1151 ± 108
$K_{M(PEP)}$ (mM)	2.1	1.5	1.4	1.1	1.4	0.8
Sensitivity to effectors						
			PEPC activity μmol mg Chl h			
No effector (control)	155 ± 18 (100)	185 ± 20 (100)	344 ±27 (100)	559 ±47 (100)	325 ±28 (100)	975 ±81 (100)
2.5 mM malate	26 + 7 (17)*	159 ± 14 (86)	69 ±6 (20)	218 ±17 (39)	69 ±5 (21)	449 ±41 (46)
2.5 mM G-6-P	267 + 24 (172)*	187 ±8 (101)	678 ±58 (197)	810 **±97** (145)	683 ±58 (210)	1248 ±74 (128)

The figures in the parentheses indicate the activity of the enzyme as the percent of respective control.

increased as a result of illumination. The catalytic activity and the extent of activation of PEPC were high with leaf discs. Dark form of PEPC was more sensitive to L-malate inhibition than the light activated enzyme. Such decrease in the sensitivity of PEPC to malate inhibition (due to illumination) was more pronounced in mesophyll protoplasts than that in leaf homogenates or leaf discs. The light form of PEPC was not stimulated by G-6-P in mesophyll protoplasts. The enzyme activity from illuminated leaf homogenates and leaf discs was stimulated by G-6-P (25-45% over control) (Table 7.1). Subsequent studies on light activation of PEPC were made in leaf discs of C3, C4 and C3-C4 intermediate species.

The extent of activation by light of PEPC found to be maximum in Zea *mays* (C_4) and low in both *Pisum sativum* (C3) and *Alternanthera ficoides* (C3-C4 intermediate) (Table 7.2). Light had no effect on the malate inhibition or G-6-P activation on the PEPC extracted from the C3 or C3-C4 intermediate species. The $K_m(PEP)$ was high but $K_m(HCO_3^-)$ was low for the enzyme prepared from Zea *mays* leaf discs compared to that of *Pisum sativum* and *Alternanthera ficoides.* The dark form of maize PEPC was more sensitive to malate (79% inhibition) than the enzyme from *Pisum sativum* (46%) or *Alternanthera ficoides* (52%). Similarly, stimulation by G-6-P of PEPC was more for the maize enzyme compared to that of C3 or C3-C4 intermediate species (Table 7.2).

Table 7.2. Properties of PEP carboxylase in leaf discs of C3, C4, and C3-C4 intermediate species

PEPC activity	*Pisum sativum* (C3)	*Alternanthera ficoides* (C3-C4)	Zea *mays* (C4)
Vmax (μmol mg Chl h)			
Dark	39 ± 2	48 + 4	388 ± 32
Light	50 ± 4	67 ± 5	998 ± 76
Light/Dark	1.3	1.4	2.6
Dark form PEPC			
Km(HCO_3^-) mM	0.68	0.74	0.41
Km(PEP) mM	0.23	0.25	0.78
5 mM malate	21 + 1 (54)*	23 ± 2 (48)	81 + 7 (21)
5 mM G-6-P	52 + 5. (133)	68 ± 6 (142)	733 ± 62 (189)

Values in parentheses indicate activity as % dark-control (taken as 100%).

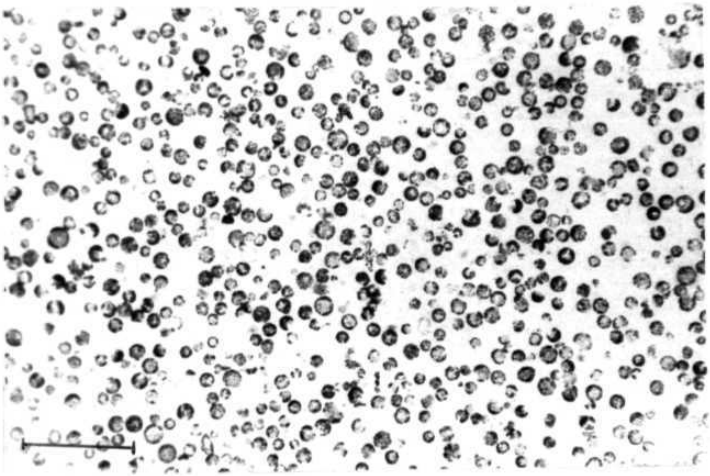
Maize mesophyll protoplasts were released when leaf pieces were subjected to digestion with a mixture of cellulase and macerozyme. These could be obtained in a relatively short time (<1 h). The protoplast preparation was quite pure without any contamination of bundle sheath cells (Fig. 7.2).

Light activation of PEPC could be demonstrated when maize mesophyll protoplasts were suspended and illuminated in low buffered (2 mM Hepes) medium (Fig. 7.3 A). If the protoplasts were kept in high buffered (20 mM Hepes) medium, there was no significant difference in enzyme activity from protoplasts kept in light or dark (Fig. 7.3 B).

To simulate the effect of pH, maize mesophyll protoplasts were resuspended in Hepes-buffer (pH 7.5) and were preincubated in Hepes-buffered media whose pH ranged from 7.2 to 8.2 and assayed at pH 7.8. Upon incubation for 10 min in darkness maximal activation of PEPC was obtained at a preincubation pH of 7.8, while the activity decreased markedly at pH of 7.2 or 8.2 (Fig. 7.4).

The effect of classical inhibitors of photosynthetic electron transport (DCMU), ATP synthesis (phlorizin) or phosphorylation (CCCP) on the light activation of PEPC from maize mesophyll protoplasts was examined. The test compounds were present during the preincubation of protoplasts *in* situ. Light

Fig. 7.2. Photomicrograph of maize mesophyll protoplasts. The horizontal bar represents 200 μm.



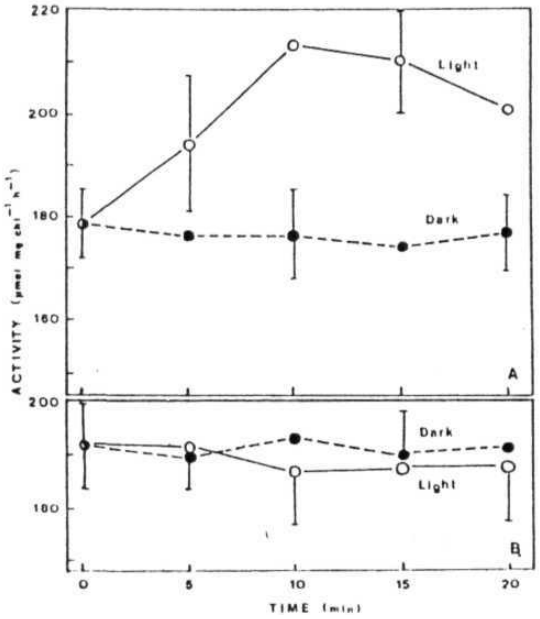


Fig. 73 The effect of buffer-strength on the light activation of PEP carboxylase. Mesophyll protoplasts isolated from maize leaves were suspended in either low (2 mM) (A) or high (20 mM) buffer (B), pH 7.2, and then exposed to light or kept in darkness. Aliquots were examined for enzyme activity at pH 7.8.

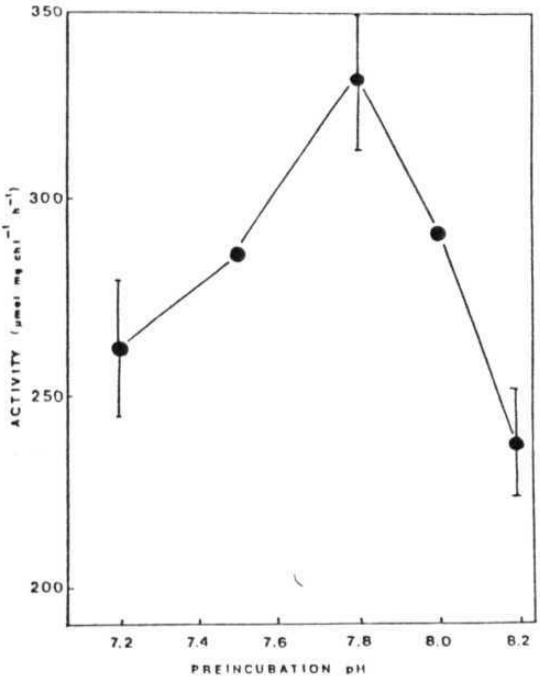


Fig. 7.1 The effect of pH during preincubation on the activity of PEP carboxylase in darkened maize mesophyll protoplasts. The protoplasts, resuspended in low buffer medium (2 mM), pH 7.5, were mixed and resuspended in 20 mM HEPES-KOH buffers, whose pH ranged from 7.2 to 8.2. Aliquots of protoplasts were taken after 10 min of incubation in darkness and assayed for PEP carboxylase activity at pH 7.8.

activation of PEPC was severely restricted by the presence of CCCP or phlorizin in the incubation medium, but not by DCMU (Table 7.3).

Discussion

There is considerable disagreement among the literature on the identity of PEPC from C3-C4 intermediates. Bauwe and Chollet (1986) reported the existence of three different isozymes of PEPC in *Flaveria* species. Although the PEPC extracted from *F. floridana* (C3-C4 intermediate) was similar electrophoretically to that of *F. cronquistii* (C3 species), the enzyme from *F. floridana* showed a two fold increase in activity upon illumination over the levels of dark adapted leaves (characteristic of the enzyme in C4 plants) (Adams *et al.* 1986). Light activation of PEPC could not be observed in leaves of C3-C4 intermediates of *F. linearis* and *F. chloraefolia* (Chastin and Chollet 1989). Although there was a two to three fold increase in the activity (over C3 plants), the kinetic properties of PEPC in the intermediates of *Panicum* were similar to those from C3 species (Holaday and Black 1981). The kinetic properties of PEPC from C3-C4 intermediates of *Flaveria pubescens*, *F. linearis* (Nakamoto *et al.* 1983), *F. floridana* and *F. chloraefolia* (Bauwe and Chollet 1986) were intermediate to those of C3 and C4 plants.

The activity of PEPC from C4 plants is regulated markedly by metabolites such as malate (negative effector) and G-6-P (positive

Table 7.3. Effect of DCMU, phlorlzln or CCCP on light activation of PEP carboxylase *in situ*. The test compounds were included in the prelncubation medium (2 mM Hepes, pH 7.2), while protoplasts were kept in darkness or light at 30 ± 1°C. After 10 min, an aliquot was examined for PEP carboxylase activity

Test compound during prelncubation	PEPC activity		Light/Dark Ratio
	Light	Dark	
	μmol mg Chl h		
None (Control)	363 + 18	290 ±29	1.3
+ DCMU (10 μM)	357 ±30	285 ± 27	1.3
+ phlorlzln (1 mM)	259 ± 21	273 ± 28	1.0
+ CCCP (1 μM)	248 ±27	256 ± 19	1.0

effector) (Huber and Sugiyama 1986, Doncaster and Leegood 1987). Further, PEPC from leaves of C4 plants is stimulated by light much more markedly than that of C3-type. The light/dark ratios of PEPC were high in C4 plants (Zea mays) and low in C3 plants (around 30/4 in *Pisum sativum*). Similarly, other characteristics of PEPC, such as $K_m(HCO_3^-)$, $K_{m(PEP)}$, inhibition by malate and activation by G-6-P, varied between C3 and C4 species. In the C3-C4 intermediate *Alternanthera ficoides*, activity levels of PEPC are high (Chapter 3 of this Thesis) compared to C3 species. Yet the light activation pattern and the kinetic properties (Table 7.1) of enzyme from this C3-C4 intermediate were similar to the C3-isoform of the enzyme. This suggests that PEPC present in the C3-C4 intermediates (at least in *Alternanthera ficoides)* may be a C3 form. Separate experiments from our laboratory on pattern of light activation and dark deactivation of PEPC in several C3 and C4 plants confirm such suggestion (Rajagopalan et *al.* 1993a).

This is the first report on the light activation of PEPC in mesophyll protoplasts and leaf homogenates of Zea *mays* (Fig. 7.1 and Table 7.2). However, maximum activation of PEPC by light occurred only in preilluminated leaf discs. This can be attributed to the need for a proper integrity and coordination of two photosynthetlc cell types (mesophyll and bundle sheath) during the light activation of PEPC (Chollet *et* aJ. 1990, Jiao and Chollet 1991, Bakrlm et *al.* 1993). It has been reported that metabolites such as PGA from bundle sheath cells can be a signal for the

activation of PEPC by light in mesophyll cells (Jiao and Chollet 1992, Bakrim et *al.* 1993).

Maize mesophyll protoplasts form a good source of PEPC (Petropoulou *et al.* 1990). Light activation of PEPC has so far not been reported in mesophyll protoplasts from leaves of C_4 plants, unlike the stimulation of PPDK or NADP-MDH (Nakamoto and Edwards 1986). The detection of the activation of PEPC by light in protoplasts only in a low buffered medium, but not in high buffered medium (Fig. 7.3), suggested an important role of pH and/or ionic strength during the activation of PEPC (Devi and Raghavendra 1992b). Studies with fluorescent dyes indicated that upon illumination, there is a marked alkalization of the cytoplasm in leaf mesophyll cells of several C_3 plants (Yin et *al.* 1990a,b). There is also a high degree of light induced alkalization in mesophyll cells of C_4 plants (Raghavendra et *al.* 1993, Yin *et al.* 1993). Since PEPC is located in the cytoplasm, such alkalization can lead to activation of the enzyme. The increase in enzyme activity upon preincubation of protoplasts in darkness at pH 7.8, but not at 7.2 (Fig. 7.4), supports the proposed role of pH.

The extent and kinetics of light actlvatlon/dark deactlvatlon of PEPC in protoplasts in situ differ slightly from those observed with leaves in *vivo* (Doncaster and Leegood 1987, Nimmo *et al.* 1987, Jiao and Chollet 1988). The lack of the DCMU effect on the light activation of PEPC observed in our experiments

(Table 7.3) and the insensitivity of the light form of the enzyme to G-6-P also are at variance with earlier reports made with leaves illuminated *in vivo* (Samaras et *al*. 1988, Reviews: Chollet *et al*. 1990, Jiao and Chollet 1991). However in at least one study the inhibition of light activation of PEPC by DCMU was only partial, when the enzyme was assayed at low substrate levels (Karabourniotis *et al.* 1983).

Although these results demonstrate that PEP carboxylase from C_3-C_4 intermediates closely resembles the C3-form, the enzyme may still play an important role, though not through typical C_4 pathway e.g. as in *Panicummilioides* (Edwards *et al* 1982). Our studies (Chapter 5 of this Thesis) using DCDP, an inhibitor of PEPC, indicted that PEP carboxylase might be significant during the *in vivo* refixation of photorespired CO_2 in leaf discs of intermediate species.

Chapter 8

Chapter 8

ACTIVITY-LEVELS OF PHOTOSYNTHETIC, PHOTORESPIRATORY AND RESPIRATORY ENZYMES IN MESOPHYLL PROTOPLASTS AND BUNDLE SHEATH CELLS OF ALTERNANTHERA TENELLA

Introduction

The inter- and intracellular location of photosynthetic and photorespiratory metabolisms is different in C3 and C_4 plants (Edwards and Walker 1983, Reed and Chollet 1985, Hatch 1987). In C_3 plants both photosynthetic and photorespiratory metabolisms operate in a single type (mesophyll) of cells (Robinson and Walker 1981). In contrast, photorespiratory glycolate metabolism and Calvin cycle occur in the bundle sheath cells, while the primary carbon assimilation through C_4 cycle is localized in the mesophyll cells of C4 plants (Reviews: Edwards and Huber 1981, Edwards and Walker 1983, Hatch 1987, 1992a,b, Raghavendra and Das 1993). Further, the location of C4-acid decarboxylation in the inner bundle sheath cells of C_4 plants builds up a very high CO_2 concentration near Rubisco, leading to a remarkable reduction in photorespiratory CO2 metabolism in these plants (Edwards and Walker 1983, Hatch 1987, 1992a,b).

A differential localization of photosynthetic and photorespiratory enzymes, if occurs, can be a significant factor responsible for reduced photorespiration in the C_3-C_4 species. The photosynthetic properties associated with reduced photorespiration were dependent on the extent of Kranz anatomy i.e. differentiation of bundle sheath and mesophyll cells, at least in some of the intermediate species like *Moricandia arvensis* (Holbrook and Chollet 1986). It has recently been shown that glycine decarboxylase, the mitochondrial enzyme which releases CO_2 during photorespiration, is confined to the bundle sheath cells in C3-C4 intermediate species from the genera of *Panicum*, *Flaveria, Mollugo*, and *Moricandia* (Hylton *et al*. 1988, Moore *et al*. 1988, Rawsthorne *et al*. 1988a). Such confinement of decarboxylation capacity of the leaf to the bundle sheath cells along with physical proximity of mitochondria and chloroplasts in these cells is proposed to form the basis for the efficient, light-dependent recapture of photorespired CO_2 in leaves of *Moricandia arvensis* (Hunt *et al.* 1987, Rawsthorne *et al.* 1988a).

The aim of the present set of experiments is to study intercellular compartmentation (if any) of photosynthetic, photorespiratory and respiratory enzymes in the C_3-C_4 intermediate species *Alternanthera tenella.* As a primary step, we isolated mesophyll protoplasts and bundle sheath cells from *A. tenella* and determined in these tissues the maximum activities of enzymes involved in photosynthetic/ photorespiratory/respiratory metabolism.

Our attempts to isolate mesophyll and bundle sheath tissues from leaves of *Alternanthera ficoides* and *Parthenium hysterophorus* were not successful.

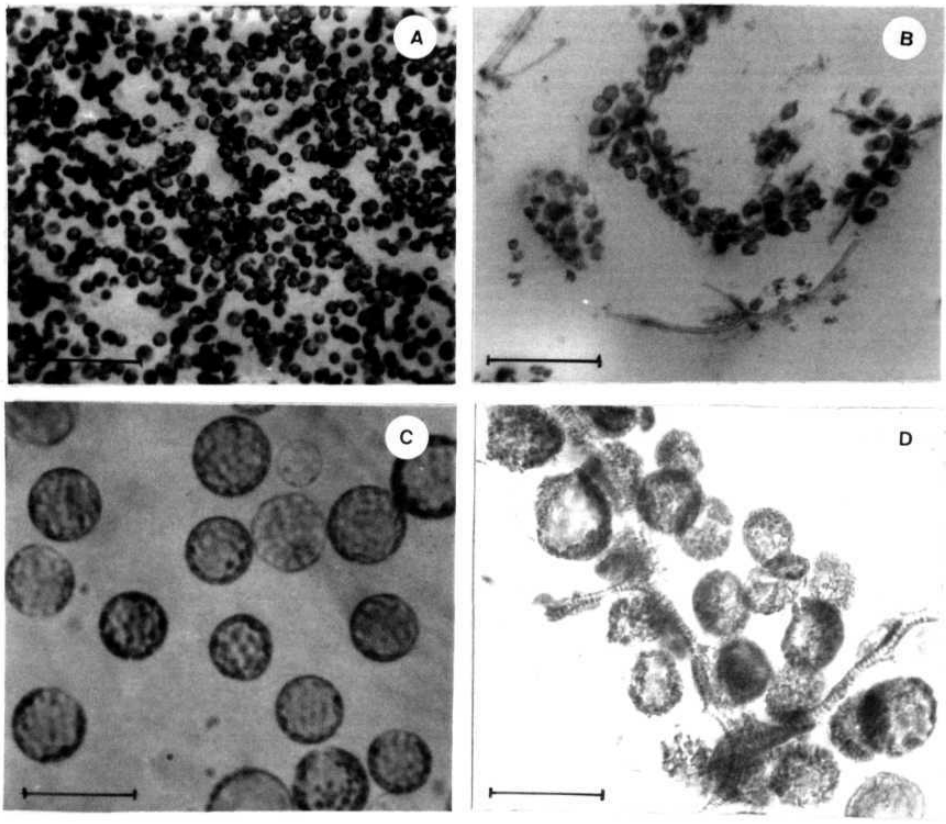
Results

Digestion of leaf pieces of *A. tenella* with cellulase/ pectinase yielded mesophyll protoplasts and bundle sheath strands. These could be separated by suitable nylon filters, due to variation in the size of these two types of tissues. The yield of bundle sheath cells was less than that of mesophyll protoplasts possibly because the Kranz anatomy was only partial in *A. tenella.*

Microscopic observations indicated very little contamination of mesophyll protoplasts with bundle sheath protoplasts/strands and *vice versa* (Fig. 8.1 A to D). Two different types of mesophyll protoplasts (20-30 /am) with slight difference in their sizes were observed, apparently corresponding to spongy and pallisade tissues. The bundle sheath cells were quite larger (>40 μm) than the mesophyll protoplasts. The contamination of bundle sheath strands with mesophyll protoplasts was very small (<10%). A single bundle sheath strand is shown in Fig. 8.1 D.

The absorption spectra of both mesophyll protoplasts and bundle sheath cells showed two major peaks corresponding to 640 nm

Fig. 8.1. Photomicrographs of mesophyll protoplasts (A,C) and bundle sheath strands (B,D) isolated from *Alternanthera tenella* (C_3-C_4 intermediate). The photomicrographs were taken at either low (A,B) or high (C,D) magnification. The bars represent 200 μ (A,B) and 50 μm (C,D), respectively.


A
B
C
D

and 440 nm (Fig. 8.2). There were no conspicuous differences in the quality of these spectra.

The rates of respiratory 02 uptake in mesophyll protoplasts were similar to the bundle sheath cells. However the rates of photosynthetic 02 evolution by mesophyll protoplasts were slightly higher (by 30%) than those of bundle sheath cells. On the other hand, the Chl *a/b* ratio was slightly higher in bundle sheath cell preparation compared to the mesophyll protoplasts (Table 8.1).

The activity-levels of some of the C4 photosynthetic enzymes like PEP carboxylase and NAD-malic enzyme were similar in both mesophyll protoplasts and bundle sheath cells. However, the activities of other photosynthetic enzymes like NADP-/NAD-malate dehydrogenases and NADP-malic enzyme were slightly lower in bundle sheath cells than those in the mesophyll protoplasts (Table 8.2). Further, the reduction in activities of NAD-MDH and NADP-malic enzyme in bundle sheath cells compared to those in mesophyll protoplasts was significant ($P < 0.005$).

The activities of key photorespiratory enzymes (GO, NADH-HPR and P-glycolate phosphatase) were similar in both mesophyll and bundle sheath cells. However, the activity-levels of catalase and PGA phosphatase were slightly higher in bundle sheath cells than in mesophyll protoplasts (Table 8.3). The ratio of PGA-/P-glycolate phosphatase was higher in the mesophyll protoplast fraction than in

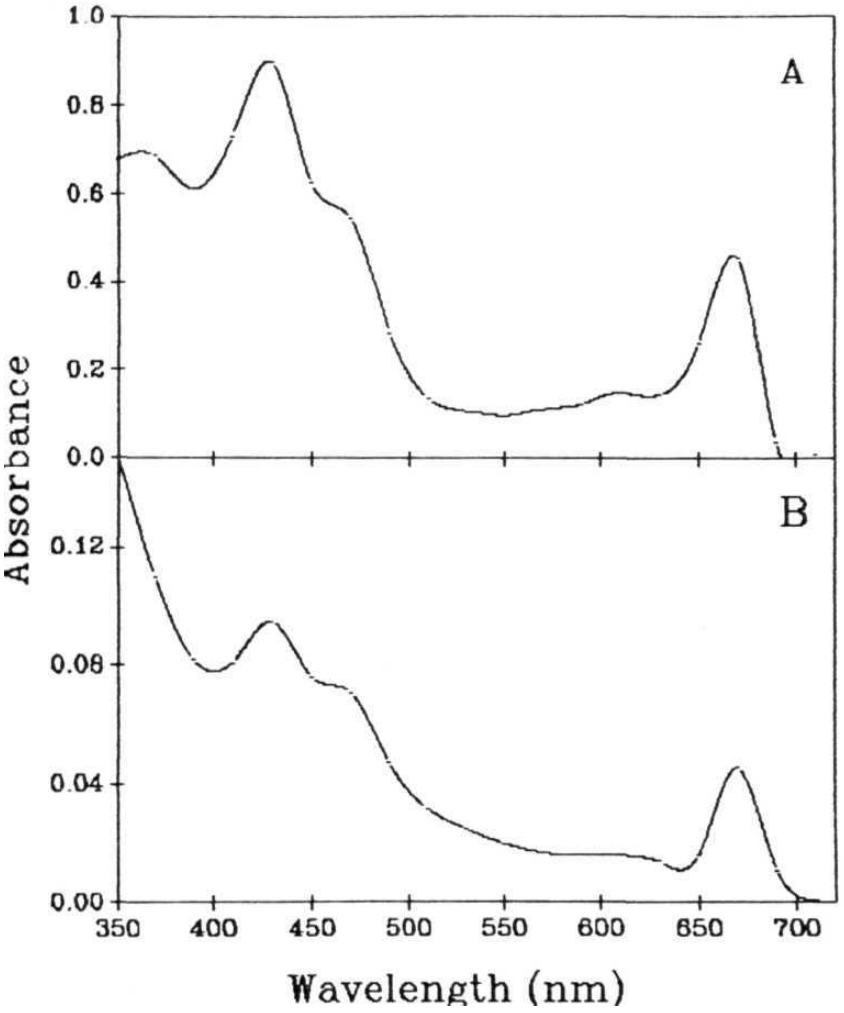


Fig. 8.2. Spectra of mesophyll protoplasts (A) and bundle sheath strands (B) isolated from leaves of *Alternanthera tenella.*

Table 8.1. Properties of mesophyll protoplasts and bundle sheath cells from *Alternanthera tenella*

Properties	*Mesophyll* protoplasts	Bundle sheath cells
Size (μm)	25 ± 5[•]	48 ± 3
Photosynthesis (μmol 02 evolved mg^{-1} Chl h^{-1})	120 ±11	93 ± 8
Respiration (μmol O_2 consumed mg^{-1} Chl h^{-1})	17 + 1	18 + 2
Photosynthesis/Respiration	7.1	5.2
Chl *a/b* ratio"	2.98	3.31
Protein/Chi"	4.17	3.85

Two different sizes (average diameters of about 20 and 30 μm) of protoplasts are observed.

[•]The values of Chl *a/b* and protein/Chi for intact leaf were 3.10 and 4.00 respectively.

Table 8.2. Activity-levels of photosynthetic enzymes in whole leaf, mesophyll protoplasts and bundle sheath strands of *Alternanthera tenella*

Enzyme	Whole leaf	Mesophyll protoplasts	Bundle sheath cells
		μmol mg Chl h	
PEP carboxylase	73 + 6	79 + 6	**69 + 5**
NAD-malic dehydrogenase	2173 + 223	2241 + 193	1858 ± 116
NADP-malic dehydrogenase	85 ± 6	95 + 8	66 + 7
NAD-malic enzyme	119 ± 10	132 + 9	111 ± 10
NADP-malic enzyme*	53 ± 4	85 ± 7	35 ± 3

The mean activities in mesophyll and bundle sheath fractions are significantly different ($P < 0.005$).

Table 8.3. Activity-levels of photorespiratory enzymes in whole leaf, mesophyll protoplasts and bundle sheath strands of *Alternanthera tenella*

Enzyme	Whole leaf	Mesophyll protoplasts	Bundle sheath cells
		µmol mg Chl h	
Glycolate oxidase	101 ± 9.3	111 ±10	96 ± 8.4
NADH-hydroxypyruvate reductase	469 + 38	486 4 41	451 *t* 39
Phosphoglycolate phosphatase	744 ± 59	799 ± 64	741 t 49
PGA phosphatase	490 ± 33	469 ± 37	543 ± 41
Catalase*	189 ± 17	169 ± 12	194 ± 16
Glycine decarboxylase	37+4	10 ± 2	72+6
Serine hydroxymethyltransferase	35 ± 3	11 ± 1	67+7

*Activity in mmol mg $^{-1}$ Chl h $^{-1}$

the bundle sheath cells. In contrast, the levels of glycine decarboxylase and serine hydroxymethyltransferase were 6-7 fold higher on Chl basis (6-10 fold on protein basis) in bundle sheath cells than those in mesophyll (Table 8.3).

The activity-levels of some citric acid cycle enzymes (citrate synthase, isocitrate dehydrogenase, succinate dehydrogenase and fumarase) and cytochrome c oxidase were 45-190% higher in bundle sheath cell preparation than in the mesophyll protoplasts on chlorophyll basis (45-210"/. on protein basis) (Table 8.4).

Discussion

All the known C_3-C_4 intermediates are characterized by a partial or near-complete Kranz leaf anatomy (Holaday and Chollet 1984, Rajendrudu *et al.* 1986, Edwards and Ku 1987, Brown and Hattersley 1989, Rawsthorne 1992). In most of the intermediates, the bundle sheath cells are not as distinct as in C_4 species (Brown and Hattersley 1989). Studies of Rajendrudu *et al.* (1986) and our own observations (Chapter 3 of this Thesis) indicated partial Kranz leaf anatomy in *Alternanthera tenella*. Therefore, an attempt is made to study the distribution of photosynthetic, photorespiratory and respiratory enzymes in mesophyll protoplasts and bundle sheath cells, by measuring their maximum catalytic activities.

Bundle sheath protoplasts can be readily distinguished from mesophyll protoplasts by their large size and the characteristi-

Table 8.4. Activity-levels of respiratory enzymes in whole leaf, mesophyll protoplasts and bundle sheath strands of *Alternanthera tenella*

	mg Chl			mg Protein		
Enzyme	Leaf	Mesophyll proto-plasts	Bundle sheath cells	Whole leaf	Mesophyll proto-plasts	Bundle sheath cells
			µmol h			
Citrate synthase	289 ± 22	217 + 17	378 ±36	69 + 6	51 ± 4	100 ± 8
Isocitrate dehydrogenase	46 ± 4	37 ± 3	55 + 5	12 ± 1	9 ± 1	13 ± 1
Succinate dehydrogenase	154 ±13	129 ±11	184 ±16	36 ± 4	32 ± 3	47 ± 5
Fumarase	59 ± 5	31 ± 4	89 ± 8	15 ± 1	8 ± 1	25 ± 2
Cytochrome c oxldase	152 ± 12	120 + 11	213 ±24	38 ± 4	28 ± 3	54 ± 5

cally asymmetric orientation of chloroplasts within the protoplasts (Fig. 8.1 A to D). Similar observations have earlier been made with mesophyll and bundle sheath protoplasts of *Moricandia arvensis* (Holaday et *al.* 1981, Winter *et al.* 1982, Holbrook and Chollet 1986). There is considerable disagreement in the literature on Chl *a/b* ratios in bundle sheath cells of C_3-C_4 intermediates. The Chl a/b ratio in bundle sheath has been reported to be either low (*Panicum milioides*; Ku *et al.* 1976), or high (*Mollugo nudicaulis*; Chellappan and Gnanam 1980) or similar to mesophyll cells *{Flaveria ramosissima*; Moore *et al.* 1988). In the present study, Chl a to b ratio was slightly higher in bundle sheath cells compared to mesophyll protoplasts (Table 8.1).

The spectral characteristics of these two cells were similar, with two major peaks at around 640 and 440 nm (Fig. 8.2). Further, the spectra resembled the absorption spectrum of maize mesophyll protoplasts (Devi et *al.* 1992b).

The rate of photosynthetic O2 evolution was high in mesophyll protoplasts compared to the bundle sheath cells in *A. tenella,* which indicates that the light reactions are more active in mesophyll cells. Respiratory O_2 uptake was similar in either cells (Table 8.1).

Similar activities of PEPC , NAD-ME and related enzymes in mesophyll and bundle sheath cells (Table 8.2) suggests that the

predominant photosynthetic metabolism is via Calvin cycle in both the cells and the operation of typical C_4 cycle is unlikely in this species. As per the literature, the pattern of distribution of photosynthetic enzymes between mesophyll and bundle sheath fractions varied among different intermediates. In *Flaveria ramosissima* and *Mollugo nudicaulis*, the activity of PEPC was high in mesophyll fractions whereas bundle sheath fraction contained high NADP-ME activity (Chellappan and Gnanam 1980, Moore *et al* 1988). The levels of NAD-MDH in bundle sheath cells were almost twice of those in mesophyll protoplasts, while NADP-MDH activities were similar in both the cells in case of *P. milioides* (Ku *et al,* 1976). The observed differences in the distribution of key photosynthetic enzymes among different intermediate species reflect the varying degree of C_4 cycle development in these species. *F. ramosissima* is likely to be more advanced C3-C4 intermediate than *A. tenella.* The enzyme distribution in mesophyll and bundle sheath cells of *A. tenella* observed in this report (Tables 8.2 and 8.3) is similar to the situation in *Panicum milioides* (Ku *et al.* 1976).

The levels of peroxisomal enzymes (GO, NADH-HPR, catalase) and related ones (P-glycolate phosphatase) were similar in mesophyll and bundle sheath preparations of *A. tenella* (Table 8.3), in agreement with the pattern reported in *P. milioides* (Ku *et al.* 1976), *Moricandia arvensis* (Rawsthorne *et al.* 1988b) and *Flaveria ramosissima* (Moore *et al.* 1988). The predominant location of two

photorespiratory enzymes (glycine decarboxylase and serine hydroxymethyltransferase) in bundle sheath cells (Table 8.3) suggests that the process of glycine decarboxylation is mostly located in the bundle sheath cells of *A. tenella.* This may be an important factor leading to a reduction of photorespiration in this species. Similar situation is reported in two intermediates, namely *H. arvensis* (Rawsthorne *et al.* 1988b) and *F. ramosissima* (Moore *et al.* 1988).

Unlike the photosynthetic or photorespiratory enzymes, a marked localization of mitochondrial (respiratory) enzymes could be seen in the bundle sheath cells of *A. tenella* (Table 8.4). Two to three fold higher activities of cytochrome c oxidase and fumarase were reported in the bundle sheath cells of *P. milioides* (Ku *et al.* 1976) and *M. arvensis* (Rawsthorne *et al.* (1988b), respectively. The high levels of respiratory enzymes may be due to the enrichment of mitochondria in bundle sheath compared to the mesophyll cells (Brown and Hattersley 1989). The result of such concentration of mitochondrial enzymes involved in glycine decarboxylation/respiration is that the C02 released in bundle sheath mitochondria either from photorespiration or dark respiration should pass over the adjacent chloroplasts (and mesophyll cells) while diffusing out of the leaf. This increases considerably the possibility of refixation of photorespired CO_2 in C3-C4 intermediates (Edwards and Ku 1987, Brown and Hattersley 1989, Rawsthorne 1992).

Any compartmentation of the photorespiratory pathway in the leaves of *A. tenella* requires also an efficient transport of metabolites between mesophyll and bundle sheath cells. According to Rawsthorne (1992) the carbon skeleton of serine (either serine itself or one of its products) returns to the mesophyll cells from the bundle sheath cells after the decarboxylation of glycine in order to keep the carbon for the operation of Calvin cycle in the mesophyll cells. The metabolites could move between the two cell types down the concentration gradients (Rawsthorne 1992, Rawsthorne et al. 1988a) as they do in C_4 species (Hatch 1987). This proposal is supported by the high levels of glycine and serine pools (four fold greater on a per-unit-leaf- area basis) observed in the intermediate species *Moricandia arvensis* (Rawsthorne and Hylton 1991). Short interveinal distances and high frequency of plasmodesmata in C3-C4 intermediates unlike in C3 species (Kanai and Kashiwagi 1975, Morgan and Brown 1979, Monson et *al.* 1984, Brown *et* al. 1983b, Rawsthorne 1992) could facilitate an efficient transfer of these metabolites.

The present study on the distribution of various enzymes in mesophyll protoplasts and bundle sheath cells of *Alternanthera tenella* suggests that there is a preferential localization of at least mitochondrial metabolism in bundle sheath cells. The compartmentatlon of photorespiratory glycine metabolism enzymes (glycine decarboxylase and serine hydroxymethyltransferase) in the bundle sheath cells helps in the reduction of photorespiration in

this species. Further experiments are needed to understand the exact implication of mitochondrial concentration in bundle sheath cells in achieving low photorespiration in C3-C4 intermediates.

Chapter 9

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SUMMARY AND CONCLUDING REMARKS

The reduced photorespiration in C3-C4 intermediates has so far been attributed to either (i) operation of a partial or near complete C_4 cycle, or (ii) efficient refixation of photorespired CO_2 (Edwards and Ku 1987, Monson and Moore 1989, Rawsthorne 1992, Raghavendra and Das 1993). The present work attempted to assess the factors responsible for the reduction of photorespiration in three C3-C4 intermediate plant species, namely *Alternanthera ficoides, Alternanthera tenella* and *Parthenium hysterophorus.*

The results of this study demonstrated that the photorespiratory metabolism was decreased in the intermediates. There was a partial reduction (about 25%, compared to the C3 species) in the maximum catalytic activities of key photorespiratory enzymes (e.g. glycolate oxidase, hydroxypyruvate reductase, catalase and glycerate kinase) in the C3-C4 intermediates. A partial reduction in some of the photorespiratory enzymes was noticed earlier in other C3-C4 intermediate species (Ku *et al.* 1976, 1991, Sayre and Kennedy 1977, Sayre et *al.* 1979, Moore et *al.* 1988, Rawsthorne et *al.* 1988b, Kumar and Abrol 1990). Yet, the present work is the first comprehensive report on activities of several photorespiratory and photosynthetic enzymes in C_3-C_4 intermediate species.

There was a partial reduction (about 25-30%) also in the levels of photorespiratory metabolites (ammonia, glycolate and glyoxylate) in these C3-C4 intermediates. The extent of such reduction in metabolites was correlated well with the reduction in activity-levels of photorespiratory enzymes. Reduced levels of photorespiratory ammonia accumulation were reported in C3-C4 intermediates of *Moricandia arvensis* and *Parthenium hysterophorus* (Kumar and Abrol 1989, 1990). Reduced incorporation of ^{14}C into glycolate was observed in *Panicum milioides* (Servaites *et al.* 1978) and in *Parthenium hysterophorus* (Moore *et al.* 1987a). However, this is the first report on glyoxylate (besides glycolate) pools in leaves of C3-C4 intermediates.

Despite the presence of a partial Kranz-anatomy, there was no indication of even a partial C_4 pathway operation in the present intermediates based on the observations on enzyme complement, photosynthetic O_2 evolution and characteristics of PEPC. The low ratios of PEPC/RuBPC and PEPC/RuBPO and PGA-/P-glycolate phosphatase, low levels of PPDK, low quantum requirements and saturation of photosynthesis at light intensities <400 µmol m s (all characteristics similar to C3) pointed at the insignificance or non-operation of C4 pathway in these intermediates. Thus, the present study confirms that development of partial Kranz-leaf anatomy and C_4 cycle are not always correlated. Similar results were shown earlier for C3-C4 intermediate species of *Seurachne minor* (Moore and Edwards 1989) and *Eleocharis pusilla* (Bruhl *et al.* 1987).

Although, the overall pattern of photosynthetic 02 evolution in C_3-C_4 intermediates was similar to that in C3 species, the response of their photosynthesis to external CO2 was significantly less than that of C3 ones. The extent of photoinhibition, light compensation point, and values of quantum yield did not change in intermediates as much as in C3 plants due to the rise in ambient CO2. Similarly, the accumulation of photorespiratory metabolites in C3-C4 intermediates was not as severely lowered as in C3 ones by 5 mM bicarbonate in the external medium. All these results indicate the possibility of a larger internal CO2 pool in leaves of the C3-C4 intermediate species than that in C3 species. An increase in internal inorganic carbon pool was suggested in some of the C_3-C_4 intermediates of *Flaveria* (Moore *et al*. 1987b).

Although the activity-levels of photorespiratory enzymes were reduced, there was no evidence of any major alteration in the form of two key enzymes involved in the photorespiration or C_4 photosynthesis. The kinetic and regulatory properties of glycolate oxidase (an important photorespiratory enzyme) from the C3-C4 intermediate species (*Parthenium hysterophorus*) were similar to that from C3 species (*Pisum sativum*). The light activation pattern and kinetic/regulatory properties of PEPC in C3-C4 intermediate *Alternanthera ficoides* were similar to the C3 form of PEPC.

In the present study, the levels of photorespiratory mitochondrial enzymes (glycine decarboxylase and serine hydroxymethyltransferase) were much more in bundle sheath than those in

mesophyll cells of *A. tenella.* The photorespiratory glycine metabolism (glycine decarboxylation) was predominantly localized in bundle sheath cells of C3-C4 intermediates of *Flaveria, Moricandia* and *Panicum* (Hylton *et al.* 1988). This indicates that the localization of glycine metabolism in bundle sheath cells can be an important factor in achieving improved refixation of photorespired CO2 in this species. In addition to the photorespiratory enzymes, the activity-levels of several TCA cycle enzymes also were high in bundle sheath cells emphasizing the enhanced mitochondrial metabolism in bundle sheath cells.

Preferential localization of glycine decarboxylase in bundle sheath cells leads to the production of photorespiratory CO2 deep within the leaf and reduces the possibility of CO2 exit. The photorespired CO_2 will be refixed by Rubisco or PEPC either in bundle sheath cells or mesophyll cells before it escapes from the leaf. Studies of the activity-levels of PEPC and inhibition pattern of photosynthesis with DCDP suggests that PEPC may play an important role in such refixation of photorespired CO_2 in the intermediates.

An important emphasis of the present investigation is the mitochondrial specialization in bundle sheath of C_3-C_4 species. Most of the C_3-C_4 intermediates, as per the literature have a tendency to localize glycine decarboxylase and serine hydroxymethyltransferase in bundle sheath cells (Rawsthorne 1992). Ultrastructural observations reveal that there is a concentration

of large mitochondria in bundle sheath cells, arranged along the cell walls adjacent to vascular tissue overlain by chloroplasts in intermediates like *Panicum millioides* (Brown and Hattersley 1989). The importance of mitochondrial metabolism is also indicated by the relative insensitivity of dark respiration to external CO_2 in C4 plants and C_3-C_4 intermediates, compared to the marked inhibition in C3 plants. We therefore feel that an altered mitochondrial metabolism may be an important feature of C_3-C_4 intermediates and C4 species.

Thus a partial reduction in the photorespiratory metabolism itself may be an important factor in the reduction of photorespiration in these intermediates. Refixation of photorespired CO_2 as suggested for other *Moricandia*, *Panicum, Flaveria* (Hunt *et al.* 1987, Hylton *et al.* 1988, Rawsthorne *et al.* 1988a,b, Morgan *et al.* 1992, 1993, Rawsthorne 1992) intermediates can further help to reduce photorespiration in the intermediates of *Alternanthera* and *Parthenium*.

A consistent feature of intermediates is their insensitivity to external CO_2, as indicated by the response of their photorespiratory metabolites or photoinhibition or dark respiration to ambient CO_2. We interpret these observations to suggest the existence of a large pool of internal CO_2 in C3-C4 intermediates compared to C3 plants. Such high internal CO_2 (which may be due to also an efficient CO_2 fixation mechanism) can lead to a further reduction in photorespiratory enzymes/metabolism.

These conclusions are summarized and incorporated into a hypothetical model (Fig. 9.1) to explain the possible mechanism of reduced photorespiration in intermediates of *Alternanthera* and *Parthenium*. This model could be a third alternative to the already proposed two possibilities, namely the operation of partial or near complete C_4 pathway and the efficient refixation of photorespired C02.

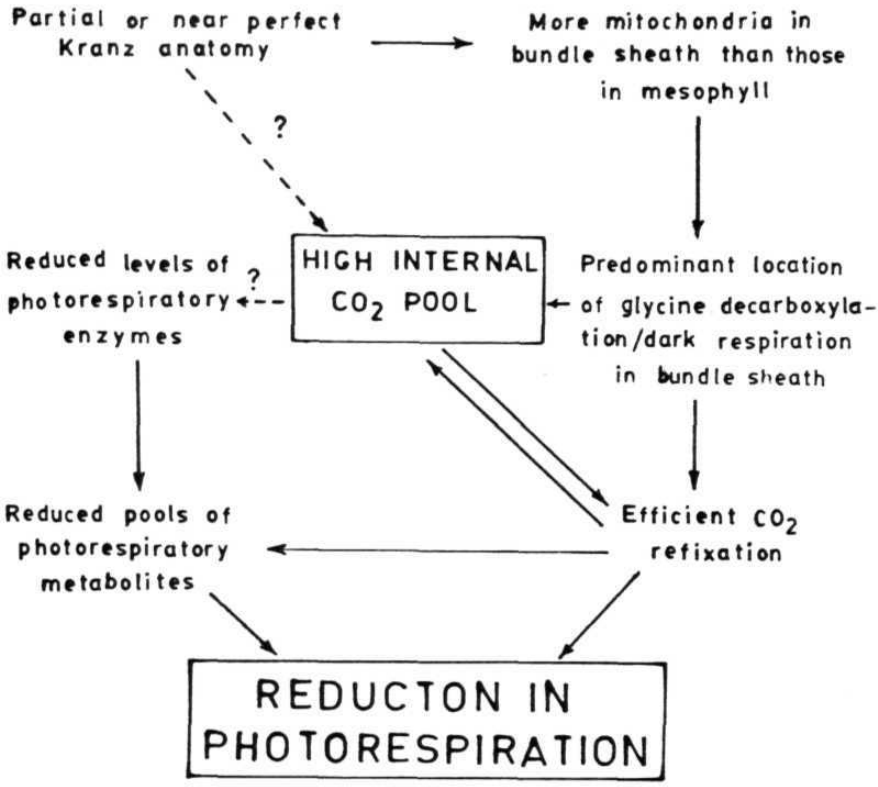

Fig. 9.1. A possible model of reduced photorespiration in **C3-C4** intermediates of *Alternanthera* and *Parthenium.* The occurrence of "partial Kranz anatomy" and **mitochondrial** enrichment in bundle sheath result **in** a marked confinement of **glycine decarboxylation** and dark respiration to these cells. As a result, the photorespiratory/dark respiratory CO2 efflux is restricted to the tissues deep within the leaf, keeping up a high pool of internal **CO2**. The efficient **refixation/internal** cycling of CO2 helps further in the maintenance of high CO2 pools. The partial reduction in activity-levels of key photorespiratory enzymes and reduced pools of metabolites form the additional factors responsible for the reduction in photorespiration in these species. Additional experiments are necessary to assess if high internal CO2 pool results in reduced activity-levels of photorespiratory enzymes or if a partial Kranz anatomy can directly facilitate the build-up of a high CO2 pools **within the leaf.**

Chapter 10

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Appendix

APPENDIX

LIST OF PUBLICATIONS

(1) **Tirumala Devi** M and **Raghavendra** AS (1992) Light activation of phospho*enol*pyruvate carboxylase in maize **mesophyll** protoplasts. *J. Plant Physiol.* **139:** 431-435.

(2) Tirumala **Devi M,** Rajagopalan AV and Raghavendra AS (1992) Structure, regulation and biosynthesis of phosphoenol-pyruvate carboxylase from C4 plants. *J. Plant Biochem. Blotechnol.* 1: 73-80.

(3) **Tirumala** Devi **M,** Vani T, **Mal** la Reddy M and Raghavendra AS (1992) Rapid isolation of **mesophyll** and guard cell protoplasts from leaves of pea and maize. *Indian J. Exp. Biol.* 30: 424-428.

(4) Tirumala Devi **M** and Raghavendra AS (1992) Reduced photorespiratory enzymes and ammonia levels **in** C3-C4 intermediates of *Alternanthera* and *Parthenium.* In *Research in Photosynthesis* (Ed. N Murata). Vol. III. pp. 887-890. **Kluwer** Academic Publishers, Dordrecht.

(5) Tirumala Devi **M** and Raghavendra AS (1993) Partial reduction in activities of photorespiratory enzymes **in C3-C4** intermediates of *Alternanthera* and *Parthenium. J.Exp. Bot.* 44: 779-784.

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(7) Tirumala Devi **M,** Rajagopalan AV and Raghavendra AS (1993) Photosynthetic and photorespiratory characteristics of C3-C4 **intermediates** of *Alternanthera* and *Parthenium.* In *Proceedings of DAE Symposium on Photosynthesis and Plant Molecular Biology.* pp. 141-146. Bhabha Atomic Research Centre, Bombay, India.

(8) **Tirumala Devi M** and Raghavendra AS (1993) **Photorespiration** in C3-C4 intermediate species of *Alternanthera* and *Parthenium:* Reduced ammonia production and increased capacity of CO2 **refixation in** the light. *Photosynth. Res:* in press.

(Continued on next page)

(Appendix continued)

(9) Rajagopalan AV, Tirumala Devi M and Raghavendra AS (1993) Pattern of phosphoenoipyruvate carboxylase activity and cytosolic pH during light activation and dark deactivation in C3 and C4 plants. *Photosynth Res:* in press.

(10) Rajagopalan AV, Tirumala Devi M and Raghavendra AS (1993) Molecular biology of C4 phosphoenoipyruvate carboxylase: Structure, regulation and genetic engineering. *Photosynth Res:* in press.

(11) Rajagopalan AV, Tirumala Devi M and Raghavendra AS (1993) Light activation and dark-deactivation of phosphoenoipyruvate carboxylase in leaves of C4 plants. *Transaction of Malaysian Society for Plant Physiol*. Vol. 3. (Special Edition): in press.

Copies are attached.

J. Plant Physiol. Vol. 139.pp. 431–435 (1992)

Light Activation of Phosphoenoipyruvate Carboxylase in Maize Mesophyll Protoplasts

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Received July 17, 1991 • Accepted **September** 18, 1991

Summary

The effect of light on phosphoenoipyruvate (I'EP) carboxylase (EC 4.1.1.31) activity in maize *(Zea mays* L. **cv.** Ganga 5) mesophyll protoplasts was examined. When protoplasts were illuminated in a low-buffered (2 mM) medium, there was a 20-35% increase in the activity of PEP carboxylase over that in darkness. There was no light activation, however, if the protoplasts were kept in a high-buffered (20 mM) medium. Maximum activation occurred after 10–15 min of **illumination** and the activation was lost **within** 8 min **after transfer** to darkness. **The** light activation of PEP carboxylase was completaly suppressed **by** phlorizin or CCCP, but not by DCMU. The characteristics of the light activated form **of PEP** carboxylase in protoplasts (*in situ*) differed slightly from those observed with leaves *(in vivo).* We suggest that **light-induced alkalization** of the cytosol and ATP formation are involved in the activation of PEP carboxylase, possibly through phosphorylation of the enzyme.

Key words: C₄ photosynthesis, light activation, mesophyll protoplasts, I'EP carboxylase, cytosolic pH, Zea mays.

Abbreviations: BSA - **Bovine** serum albumin; CCCP - Carbonyl cyanide m-chlorophenylhydrazone; Chl - **Chlorophyll!** DCMU - 3-(3',4'-Dichlorophenyl)-1,1-dimethylurea; HEPES = N-2-I lydroxy-ethyl **piperazine-N'-2-ethanesulphonic** acid; MDH - Malate dehydrogenase; MES = 4-Morpholi-noethane sulphonic acid; PEP = Phosphoenoipyruvate; PPDK = Pyruvate, phosphate dikinase.

Introduction

Phosphoenoipyruvate (PEP) carboxylase is a key enzyme involved in primary CO; fixation by C_4 and CAM plants (Andreo **et al.,** 1987). The enzyme is also present in C, **plants, algae and** bacteria (O'Leary, 1982). Apart from photosynthetic carbon assimilation, other functions of PEP carboxylase include replenishment of tricarboxylic acid cycle intermediates, generation of NADPH, refixation of respired CO_2, nitrogen assimilation, amino acid synthesis and maintenance of pH/electroneutrality **(Latzko** and Kelly, 1983). PEP carboxylase is **a** cytosolic enzyme and occurs in **leaves as well** as non-photosynthetic organs. In C_4 species, **PEP carboxylase** is located primarily in mesophyll cells **(Edwards and Walker,** 1983).

There have been several studies on the regulation of PEP **carboxylase. In C_4 plants,** the enzyme is regulated by factors such as light, temperature and pH. The activity of the enzyme is modulated by metabolites, particularly glucose-6-phosphate and **glycine,** and is subject to feedback inhibition by organic acids like L-malate or aspartate. Compounds that are known to inhibit the enzyme include analogues of pyruvate or PEP such as bromopyruvate, phospholactate or phosphomalate (Gonzalez and Andreo, 1989), glyphosine (Podesta et al., 1987), and 3,3-dichloro-2-dihydroxyphosphinoylmethyl-2-propenoate (Jenkins **et** al., 1987). A strong interaction exists between light, temperature and pH in regulation of PEP carboxylase, as shown in the case of *Amaranthus paniculatus* and *Zea mays* (Selinioti et al., 1986; Karabourniotis et al., 1985; Grammatikopoulos and Manetas, 1990).

Photosynthetic enzymes like ribulose-5-P kinase, NADP-malate dehydrogenase (MDH), NADP-triose phosphate dehydrogenase and fructose-l,6-bisphosphatase are regulated

by light through electron transport and the ferredoxin-thioredoxin system (Buchanan, 1980). Similarly, light modulates some of the enzymes involved in C_4 photosynthesis. Upon illumination, the activities of pyruvate, phosphate dikinase (PPDK) and NADP-MDH are stimulated several fold in leaves or mesophyll protoplasts of C_4 plants (Nakamoto and Edwards, 1986). During greening, there is a marked increase in the activity of not only PPDK and NADP-MDH, hut also PEP carboxylase (Hauge and Sims, 1980). Such enhancement in PEP carboxylase is due to an increase in the *de novo* synthesis of the enzyme.

I he interest in light regulation of PEP carboxylase was revived with recent reports on the modulation by light of PEP carboxylase activity and malate sensitivity in leaves of maize (Karabourniotis et al., 1985; Doncaster and Leegood, 1987; Chollet et al., 1990), *Amaranthus* (Sclinioti et al., 1986) and *Sedum praealtum* (Manetas, 1982). However, the role of light in the activation of PHI[1] carboxylase remained ambiguous, since the promotive effect of light could not be demonstrated if the enzyme was extracted and assayed at pH 8.0. Similarly, light activation of PEP carboxylase has so far not been reported in mesophyll protoplasts from leaves of C4plants, unlike the stimulation of PPDK or NADP-MDH (Nakamoto and Edwards, 1986).

In the present report, mesophyll protoplasts from leaves of maize were used to reexamine the effect of light on PEP carboxylase activity. Activation of the enzyme occurred only when protoplasts were suspended in a low-buffered medium, indicating that the high buffering capacity normally used during experimentation prevented the detection of light activation of PEP carboxylase. We also present some evidence to indicate that cytosolic pH could be involved in light activation of PEP carboxylase. While this article was under preparation, a report appeared suggesting the usefulness of maize mesophyll protoplasts as a source of PEP carboxylase (Petropoulou et al., 1990).

Materials and Methods

Plant material

Plants of maize (*Zea mays* L. cv. Ganga 5) were raised in soil supplemented with farm-yard manure in 15-cm diameter plastic tubs. Plants were grown outdoors under a photoperiod of approximately 12 h and mean temperatures of 30 °C day/20 °C night. Fully expanded second and third leaves were taken from 8- to 10-day-old plants between 8.00 a.m. to 9.00 a.m. (2 to 3h after exposure to light).

Isolation and incubation of mesophyll protoplasts

The leaves were cut into 1-mm thick pieces and kept in the digestion medium in a petri dish containing 2% (w/v) Cellulase «Onozuka R-10», 0.2% (w/v) Macerozyme, 0.2% (w/v) BSA, 10 mM MES-KOH, pH 5.5, 0.5 M sorbitol, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.2mM KH_2PO_4 for 1 h at 30 °C. The digestion medium was sucked off with the help of a Pasteur pipette. A few mL of fresh resuspension medium, consisting of either 2 mM or 20 mM HEPES-KOH, pH 7.2, 0.5 M sorbitol, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM KH_2PO_4, were added to the petri dish to submerge the leaf pieces. The pctri dishes were lapped gently to release the mesophyll protoplasts. The medium containing released protoplasts was passed successively through 500-, 85- and 60-um nylon filler cloths. The final filtrate was centrifuged at 100 g for 5min and the pellet was suspended in the resuspension medium. Further details are described elsewhere (Tirumala Devi et al., 1991).

Protoplasts were washed and resuspended in a low (2 mM) or high (20 mM) HEPES-buffered resuspension medium (described above) in such a way so as to have the equivalent of 200 ug Chl mL^{-1}. The suspension was kept in darkness for at least 1 h at 0 °C and then either maintained in darkness at 30 °C or illuminated with tungsten lamps (Philips Comptalux 75 W) to provide an intensity of 1000 $\mu mol\, m^{-2} s^{-1}$. A water filter was placed between the light source and the protoplast suspension to minimize healing. At the required time, a 50-uL aliquot of protoplasts, equivalent to 10 ug chlorophyll, was added to the reaction mixture to assay the enzyme.

Assay of PEP carboxylase (EC 4.1.1.31)

The activity ol PEP carboxylase was followed spectrophotometrically in a coupled system using NAD-MDH. The reaction mixture contained 20mM Tricine-KOH, pH 7.8, 5mM $MgCl_2$, lOmM $NaHCO_3$, 2 units NAD-MDH, 0.2mM NADU and 2.5 mM PEP in a final volume of 1.0 ml. The oxidation of NADH was measured at 340 nm and 30 °C in a Hitachi (Model 150-20) spectrophotometer. The protoplasts were completely broken upon addition to the hypotonic reaction medium. During preliminary trials, the addition of Triton X-100 (0.05 % or 1 %, v/v) did not increase activity.

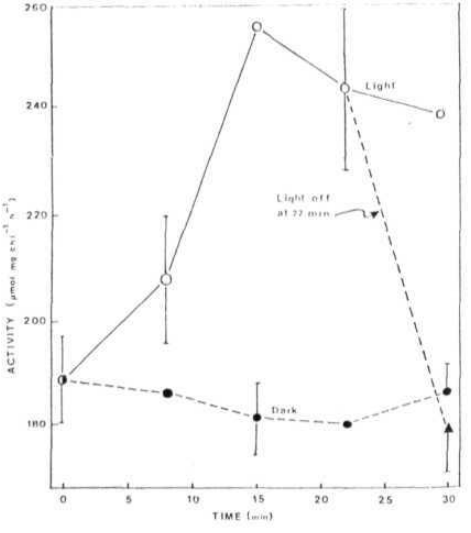

Fig. 1: Light activation o(PEP carboxylase in maize mesophyll protoplasts, The protoplasts were kept in darkness or under illumination in low (2 mM) buffer at pH 7.2 and 30 °C. An aliquot of protoplasts was taken and assayed for en7ymc activity. The activity of PEP carboxylase increased during illumination. When light was switched off at 22 min, the activity fell to the dark level (closed triangle). There was only a slight decrease in the activity of the enzyme at 30 min in protoplasts kept under light (open circles).

Triton X was therefore not used in subsequent experiments. Similarly, the presence or absence of 5 mM DTT also had no effect on enzyme activity.

Test compounds were included either in the reaction mixture or during protoplast incubation as indicated in Results. The data presented in this report are the averages (± SD) of at least three experiments.

Chemicals

Cellulase «Onozuka R-10» and Macerozyme R-10 were from Yakult Honsha Co. Ltd., Nishinomiya, Japan; Phlorizin, DCMU, CCCP and MDH were from Sigma Chemical Co., USA. All other chemicals were of analytical grade from Glaxo Laboratories or Sisco Research Labs, Bombay, India. Nylon filter cloths were from Dadia Textiles, Bombay.

Results

The activity of PEP carboxylase in maize mesophyll protoplasts was stimulated by illumination (Fig. 1). After 10–15 min of illumination, the activity of PEP carboxylase was about 35% more than that in the protoplasts kept in darkness. There was a slight decrease in the activity after 15 min of illumination. There was not much change in PEP carboxylase activity when protoplasts were kept in darkness. When the illuminated protoplasts were returned to darkness, the activity of PEP carboxylase fell within 8 min to the level observed in darkness.

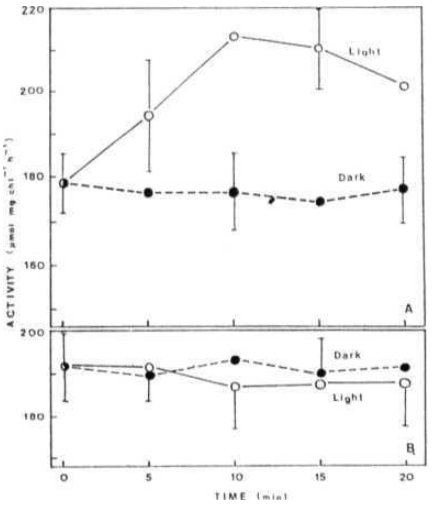

Fig. 2: The effect of buffer-strength on the light activation of PEP carboxylase. Mesophyll protoplasts isolated from **maize** leaves were suspended in either low (2 mM) (A) **or** high (20 mM) **buffer** (B), pH 7.2, and then exposed to light or kept in darkness. Aliquots were examined for enzyme activity at pH 7.8.

Table 1: The characteristics of PEP carboxylase in maize mesophyll protoplasts kept in either dark or light for 10 min at 30 °C. An aliquot of protoplasts after preincubation (low buffer, 2 mM) at pH 7.2 was added to the reaction medium (pH 7.8). Malatc or G-6-P was included in the reaction medium used for the assay of PEP carboxylase. **The** figures in parentheses represent the activity of the enzyme as the percent of respective control.

Parameter	Preincubation	
	Light	Darkness
	Activity	µmol mg Chl^{-1} h^{-1}
Kinetic properties		
V_{max}	213±22	195±21
K_m (PEP) mM	1.5	2.1
Sensitivity to effectors		
Control	185±20 (100)	155±18 (100)
2.5 mM malate	159±14 (86)	25± 7 (16)
2.5 mM G-6-P	187± 8 (101)	267±24 (172)

Table 2: Effect of DCMU, phlorizin or CCCP on light activation of PEP carboxylase *in situ*. The test compounds were included in the lower-buffer (2 mM) preincubation medium, pH 7.2, while protoplasts were kept in darkness or light at 30 °C. After 10 min, an aliquot was examined for PEP carboxylase activity,

Test compound during preincubation	Activity (µmol mg Chl^{-1} h^{-1})		Ratio of activity
	Light	Dark	Light/Dark
None (Control)	363±18	290±29	1.25
+ DCMU (10 µM)	357±30	285±27	1.25
+ phlorizin (1 mM)	259±21	273±28	0.95
+ CCCP (1 µM)	248±27	256±19	0.97

The light activation of PEP carboxylase was found only when the protoplasts were suspended and illuminated in low-buffered (2 mM) medium (Fig. 2 A). If the protoplasts were kept in high-buffered (20 mM) medium, there was no significant difference in enzyme activity from protoplasts kept in light or darkness (Fig. 2 B). Among different combinations of incubation and assay pHs (7.0 to 8.5), incubation in pH 7.2 and assay at pH 7.8 were optimal for the light response of PEP carboxylase.

The properties of PEP carboxylase from illuminated protoplasts were different from those in protoplasts kept under darkness (Table 1). The affinity of PEP carboxylase for PEP was higher in light than that **in** the darkness, whereas the V_{max} was only slightly altered. The enzyme extracted from illuminated protoplasts was less sensitive to L-malate (14% inhibition by 2.5 mM L-malate) or glucose-6-phosphate (no activation by 2.5 mM G-6-P) than that from protoplasts kept in darkness (84% inhibition by malate; 72% activation by G-6-P).

The effect of classical inhibitors **of** photosynthetic electron transport (DCMU), ATP **synthase** (phlorizin) or phosphorylation (CCCP) were examined. These test compounds were present during preincubation of protoplasts **in** *situ*. The

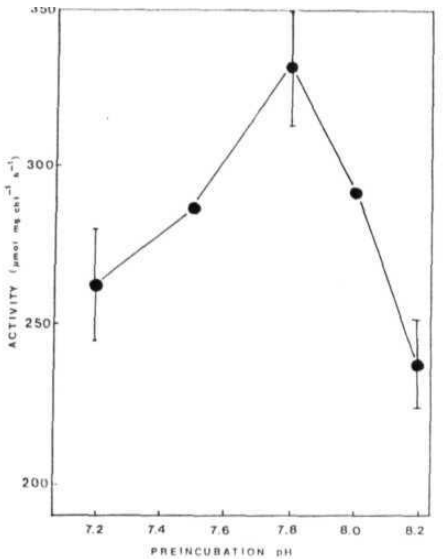

Fig. 3: **The effect** of **pH during** preincubation **on** the activity of PEP carboxylase in darkened maize mesophyll protoplasts. The protoplasts, resuspended in low buffer medium (2 mM), pH 7.5, were mixed and resuspended in 20 mM HEPES-KOH buffers, whose pH ranged from 7.2 to 8.2. Aliquots of protoplasts were taken after 10 mm of incubation in darkness and assayed for **PEP** carboxylase activity at pH 7.8.

light activation of PEP carboxylase was severely restricted by CCCP or phlorizin, but not by DCMU (Table 2).

In an **experiment designed** to simulate the **effect of** pH, protoplasts resuspended at pH 7.5 were preincubated in HEPES-buffered media whose pH ranged from 7.2 to 8.2 and then assayed at pH 7.8. Upon incubation for 10 min in darkness, maximal activity of PEP carboxylase was achieved at a preincubation pH of 7.8, while the activity decreased markedly at pH 7.2 or 8,2 (Fig. 3).

Discussion

This is the first report on light-modulation of PEP carboxylase in mesophyll protoplasts from leaves. The activation of PEP carboxylase by light in leaves of C_4 plants is known, but could be detected only when the enzyme was extracted and assayed at suboptimal pH. The extent of activation of PEP carboxylase by light was modified by other factors such as pH or temperature. Our ***in situ*** results confirm that light activates **maize** PEP carboxylase activity ***in vivo*** (Fig. 1). Thus, illumination promotes not only *de novo* synthesis of C_4 PEP carboxylase (Goatly et al., 1975; Hauge and Sims, 1980), but also the activity of the enzyme in C_4 leaves (e.g. Karabourniotis et al., 1985; Chollet et al., 1990) and **protoplasts (present report).**

The detection ol activation ol **the enzyme** by light in protoplasts only in a **low-buffered medium, but** not in **high-buffered** medium (Fig. 2), **suggested an** important **role of** pH **and/or** ionic **strength** during **activation ol** PEP **carboxylase,** Studies with fluorescent probes indicated that upon illumination, there is a **marked alkalization** of **the cytoplasm in** leaf mesophyll cells of several *C,* plants (Yin **et** al., 1990 a, b). There is also a high **degree** of light induced alkalization in mesophyll cells of C_4 plants (RAGHAVENDRA, A. S., Z.-H. YIN, and U. HEBER, unpublished observations). Since PEP carboxylase is located in the cytoplasm, such alkalization can lead to activation of **PEP** carboxylase. The increase in enzyme activity upon preincubation in darkness at pH 7.8, but not at 7.2 (Fig. 3), supports the proposed **role** of

1here have been several reports **indicating changes** in **the** kinetic and **allosteric** properties of PEP carboxylase in leaves upon **illumination** (Karabourniotis et **al.,** 1983; **Doncaster and** Leegood, 1987; Nimmo **et** al., 1987; Jiao **and** Chollet, 1988, 1989; Chollet et **al,,** 1990). The **light-activated form has .1 greater affinity** for PEP **and** is less **sensitive** to **nutate** inhibition than the dark adapted **form** ol PEP carboxylase. Our results confirm that upon illumination, while the activity of PEP carboxylase is stimulated, the sensitivity of the **enzyme** to effectors (such as malate or G-6-P) is severely reduced (Table **1).** In illuminated leaves, PEP carboxylase would therefore be able to tolerate an increase in malate **concentration.**

The extent and **kinetics of** light **activation/dark inactivation** of PEP carboxylase **in** protoplasts ***in situ*** (observed in present study) differ slightly from those observed **with** leaves *in vivo* (e.g. Doncaster and Leegood, 1987; Nimmo et al., 1987; Jiao and Chollet, 1988). The lack of the DCMU effect on light activation ol PEP **carboxylase in** our experiments (*in situ)* and the **insensitivity** ol the light **form** of enzyme to G-6-P also are at variance with earlier **reports** made **with leaves illuminated** *in vivo* (Samaras **et al.,** 1988; Review: Chollet et al., 1990). However, in at least one study, the inhibition of light activation of PEP carboxylase by DCMU was only partial, when the enzyme was assayed at low substrate levels (Karabourniotis et al., 1983). It is possible that during isolation the photosynthetic system in protoplasts gets partially affected. Isolated mesophyll protoplasts from C_4 plants neither **assimilate** carbon nor take up external **metabolites** such as PEP but can evolve oxygen in the presence of benzoquinone (cf. Edwards and Walker, 1983).

A likely **mechanism** that is involved in the light-dark regulation of **PEP** carboxylase activity from C_4 and CAM **plants** is the seryl-phosphorylation of the enzyme (Jiao and Chollet, 1988, 1989; Chollet et al., 1990; Brulfert et al., 1986; Nimmo et al., 1986). The suppression of light activation of PEP carboxylase in maize mesophyll protoplasts by CCCP or phlorizin (but not by DCMU) suggests that phosphorylation is essential to bring about the activation by light. The **PEP carboxylase** in **mesophyll protoplasts** resembles PPDK in this respect and differs from NADP-MDH, which is **light** activated due to the electron transport based ferredoxin-thioredoxin system (Nakamoto and Edwards, 1986). Further, **the**

protonophore CCCP could also dissipate the proton gradient across the membrane and disturb the pH of the microenvironment. Therefore, our suggestion that the activity of PEP carboxylase could be regulated through changes in cytosolic pH remains a distinct possibility.

While demonstrating the role of light in rapid modulation of PEP carboxylasc in mesophyll protoplasts of maize, we suggest that light may be acting through regulation of cytosolic pH and/or phosphorylation of the enzyme. It is possible that the protein kinase involved in phosphorylation of PEP carboxylase responds to pH.

Acknowledgements

Pan of this work was supported by a research grant (No. SP/SO/A43/88) from the Department of Science and Technology, New Delhi.

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J. Plant Biochemistry i Biotechnology Vol.1, 73-80, July 1992

Mini Review

Structure, Regulation and Biosynthesis of Phosphoenolpyruvate Carboxylase from C_4 Plants

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This review attempts to summarize the large body of Information on the structure, regulation and biosynthesis of the enzyme phosphoenolpyruvate carboxylase in C_4 plants which has accumulated particularly since the appearance of the last review in 1987. Among the major discoveries are the involvement of protein **phosphorylation-dephosphorylation** cascade **in** the light activation of the enzyme, extraction and characteristics of PEPC-protein **serine kinase,** dynamic changes **in oligomeric** state of the enzyme in response to pH or temperature, **isolation** of multiple cDNAs encoding **different** forms of PEPC and cloning and expression of **maize/sorghum** PEPC **in transgenic** tobacco or transformed *E. coll* cells. Further experiments using advanced techniques of biochemistry and molecular biology would help In understanding the molecular mechanism of reaction, regulation of enzyme activity, gene expression and evolutionary pattern of C_4 PEPC.

Phosphoenol pyruvate carboxylase {PEPC, Orthophosphate : oxaloacetate carboxylase (phosphorylating), EC 4.1.1.31} catalysing practically the irreversible |l-carboxylation of PEP, is an ubiquitous enzyme and is widely distributed in many plants and microorganisms. This is a key enzyme, accomplishing the primary carboxylation of PEP, in plants with C_4 or CAM pathway of CO., fixation. In view of the importance of the enzyme, PEPC was the focus of several reviews (1-6), and a special issue of an entire journal (*Physiologie Vegetate,* Vol. 21, No. 5, 1983). The regulatory properties of this enzyme have been discussed in reviews dealing with C_4 metabolism (7-9), Calvin cycle (10) or CAM (11).

The last comprehensive review on higher plant PEPC appeared in 1987 (4). Since then, a number of reports have appeared, particularly on the regulation of the enzyme by light and temperature, its gene and mRNA structures and other regulatory properties. This review concerns mainly with the properties of the C_4 enzyme. At places, the characteristics of PEPC from C_3 or C_3-C_4 intermediates or CAM species are also discussed to emphasize the nature of PEPC from plants with the C4 pathway of photosynthesis.

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Abbreviations : CAM — Crassulacean acid metabolism, OAA — Oxaloacetate, PEPC PK — Phosphoonol pyruvate carboxylase protein kinase, Rubisco — Ribulose-1, 5-bisphosphate carboxylase/oxygonase.

Occurrence

The kinetic and regulatory properties of the enzyme vary considerably with the source, *viz.* organ/plant. Although present in most of the plants, the activities of PEPC ih C_4 plants are very high and are several fold higher than those in C_3 ones (2, 4). While the enzyme ftom C_4 and CAM plants catalyzes the primary carboxylation step, many auxiliary functions are attributed to the enzyme from C_3 plants (2, 4, 10).

Besides the C_4 specific PEPC, other isozyme-forms of the enzyme occur in C_3 plants or etiolated C_4 leaves. These forms can be distinguished by chromatographic, *immunological* and kinetic properties (2, 4). The enzyme is also reported from non-photosynthetic tissues (12), fresh water/marine aquatic plants (13), the legume root nodules (14), and prokaryotic cyanobacteria (15).

The enzyme is extra-chloroplastic and located in the cytosol of mesophyll cells in C_4 and CAM plants (16). The enzyme from C_3 plants is distributed in cytosol as well as in chloroplasts (10, 16). The enzyme is purified from several sources (2) and its properties are extensively studied (4).

Significance of the enzyme

The most important function of PEPC is the primary CO_2 fixation in both C_4 and CAM plants. The action of this enzyme along with others of C_4 pathway achieves the concentration of CO_2 (several fold higher than the atmospheric levels) in bundle sheath cells, the site of Rubisco. The high concentration of CO_2 reduces the oxygenase activity of Rubisco, and thus minimizes the photorespiratoy CO_2 loss. PEPC is well

suited for this role of CO_2 concentrating mechanism in C_4 plants. With a low Km for HCO_3^- of about 7 μM, it is sufficiently active even at the very low CO_2 concentration found in the mesophyll, the site of PEPC (7, 9). Thus in C_4 photosynthesis and as well as in CAM, PEPC plays the major role of primary assimilation and concentration of CO_2.

PEPC is more active in several C_3-C_4 intermediate species of *Flaveria* than the C_3 ones, but the exact role of this enzyme in these intermediates is still uncertain. The studies on characteristics of PEPC from C_3-C_4 intermediates are rather limited (17-19) and further studies may help us to understand the mechanism of C_4 pathway evolution. It is proposed that at least in some C_3-C_4 intermediates, active PEPC and partial operation of the C_4 cycle is responsible for the refixation of the photorespired CO_2 and reduction of photorespiration (17, 19).

Several secondary functions are proposed to be carried out by PEPC from C_3 plant species. Among them are : (i) replenishment of TCA cycle intermediates, (ii) providing malate for decarboxylation and generation of NADPH, (iii) refixation of respired CO_2, (iv) "malate fermentation", (v) nitrogen and amino acid metabolism, and (vi) maintenance of cytosolic pH and electroneutrality (10).

Structure of PEPC

The active form of PEPC is a homotetramer with a molecular mass of about 400 kD (2, 4). However, the enzyme can exist in different oligomeric forms depending on pH (20), ionic strength (21) and temperature (22). The enzyme dissociates into dimers (at pH 7.0) or dimers and monomers (at pH 8.0) in the presence of NaCl (23). The presence of PEP and Mg^{2+} or glucose-6-phosphate (21) prevents the dissociation of the enzyme. Presence ol glycerol (24) or high protein concentration (25) shifts the equilibrium to the larger and active tetrameric form but glycerol does not protect the enzyme against salt induced dimerization (24).

The dimer form ol the enzyme is partially active (23), whereas the monomeric form is totally inactive (26). The Km for PEP increases during the dissociation of PEPC. Conditions which promote enzyme dissociation (pH 7.0, NaCl, absence of dithiol reductants and temperature) induce the sigmoidal kinetics for PEP binding (24, 27, 28). At optimum pH (8.0), the enzyme exists in tetrameric form and the response of activity to increasing PEP concentrations is hyperbolic (24, 27). Studies with fluorescent probes showed that each of the enzyme's subunit contains at least one site for substrate binding (29).

The primary structure of PEPC from leaves of a few C_4 plants has been studied in detail (30, 31). The maize enzyme is composed of 935 amino acid residues compared to 883 amino acid residues of *E. coli* enzyme (30). The deduced N-terminal sequence for maize and *E. coli* enzymes are NH_2-Met-Ala-Ser-Thr-Lys-Ala-Pro-Gly-Pro-Gly-Glu-Lys-His-Ser(P)-and NH_2-Met-Asn-Glu-Gln-Tyr-Ser-Ala-Leu-Arg-, respectively (30-32).

There is a 43% sequence homology of maize PEPC with that of *E. coli* (30). On the other hand, up to 60% of the residues are identical among the PEPC of three C_4 (*Flaveria trinervia, Zea mays* and *Sorghum bicolor*) and the CAM and C_3 isoforms of the facultative CAM plant *Mesembryanthemum crystallinum* (33). At least four types of amino acid residues (cysteine, arginine, lysine and histidine), essential for the catalytic activity of the enzyme, have been identified to be present in the active site. Maize PEPC protein consists of 32 cysteine residues, but none of them seems to be conserved (31). The reduction of two disullide bonds per tetramer seems to be involved in the activation of PEPC by DTT and other thiol compounds (34, 35).

Chemical modification using phenylglyoxal (36) or pyridoxal 5'-phosphate (37) of maize PEPC showed that two arginine and four lysine residues were essential tor activity and that these groups were protected by the presence of the substrate, PEP. Kinetic studies (3) and experiments with diethyl pyrocarbonate (38) have shown that two histidine residues are important for the PEP and/or Mg^{2+} binding to the maize enzyme. The data on the essentiality of the lour amino acids, namely arginine, histidine, lysine and cysteine, for catalytic activity matches well with the information available on the amino acid sequences of PEPC from different C_4 species (e.g. Ref. 33). The location of most important amino acids, important for either regulation or actual reaction, in typical C4 species is indicated in Table 1.

Six conserved cysteine residues wore detected in the enzyme from F. *trinervia,* which could be involved in subunit/subunit interactions (33). Three lysine and seventeen arginine residues are conserved in the amino acid sequences of the PEPC from diflerent sources (31). Four histidine residues are conserved in the PEPC from *E. coli,* maize, and *Anabaena variabilis,* one of them in the region between residues 603 and 616 of the maize enzyme (39).

The regions surrounding the lysine-600 residue of *F. trinervia* (residues 594-603 and 632-645) are highly conservod among all eukaryotic and prokaryotic PEPC analyzed so far (31-32). The available evidence suggests that this part of the enzyme may be involved in PEP binding and catalytic activity. Residues of valine-725 and serine-773 (of the *F. trinervia* enzyme) are specific for both mono- and dicotyledonous C_4 PEPCs, whereas these are absent in PEPC from CAM and C, plants. The significance ol this finding is unclear (33).

Table 1. Some important amino acid residuos and their location in PEPC from three C_4 plant species

Amino acid	Location as the number from N-terminal		
	Zoa mays	*Sorghum* bicolor	*Flaveria* trinervia
Serine*	15	8	11
Cysteine	196	185	191
	307	297	303
	334	324	330
	419	409	413
	424	414	418
	426	416	420
	687	676	681
Lysine	606	595	600
Valine	731	716	725
Serine	780	766	773
PEP binding[a] and	600-609	589-598	594-603
catalytic site[b]	638-651	627-640	632-645
Reference	70	88	33

*Phosphorylated in light
[a]-Gly-Tyr-Ser-Asp-Ser-Gly-Lys-Asp-Ala-Gly-
[b]-Phe-His-Gly-Arg-Gly-Gly-Thr-Val-Gly-Arg-Gly-Gly-Gly-Pro-

Metabolic Regulation

Many internal and external factors modulate the activity of the enzyme (40). This is one of the most important enzyme of C_4 photosynthesis and is subjected to classic post-translational regulation by light (6, 41). Further the activity of PEPC is remarkably regulated by pH, temperature or metabolites. Only the most important and recent aspects of these regulations are discussed here. The readers are referred to earlier reviews (2, 4, 6) for other details.

(I) Light— Like many of the C_3 or C_4 photosynthetic enzymes, the kinetic and regulatory properties of C_4 PEPC are modulated considerably by illumination. The enzyme extracted from preilluminated C_4 leaf tissue exhibits two to three fold more activity than the dark form when assayed under suboptimal but physiological pH and substrate concentration. Besides an increase in V , there is a decrease in sensitivity of PEPC to malate and an increase in Km for PEP in the light form (28, 40, 42, 43). As the amount of PEPC remains constant during these short-term dark/light transitions, it is definite that post-translational modification ol the enzyme is responsible for increased activity (43). Recently, photoactivation of PEPC has been demonstrated in maize mesophyll protoplasts (44) as well as in reconstituted system with an artificial photosensitive dye (45).

Two mechanisms of post-translational reversible covalent modification of the enzyme are proposed to explain the photoactivation of C_4 PEPC. The first one is oxidation-reduction of -SH groups (28, 35) and the second one is phosphorylation/dephosphorylation of seryl residues (46-50). The first mechanism plays an important role in the modulation of some photosynthetic enzymes, but has not been proven in case of PEPC.

There are several evidences confirming the involvement of phosphorylation/dephosphorylation cascade in the regulation of PEPC on illumination (6, 51-53). The studies of Jiao and Chollet (49) proved that the reversible seryl phosphorylation of the maize leaf PEPC is a major molecular mechanism by which the reversible light-induced changes in the kinetic and regulatory properties of the C_4 enzyme are brought about. The *in vitro* phosphorylation site in the dark-form PEPC is located at Ser-15 in maize (32, 54) and Ser-8 in sorghum (6, 52). The phosphorylation site is only two residues away from a basic amino acid (Lys-12) in the primary sequence, a feature similar to that of various protein serineAhreonine kinase substrates (51). The structural motif Lys/Arg-X-X-Ser- of the phosphorylation site is shared by the C_4 and CAM isoforms but is lacking in the C_3 enzyme. The specific phosphorylation site is accessible to both homologous and heterologous protein kinases (51).

Leaves of sorghum and maize contain an active PEPC-protein serine kinase (PEPC-PK, Ref. 50, 51), which is activated by light and inactivated by dark *in vivo* (5). Partially purified PEPC-PK from maize leaf is not affected by putative light modulated cytoplasmic effectors like Ca^{2+}/calmodulin, fructose-2, 6-bisphosphate, PPi, and thioredoxin *n* (49). Cycloheximide, the eukaryotic cytosolic protein synthesis inhibitor, blocked the light activation of PEPC, suggesting that a continuous synthesis of PEPC-PK was necessary for light activation/seryl phosphorylation of PEPC from C_4 plants (52). It was also shown that type 2A protein phosphatase was active in the light. Thus a net turnover of PEPC-PK or a putative modifying protein in the light is necessary and the insufficient synthesis of PEPC-PK leads to a net dephosphorylation and a decrease in activity of PEPC (52).

In CAM plants, a large diurnal variation exists in the activity of PEPC. PEPC activity extractable during the day was only a fraction of that extracted in night (55). Diurnal changes in malate sensitivity and quarternary structure of CAM PEPC may be due to phosphorylation (night-fo. n)/dephosphorylation (day-form) of both seryl and threonyl groups in the protein (6, 46, 56).

(II) pH—The activity of PEPC is regulated by cytosolic pH. At a pH of around 8.0, the enzyme is very active producing malic acid in the system. When the pH becomes acidic due to the excessive production of malic acid, the enzyme becomes inactive, thus keeping the system in balance. At higher pH (7.0 to 8.0), there is an increase in the affinity of the enzyme for PEP and Mg^{2+} (4). The inhibition of PEPC by malate

decreased with increase in pH and concentrations of PEP and Mg^{2+}. Malate inhibition was competitive at higher pH (pH 8.0), whereas it was non-competitive at lower pH (pH 7.0) (57).

(III) ***Temperature***—Temperature is one of the important factors regulating the activity of PEPC in C_4 and CAM plants. At low temperature (e.g. 2°C), the activity is stable when assayed at suboptimal pH (5.8-6.8). Cold inactivation, particularly at alkaline pH (above **7.0), was observed** in *Cynodon dactylon, Atriplex halimus,* and *Zea mays* (58). The C_4 enzyme loses activity gradually at **4°C** (22).

One of the possible effects of temperature on PEPC activity is dissociation (in case of CAM) or aggregation (in case of C_4) of the enzyme (22). At 17°C, the CAM enzyme shows the maximum affinity for PEP. This is the normal temperature encountered by CAM plants during their nocturnal CO, fixation. Glucose-6-phosphate minimizes the effect of temperature on the overall *in vitro* activity of the enzyme (59). The Km (MgPEP) and Vmax for PEPC isolated from maize (C_4) and *Crassula argentea* (CAM) are altered with a change in temperature. In case of CAM enzyme, Vmax as well as Km for PEP increases with increasing temperature from 10-35°C with no inflection being apparent over this range, whereas the maize enzyme shows a discontinuity in the slope of Arhenius plot at 25°C. With increasing temperature, conformational or aggregational change may occur in the C_4 enzyme. The Km for PEP of C_4 enzyme decreases with increase in temperature (22). As C_4 plants generally fix CO_2 under high temperature conditions, this may be an important factor in the regulation of the enzyme.

(iv) Metabolites/enzyme inhibitors—The enzyme exhibits a feedback regulation and is inhibited by the products of the carboxylation reaction *in vivo.* L-malate, the primary product of carboxylation, and aspartate are normally non-competitive inhibitors of C_4 PEPC, while OAA inhibits the enzyme in a competitive manner (40, 43). Malonate (60) and SO_3^{2} (61) are among some effective inhibitors of C_4 enzyme reported earlier. Phosphonic acid substrate analogues are potent inhibitors of C_4 PEPC (62). One of these compounds is DCDP [3, 3-dichloro-2(dihydroxyphosphinoylmethyl) propenoate] (63). Several analogues of PEP or pyruvate are also capable of inhibiting the enzyme (5).

Glucose-6-phosphate is an allosteric activator of the C_4 enzyme and acts by decreasing the Km for PEP (27). Glycine activates the C_4 enzyme from monocots but not dicots (64) for reasons, yet unknown. The activity is also increased by other phosphorylated compounds such as 3-phospoglycerate, fructose-1, 6-bisphosphate (65), carbamyl phosphate and acetyl phosphate (66). Inorganic phosphate, one of the product of carboxylation also stimulates the PEPC activity at low PEP levels (67). Thiol compounds (34, 35) and nitrate (68) enhances PEPC activity. The recant information on the effect of inhibitors or allosteric activators on PEPC is included in Tables 2 and 3, respectively.

Table **2.** Inhibition of C_4 PEPC by different metabolites/chemicals as indicated by the increasing order of inhibition constants (K_i)

Inhibitor	K	Source ot PEPC	Reference
1-Hydroxy cyclopropane carboxylic acid phosphate	7 µM	*Zea mays*	5
Phosphoenol-3-bromo pyruvate	7 µM	*Zea mays*	5
Z-Methylphosphoenol-pyruvate	17 µM	*Zea mays*	5
Malate	20 µM	*Zea mays*	40
3. 3-Dichloro 2 (dihydroxy phosphinoy-lmethyl) propeonate	80 µM	*Zea mays S Panicum sp*	63
L-Phospholactate	100 µM	*Zea mays*	5
E-methyl phosphoenol-pyruvate	110 µM	*Zea mays*	5
Phosphoglycolate	200 µM	*Zen mays*	5
Dimethyl phospho-enolpyruvate	380 µM	*Zea mays*	5
Phosphoenolpyruvate phosphonatos	400 µM	*Zoa mays*	5
Aspartate	900 µM	*Zoa mays S Sorghum*	2
Oxalacetate	1.0 mM	*Zea mays S Solaria italica*	2
E-3-Cyanophosphoenol pyruvate	1.4 mM	*Zea mays*	5
Phosphoenolthio-pyruvate	2.0 mM	*Zoa mays*	5
Phosphonoacetate	2 0 mM	*Zea mays*	5
Malonate	2 5 mM	*Solaria italica*	60
DL-2-phosphomalate	2 7 mM	*Zea mays*	5
Malcate	3 5 mM	*Zea mays*	90
Inorganic phosphate	5 0 mM	*Zoa mays*	40
Sulphite	5.0 mM	*Zea mays*	61
Glyphosine (N, N-bis-(phosphomethyl glycine)	5 0 mM	*Zea mays*	91
Phosphonopropionate	10 0 mM	*Zea mays*	5

Molecular biology ol PEPC

In recent years, molecular biological techniques have been used to elucidate the structure and regulation of the PEPC gene and its mRNA. An attempt has been made in this section. to summarize the recent advances in our knowledge of the molecular biology of PEPC.

Table 3. Activation of C_4 PEPC by different metabolites/ chemicals as indicated by the increasing order of activation constants (KA)

Metabolite	K_A (mM)	Source of PEPC	Reference
Fructose-2, 6-bisphosphate	0.1	*Zea mays*	40
Inorganic phosphate	0 4	*Amaranthus viridis*	67
Glucose 6 phosphate	1.0	*Zea mays*	40
Dihydroxy acetone phosphate	2.0	*Zea mays*	40
Fructose-6-phosphate	2 0	*Zea mays*	40
Glycine	5 0	*Zea mays*	64
Alnnine	5 0	*Znn mnys*	40
Serine	5 0	*Zea mays*	40
Cysteine	5 0	*Zea mays*	40
Thiol compounds/ Dithiothreitol	50 0	*Zea mays*	34

(I) ***Isozymes***—Several isozymic forms of PEPC have been identified in different organs of C_4 and C_3 plants. The various forms are encoded by different genes (54. 69-71). Two isoforms of PEPC have been characterized from sorghum; the E-PEPC form, which occurs in the etiolated leaves and shows characteristics of C_3 form, and G-PEPC form which is present in the green leaves and shows the typical kinetic and regulatory properties of C_4 form (69). The G-PEPC is the light activated. No interconversion occurs between G-PEPC and E-PEPC indicating that these two forms are encoded by two different mRNAs (71). In maize, the leaf specific form (C_4 PEPC) is encoded by a single gene where as in *F. trinervia* even the leaf specific isoform is encoded by multiple gene family (54, 71).

(II) Structure of PEPC gene— The PEPC gene in maize is 5.3 kb long and consists of 10 exons and 9 introns (54). The size of exons varies from 85 to 999 bp, while that of introns range from 97 to 872 bp (72). The PEPC gene in maize contains several short repetitive sequences at the 5' -flanking side which is also characteristic feature for the light regulated gene encoding small-subunit of Rubisco. Some of the 5' -flanking sequences of maize gene are similar to other light-regulated genes from maize and wheat. In the 5' -flanking sequence of PEPC gene, no typical TATA or CAAT box sequences, which are characteristic features of eukaryotic genes, were found (32, 54).

The exon/intron junction region follows the GT/AG dinucleotide rule, and the regions adjacent to the junction also show high similarity with other eukaryotic genes. The poly (A) adenylation signal (AATAAT) is exactly similar to the conserved sequences proposed lor plants. Maize PEPC gene contains CCTTATCCT sequence, at positions -651 to -659, which may play a role in the control of the gene expression by light (72).

The cDNAs for PEPC of *F. trinervia* (33, 71), sorghum (73), and maize (32, 74) were prepared and studied in detail. The isolation of multiple cDNAs in *F. trinervia* supports the idea that the C_4 isoform of PEPC is encoded by a separate sub-family of genes in this species (33).

The cDNA of PEPC from sorghum consists of 3161 nucleotides. It has an open reading frame which encodes a polypeptide of about 952 amino acid residues with a molecular weight of about 108 kD. The coding region of sorghum cDNA shows about 90% sequence homology with that of maize whereas the 3' -noncoding region shows 74% sequence homology. Izui *ot al* (31) reported that the regions corresponding to the residues 401-826 in maize cDNA exhibited a 41% sequence homology with the *A. nidulans* and upto 51% with that of the *E. coli* one. From the cDNA analysis it was found that most of the conserved sequences are concentrated in the C-terminal half of the maize enzyme suggesting that this region may comprise part of the catalytic site. The domains formed by the N-terminal halves are involved in the allosteric regulation of the enzyme (31).

The structure and regulatory features of the PEPC mRNA was studied in F. *trinervia. Z. mays* and S. *bicolor* (69) or *F. trinervia* (71). The mRNA of F. *trinervia* PEPC contains a long open reading frame of 2898 bp, consisting of 50 bases of 5' non-translated sequence, a 222 bp 3' -noncoding region and a 9 nucleotide poly (A) tail (33). The translatable part of the open reading frame in maize results in a polypeptide of 966 amino acid residues with a molecular weight of about 110 kD (75).

(III) Regulation of gene ***expression***—The expression ot gene encoding the C_4 PEPC isoform from *F. trinervia* is restricted only to tissues exhibiting C_4 pathway (71). This situation is in contrast to that in maize, where the expression of the gene occurs in leaf, inner leaf sheath, tassels and husks (54).

Immunochemical studies with monoclonal antibodies (69, 70) led to the conclusion that at least two RNA species encoding PEPC isozyme occur in green leaves of maize and the increase in activity of PEPC during greening was accompanied by both accumulation of the G-PEPC form and a higher translatability of the corresponding mRNA. The specific mRNA was isolated and the accumulation of it during the greening was shown (69, 70, 76).

There is a further control of PEPC gene expression by light at the transcriptional level. The studies on sorghum PEPC showed that phytochrome is responsible for the accumulation of both PEPC mRNA and G-PEPC protein (69, 70, 77). These results suggest that phytochrome controls the enzyme induction during the greening of leaves.

(Iv) *Genetic* ***engineering related to PEPC—*** The gene(s) encoding C_4 PEPC was cloned and expressed successfully in both prokaryotes and higher plants. Cretin *et al* (73) were able to clone PEPC-deficient strains of *E. coli* with sorghum PEPC. The enzyme expressed in transformed *E coli* cells was active and could be phosphorylated *in vitro* by sorghum PEPC-PK. Transgenic tobacco plants, carrying sorghum (78) or maize (79) PEPC gene, were produced. The expression and characteristics of PEPC in the transformed plants were studied along with their photosynthetic characteristics. The levels of PEPC in transgenic tobacco plants were two-fold higher than that in normal plants, but these levels were less than those in maize (79). Two different isoforms of PEPC, were identified in transgenic tobacco plants with maize PEPC gene, based on the etfect of glucose-6-phosphate on the affinity ot the enzyme for the substrate, PEP. One form was like the tobacco enzyme with low $Km_{(PEP)}$ (of about 25 u.M), whereas the second form was like that of maize enzyme with high Km_{PEP} (of about 2.2 mM). In transgenic tobacco plants the malate content was higher than the normal control plants indicating the higher activity of PEPC and their inability to decarboxylate malate (79).

In transgenic tobacco plants, with maize PEPC gene, the size of the transcript was 4.2 kb, which is 700 bp larger than the mRNA transcript from the same gene in maize (79). But in case of sorghum, transcript size was same both in the parent plant and the transgenic plant. The increase in the transcript size in transgenic plants with maize gene may be due to an inaccurate transcription initiation (79).

The successful cloning and expression of C_4 PEPC in other organisms provides several exciting opportunities. Site directed mutagenesis of cDNA for C_4-PEPC is being used to elucidate the importance of amino acid residues in the catalytic/regulatory properties of the enzyme and role in phosphorylation/ dephosphorylation of the PEPC-protein (80, 81).

Concluding Remarks

PEPC is one of thn **Important** target enzymes for breeding by genetic manipulation, since an increment of PEPC together with other C_4 pathway enzymes can bring about a marked enhancement in photosynthetic carbon fixation ability of the plants.

The salient features of PEPC and its regulation by light and metabolites are summarized in Fig. 1. In recent years, a **lot** of attention is paid towards the structural features of the enzyme, its regulation and gene expression. Significant gaps still remain in our knowledge regarding the details of the catalytic mechanism, the three dimensional structure of the enzyme and the primary/secondary messengers during light activation of the enzyme. Reasons for the ability of glycine to activate PEPC from only C_4 **monocots** but not C_4 dicots are not understood and future studies in this aspect may help in understanding the evolution of C_4 monocots and dicots.

Further experiments are needed to understand the mechanism of the modulation of the enzyme by light or temperature and **genetic basis of** its **selective gene expression** only in **mesophyll cells** ol C_4 **plants.** Site directed mutagenesis would be an useful tool to constitute and express PEPC in transgenic plants or transformed *E. coli* cells, and assess the specific role of important amino acid residues in structure and function of the enzyme. The application of such advanced molecular biological techniques could lead to exciting new information about the structure, regulation and evolution of PEPC. The progress in our knowledge of PEPC would provide a means of improving the efficiency of not only C_4 photosynthesis but also agricultural productivity.

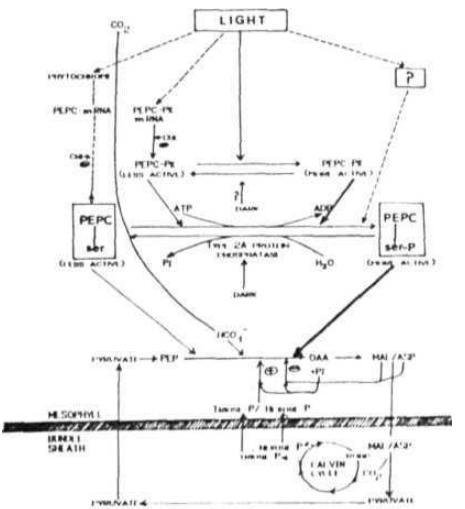

Fig. 1. Regulation ot PEP carboxylase (PEPC) hy **light** Tho phosphorylated form ot PEPC is much more active than the dephosphorylated form Light promotos the **activation** ol PEPC-protein kinaso which accomplishes phosphorylation ot PEPC. The dephosphorylation of PEPC in dark is catalyzed by a type 2A protein phosphatase Illumination promotes the biosynthesis of PEPC or PEPC PK, which can bo inhibited by cycloheximide (CHI) The enzyme is subjected to also classic feedback regulation through inhibition by its products malate (MAL), aspartato (ASP), OAA or Pi. Tho inhibition by these metabolites is indicated by (—) and tho stimulation by (+), The thickened arrows represent strong action

Acknowledgements

The preparation of this articlo and a part of our work on PEPC was supported by a grant (No. SP/SO/A43/88) from Department ol Science and Technology, Now Delhi. MTD and AVR were recipients of Sonior and Junior Research Fellowships, from Council ot Scientific and Industrial Research and University Grants Commission, New Delhi, respectively.

Received 12 March, 1992; Revised 27 May, 1992

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Indian Journal of Experimental Biology
Vol 30, May 1992, pp 424-428

Rapid isolation of mesophyll and guard cell protoplasts from pea and maize leaves

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Received 10 May 1991; revised 24 February 1992

A simple method is described for rapid isolation of mesophyll and guard cell protoplasts from leaves of pea (*Pisum sativum*) and maize (*Zea mays*). The time required for the release of protoplasts is less than an hour as compared to earlier methods. The isolated protoplasts are intact and metabolically very active, as indicated by their respiratory oxygen uptake and photosynthetic oxygen evolution. The important factors during the preparation of protoplasts are short time of digestion, composition of medium, filtration through nylon nets, centrifugation at low speed and handling protoplasts with utmost care. The ease of growing pea or maize and the technique of rapid isolation make these protoplasts an ideal system for studying various metabolic activities.

Isolated protoplasts permit the study of various metabolic processes. An ideal technique for protoplast preparation aims at achieving rapid isolation with a maximum yield and high metabolic integrity of protoplasts. In this paper, an easy and rapid method is reported for the isolation of large batches of protoplasts from the epidermis as well as mesophyll of pea (using same leaves) and maize leaves by enzymatic digestion with cellulase and macerozyme.

Materials and Methods

Chemicals - Cellulase Onozuka R-10 and macerozyme R-10 were from Yakult Honsha Co Ltd. Medicine Dept. Enzyme Division. 1-1-19 Higashi-Shinbashi, Minatoku, Tokyo 105, Japan. Evans blue and neutral red were from Loba Chemie, Bombay. Coomassie brilliant blue-G was from Bio-rad and other chemicals were from Sisco Research Laboratories, Bombay.

Plant material – Pea (*Pisum sativum cv.* Arkel) and maize (*Zea mays cv.* Ganga 5) were raised on soil supplemented with farm-yard manure in plastic tubs (30 cm diam.). Plants were grown either in the field or in the laboratory under a photoperiod of approximately 12 hr and mean temperature of 30°C day/20°C night. Plants were watered daily. Fully expanded third and fourth leaves were picked from 8 to 10 day-old plants, between 0800 and 0900 hrs.

Preparation of mesophyll cell protoplasts (MCP) – The lower epidermis of pea leaves was removed and the leaf was cut into 0.5 cm pieces. Leaf pieces were floated on a preplasmolysis buffer (10 mM MES-KOH *p*H 6, 270 mM mannitol and 1 m*M* $CaCl_2$) for 30 min. These were then transferred to a petridish containing digestion medium of cellulase "Onozuka R-10" (2% w/v), macerozyme (0.2% w/v), BSA (0.25% w/v). 10 m*M* MES-KOH. pH 5.5, 500 mAf mannitol, 1 m*M* $CaCl_2$, 0.25 m*M* EDTA. 10 m*M* sodium ascorbate. After incubation for 40 min at 30°C, the digestion medium was gently removed with the help of a Pasteur pipette. The washing medium (10 m*M* MES-KOH. pH 6, 500 m*M* mannitol and 1 m*M* $CaCl_2$) was added and the petridish was tapped and shaken gently. The medium containing mesophyll protoplasts was filtered successively through 300, 80 and 60 μm nylon filter cloths (Saryu Textiles. 614 Cotton Exchange Bldg, Kalbadevi Road, Bombay-400 002, India) and the filtrate centrifuged at 50 *g* for 10 min. The pellet was resuspended in washing medium and protoplasts were collected by centrifugation again at 50 *g* for 5 min. The pellet was finally resuspended in the reaction medium (10 m*M* HEPES-KOH, pH 7.5, 500 m*M* mannitol. 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$).

Leaves of maize were cut into 0.5-1 mm thick pieces and floated in the preplasmolysis buffer for 30 min. Leaf pieces were incubated in a petridish containing digestion medium, under illumination at 30°C. After an hour, the digestion medium was removed with a Pasteur pipette. Fresh washing medium was added and the petridish was agitated gently to liberate protoplasts into the medium. The medium was passed successively through nyl-

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on filter cloth (Saryu Textiles, Bombay, India) of pore size 500 and 85 *p.m.* The filtrate was centrifuged at 50 *g* lor 5 mill. The pellet was resuspended in washing medium, centrifuged and suspended in the reaction medium.

The preplasmolysis/digestion/washing/reaction media used with maize leaves/protoplasts were similar to those used with pea except that sorbitol replaced mannitol and in addition also contained 1 m*M* MgCl, and 0.2 m A/KH_2PO_4.

Preparation of guard cell protoplasts (GCP) – Lower epidermis was peeled off IIOIII leaves of pea and epidermal strips were kept in preplasmolysis buffer for 45 *min*. The strips were transferred into petridishes containing digestion medium of cellulase 'Onozuka' R-10 (3% w/v), macerozyme R-10 (0,3% w/v). the remaining components were the same as those in the digestion medium used for isolation of pea mesophyll protoplasts. The petridishes were subjected to an orbital shaking for initial 10/15 min at 60 rpm. Alter intubation lor 45 min, the digestion medium containing guard cell protoplasts was removed with a Pasteur pipette. More protoplasts were released by shaking the digested stiips in petridishes with excess of washing medium for 5 min. The pellet of protoplasts was suspended in washing medium. The pooled protoplast suspension in washing medium was passed successively through 100 and 30 *fun* nylon mesh (Nylon nets below 30 μm – Nybolt, Swiss Silk Bolting Cloth Mfg Co Ltd, Postfach, CH-8027 Zurich 2, Switzerland). The filtrate containing guard cell protoplasts was centrifuged at 50 *g* for 5 min and finally resuspended in a small volume of reaction medium (10 m*M* HEPES-KOH *pH* 7, 500 m*M* manitol, 1 m*M* $CaCl_2$, 10 m*M* KCl and 0.5 m*M* $MgCl_2$).

Preplasmolysis/washing media were the same as those employed with pea mesophyll protoplasts.

Protoplast characteristics – The number of protoplasts in the preparation was counted using a haemocytometer. The size of the protoplasts was measured with a precalibrated occular micrometer using a research microscope (Wolfe, Japan). The viability and intactness of protoplasts were checked using neutral ted and Evans blue.

Chlorophyll was determined after extraction into 80% (w/v) acetone[1]. Protein was estimated[2] by dye binding, using Coomassie brilliant blue G-250.

Oxygen uptake/evolution – The uptake or evolution of oxygen by protoplasts was monitored at 30V using a Clark type oxygen electrode (Model DW2, Hansatech Ltd, UK). Calibration of oxygen content in the electrode chamber was made with air saturated water'. Further details of measuring photosynthesis or respiration arc described elsewhere[4].

Results

Mesophyll and guard cell pioloplasts were released when the leaf snips were subjected to digestion with a mixture ot cellulase and macerozyme for <1 hr (Fig. 1). Further increase in digestion time beyond 40-45 min increased the yield of pioloplasts, but decreased the metabolic quality of protoplasts as indicated by their respiratory and photosynthetic oxygen exchange (Table 1). Protoplasts were metabolically very active when digestion medium contained USA, EDTA and ascorbate. Omission of any of these compounds reduced the metabolic activity of isolated pea mesophyll protoplasts (data not shown).

The mesophyll and guard cell pioloplasts prepared from pea or maize leaves, possessed high metabolic activity as indicated by photosynlhetic O_2 evolution and respiratory O_2 uptake. Properties and metabolic activities of protoplasts are given in Table 2.

The absorption spectra of both pea and maize mesophyll protoplasts exhibited two major peaks, one in the blue legion around 440 nm and the other in the red region near 680 nm, with shoul-

Table 1 – Effect of duration of digestion on the yield and metabolic activity of pea mesophyll protoplasts

Duration of digestion (min)	Protoplast yield per g leaves		Metabolic activity (μmol O_2 mg Chl $^{-1}$ hr $^{-1}$)	
	Chlorophyll (μg)	Number (1×10^6)	Respiration (O_2 uptake)	Photosynthesis ((O_2 evolution)
2(	119	1.7	IV.4	88
30	140	20	22.4	86
40	154	22	204	137
60	168	2.4	15.4	128

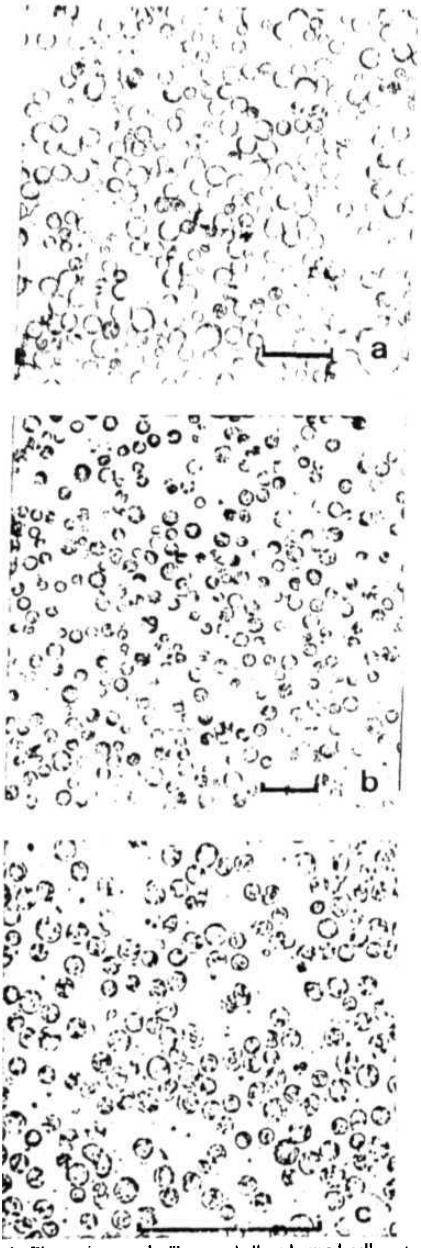

Fig. 1 - Photomicrographs "I mesophyll and guard cell protoplasts (a) Pea - mesophyll |bj Maize mesophyll |c) Pea guard cells. The (horizontal) bar represents 100 /im.

Table 2 - Properties and metabolic activities of protoplasts prepared from leaves of pea and maize

Properties	Pea		Maize mesophyll
	Mesophyll	Guard cell	
Size (μm)			
Palisade	31.9 ± 3.2		36.7 ± 1.0
Spongy	20.4 ± 3.7	12.0 ± 1.0	23.2 ± 2.9
Chlorophyll (pg)	70.0 ± 6.0	1.2 ± 0.1	80.0 ± 8.0
Protein (pg)	407.0 ± 51.0	63.0 ± 3.0	384.0 ± 40.0
Metabolic activity (μmol mg chl $^{-1}$ hr $^{-1}$)			
Respiratory O_2 uptake	10.0 ± 0.3	280 ± 10.0	5.5 ± 0.5
Photosynthetic O_2 evolution	137.0 ± 11.0	120 ± 6.0	3.0 ± 0.37*
			20.0 ± 0.51**

*With bicarbonate; **With benzoquinone

ders at 475 and 650 urn (data not shown). The absorption spectrum of the guard cell protoplasts exhibited very high absorption in violet/ultraviolet region, besides the peaks in blue and red regions.

The pea incsophyll protoplasts consumed <>, in darkness and evolved O_2 on illumination. Such photosynthctic O_2 evolution was stimulated Bv the presence ol bicarbonate, ()AA or PGA in the medium (data not shown). Maximum photosynthetic O_2 evolution was in the presence of 2 mM bicarbonate. Rates ol O_2 consumption by guard cell protoplasts ol pea were very high. However, their activity ol oxygen evolution in light was similar to that ol M(Ps. Mai/e mesophyll protoplasts could evolve significant amounts ol oxygen in presence of benzoquinone (Table 2).

Discussion

In the present study, mesophyll protoplasts from pea leaves were liberated within a short span of 20 min (Table 1) compared to many hours earlier (Table .1).

Isolation and use of guard cell protoplasts is a relatively new phenomenon[5]. Again, the isolation ol guard cell protoplasts usually required very long periods, ranging from a few hours to even a day (Table 4). In this report, guard cell protoplasts were isolated from the abaxial epidermis ol pea leaves within 45 min of digestion. An additional advantage of the approach is the prepara-

Table 3 — List of selected reports on the isolation of mesophyll protoplasts from leaves (Reports are listed as per the chronological order of appearance in the literature).

Plant material	Digestive enzymes	Duration of digestion (hr)	Purification	Ref.
Rye, wheat, barley, avena	4% Cellulase 1% Macerozyme	18	Sucrose gradient	9
Maize	2% Cellulase	6	Dextran and PEG two phase system	10
Wheat	2% Cellulase 0.3% Pectinase	3	Sucrose-sorbitol gradient	11
Spinach	3% Cellulase 0.5% Macerozyme	3	Sucrose-sorbitol gradient	12
Maize	2% Cellulase 0.2% Macerozyme	2.25	Sucrose gradient	6
Arabidopsis thaliana	1.6% Cellulysin 1.6% Macerase	3	Flotation on percoll	7
Wheat	2.5% Cellulase 0.5% Macerase	3.5	Raffinose-sorbitol gradient	13
Panicum miliaceum	1% Cellulase 0.05% Pectolyase	3.5	Sucrose-sorbitol gradient	14
Soybean	2% Cellulase 0.1% Pectolyase	1.5	Metrizamide-sorbitol gradient	15
Haworthia ramosissima	2.5% Cellulase 0.3% Macerozyme	5	Centrifugation and dextran step gradient	16
Pisum sativum *Zea mays*	2% Cellulase 0.2% Macerozyme	< 1 (40 min)	Filtration and centrifugation	Present report

Table 4 — A list of selected reports on the isolation of guard cell protoplasts from leaves (Reports are listed as per the chronological order or appearance in the literature)

Plant material	Digestive enzymes	Duration of digestion (hr)	Purification	Ref.
Allium cepa, *Nicotiana tobacum*	4% Cellulysin	10-12	Sequential washing	17
Vicia faba, *Allium cepa*	4% Cellulysin	4	Centrifugation	18
Vicia faba	4% Cellulysin	5	Sequential filtration	19
Vicia faba	4% Cellulase	20-24	Filtration and centrifugation	20
Vicia faba	4% Cellulase 1% Macerozyme	6-7	Sequential centrifugation	21
Commelina communis	1% Cellulysin 0.005% Pectolyase	4	Percoll gradient centrifugation	22
Commelina communis	1% Cellulysin 0.05% Pectolyase	3	Percoll gradient centrifugation	23
Vicia faba	2% Rohament TC 0.5% Rohament P	1.5	Filtration and centrifugation	24
Pisum sativum	3% Cellulase 0.3% Macerozyme	45 min	Filtration and centrifugation	Present report

lion of both mesophyll and guard cell protoplasts from the same leaves, facilitating an appropriate comparison of these two types of protoplasts. Conventionally, protoplasts are purified by centrifugation on density gradient media formed of compounds like dextran, PEG, percoll or sucrose. Whereas presently a simple filtration through nylon nets is employed followed by washing with excessive medium and quick centrifugation.

Addition of USA, calcium. EDTA and ascorbate to the digestion medium improved the yield as well us the quality of mesophyll protoplasts. Day *et al.*[6] reported that $CaCl_2$ and HSA were essential for rapid and linear rates of O_2 evolution and omission of HSA, $MgCl_2$, and KH_2PO_4 together was deleterious to the protoplast quality. Calcium prevents clumping of protoplasts and further inhibits photosynthesis by chloroplasts, which arc released due to protoplast rupture[5]. The inclusion of 10 *mM* EDTA as a chelator in the isolation medium is an absolute requirement for the recovery of active protoplasts from leaves of *Arabidopsis*[7]. Ascorbate is a protective agent and its inclusion in the digestion medium prevents damage to the intact protoplasts'.

Protein and chlorophyll contents of mesophyll protoplasts of pea and mai7c were similar. Hut the guard cell protoplasts had very low levels of protein and chlorophyll (Table 2). The mesophyll protoplasts had nearly six times more protein and 60-70 times more chlorophyll than that guard cell protoplasts. The low chlorophyll levels of guard cell protoplasts is known".

The absorption spectra of mesophyll protoplasts of both pea and maize were similar with a major peak in blue region and another peak in the red region. However, the guard cell protoplasts were different and absorbed heavily in the UV region, besides the two peaks in blue and red regions. The UV absorbing components of guard cell protoplasts are not yet identified.

The uptake of oxygen in darkness and the oxygen evolution in light by mesophyll and guard cell protoplasts indicated the operation of respiration and photosynthesis, respectively. These observations demonstrate that the isolated mesophyll and guard cell protoplasts arc mctabolically very active. Guard cell protoplasts showed high rates of respiration, but their rates of photosynthesis were similar to mesophyll protoplasts. The inability of mesophyll protoplasts of maize to exhibit CO_2 dependent oxygen evolution is known. Maize nicsophyll protoplasts exhibited low rales of oxygen evolution with substrates such as 3-phosphoglycerate, oxalacetate or pyruvate, even at higher concentrations. Hut they could evolve significant amounts of oxygen either with benzoquinone or after disruption of the protoplasts".

Acknowledgement

The work was supported by grants hum CSIR, New Delhi (No. 38 (588)/86-EMR II) and Department of Atomic Energy, Bombay (No. 4/6/89-G). M.T.D. and M.M.R. were supported by (CSIR (SKI) and UGC(I IP) fellowships, respectively.

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Journal of Experimental Botany, Vol. 44, No. 261, pp. 771–774, April 1993

Partial Reduction in Activities of Photorespiratory Enzymes in C_3–C_4 Intermediates of *Alternanthera* and *Parthenium*

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Received 20 October 1992; Accepted 12 January 1993

ABSTRACT

The maximum catalytic activities of several photorespiratory and photosynthetic enzymes were determined in leaf extracts of three C_3–C_4 intermediates (*Alternanthera ficoides*, *A. tenella* and *Parthenium hysterophorus*) and were compared to those of C_3 (*A. sessilis*, *Pisum salmon*) and C_4 ((*A. pungens*, *Zea mays* and *Amaranthus hypochondriacus*) species. The activity levels of key photorespiratory enzymes, glycolate oxidase, catalase, NADH-hydroxypyruvate reductase and glycerate kinase were less (28 to 35% reduced) in intermediates than those of typical C_3 species. Similarly, the activities of photorespiratory aminotransferases in the C_3–C_4 intermediates were also partially reduced (23 to 37% reduction). The activities of phosphoenolpyruvate carboxylase (PEPC), pyruvate, orthophosphate dikinase and NAD-malic enzyme were higher (2 to 7 times) in leaf extracts of the intermediates than those of C_3 species. But the ratios of PEPC/rubisco in the C_3–C_4 intermediates were more like C_3 than C_4 species. We draw attention to the partial reduction in enzyme activity of photorespiratory metabolism, which could be an important factor for restriction of photorespiration in the C_3–C_4 intermediate species, in addition to enzyme compartmentation and/or operation of a 'C_4-like' cycle.

Key words: C_3–C_4 intermediates, C_4 pathway, enzyme profile, glycolate metabolism, photorespiration, photosynthesis.

INTRODUCTION

Photorespiration plays an important role in the carbon and nitrogen metabolism of the leaf (Lea *et al.*, 1990). The level of the CO_2 compensation point (Γ) is taken as an indication of the extent of photorespiralion in leaves. The intermediate values of the CO_2 compensation point (5–39 mm³ dm⁻³), indicate that the process of photorespiration is partly reduced in the C_3–C_4 intermediate species. Currently, 24 species belonging to 8 genera of 6 families have been identified as naturally occurring C_3–C_4 intermediates (Rawsthorne, 1992).

C_3–C_4 intermediate species offer a good model to study the mechanism of photorespiration and evolution of the C_4 photosynthetic pathway (Raghavendra, 1980; Edwards and Ku, 1987; Monson and Moore, 1989; Raghavendra and Das, 1993; Rawsthorne, 1992). These intermediates presumably represent a transitory stage between C, and C_4 species during the course of evolution, C, C_4 intermediates seem to have arisen during the process of evolution of C_4 plants from C, species, since C_3 photosynthesis is believed to be the phylogenetic precursor of the C_4 pathway (Moore, 1982).

Among the C_3–C_4 intermediate plants reported recently are the species of *Alternanthera* (Rajendrudu *et al.*, 1986) and *Parthenium* (Hegde and Patil, 1981; Moore *et al.*, 1987). Although photosynthetic metabolism in *Parthenium hysterophorus* has been studied (Moore *et al.*, 1987), information on the photosynthetic and photorespiralory features of *Alternanthera tenella* and *A. ficoides* is very limited.

The major objective of the present work was to examine the mechanism of reduced pholorespiration in C3-C4 intermediate species of *Alternanthera* and *Parlhenium*. We have initially attempted lo determine the complement of photosynthetic and photorespiratory enzymes in leaves. Our results indicate a partial, but significant, reduction in the maximum catalytic activities of key photorespiratory enzymes, such as glycolate oxidase, NADH-hydroxypyruvate reductase and glycerate kinase in the C_3–C_4

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intermediates, We suggest **that** the reduction **in** enzymic capacity may be an important factor, in addition to **a** C_4-like cycle and/or compartmentation **of** glycine decarboxylase during the **reduction** of photorespiration observed in these intermediates.

MATERIALS AND METHODS

Plant material

Plants of *Amaranthus hypochondriacus* (cv. AG-67), *Pisum sativum* (cv. Arkel) and *Zea mays* (cv. Ganga 5) were raised **from** seed. *Alternanthera ficoides*, *A. pungens*, *A. sessiles*, *A. tenella* and *Parthenium hysterophorus* **were collected from** the **University** campus and were multiplied by transplantation of cuttings/seedlings. The seedlings/plants were grown on soil, supplemented with farmyard manure, in 30 **cm** diameter plastic tubs. The tubs were kept in the field under **a** natural photoperiod of approximately 12 h and average temperature **of 30°C** day/20 C night. Plants were watered daily. Fully-expanded 3rd and 4th leaves were picked from 3- to 4-week-old plants between 8 00 a.m. and 900 a.m. and were used for experiments.

Extraction and assay of enzymes

Leaves were cut into pieces of *c.* 10 to 20 nun^2 and quickly homogenized at 4 °C in a mortar and pestle using the appropriate extraction buffer. The extract was filtered through four layers of cheese cloth and clarified **by** centrifugation at 15 000 *g*, unless otherwise specified. An aliquot of the **filtered** extract was kept prior to centrifugation for **chlorophyll determination** (Arnon, **1949**). The **enzyme** assays were carried out within 30 min of extraction.

The extraction and assay of the photosynthetic and photorespiratory enzymes were based on the published procedures, with minor changes, as follows.

RuBP carboxylase (RUBPC, EC 4.1.1.39) (Rajendrudu *et al.*, 1986); **PEP** carboxylase **(PEPC. EC** 4.1.1.31) **(Iglesias** and Andrco, 1989): Extraction medium consisted of 50 mol m^{-3} **HEPES KOH** (pH 7 2). 10 mol m^{-3} $MgCl_2$, 2 mol m^{-3} K_2HPO_4, 1 mol m^{-3} **EDTA,** 20% glycerol and 10 mol m^{-3} β-mercaptoethanol; assay **medium** (1 cm^3) consisted of 20 mol m^{-3} Tricine KOH (pH 7·8), 5 **mol** m^{-3} $MgCl_2$, 10 mol m^{-3} $NaHCO_3$, 2 units of NAD-MDH, 0 2 mol m^{-3} NADU and 50 mm^3 of 50 mol m^{-3} PEP was added prior to initiation of the reaction; pyruvate, orthophosphate dikinase (PPDK, EC 2.7.9.1) (Aoyagi and Bassham, 1983); NAD-malic enzyme (NAD-ME, EC 1.1.1.39) (Hatch *et al.*, 1982); carbonic anhydrase (CA, EC 4.2.1.1) (Hatch and Burnell, 1990).

RuBP oxygenase (RUBPO, **EC** 4.1.1.39) (Lorimer *et al.*, 1977); **Extraction** medium consisted of 50 mol m^{-3} HEPES KOH (pH 8.5), 20 mol m^{-3} $MgCl_2$, 5 mol m^{-3} DTT and 1% (w/v) PVP; assay medium consisted of 50 mol m^{-3} **HEPES** KOH (pll 8.5), 1 mol m^{-3} $MnCl_2$ and a final concentration of 0·5 mol m^{-3} RuBP; preactivation of enzyme at room temperature for 10 min, in 10 mol m^{-3} NaHCO, and 10 mol m^{-3} $MgCl_2$ 3-PGA phosphatase (**EC** 3.1.3.20) and P-glycolate phosphatase (EC 3.1.3.18) (Osmond and Harris, 1971); glycolate oxidase (GO, **EC** 1.1.3.1) (Moore *et al.*, 1988): Extraction medium consisted of 100 mol m^{-3} HEPES KOH (pH 8.3), 2 mol m^{-3} $MgCl_2$, 2 mol m^{-3} $MnCl_2$, 1 mol m^{-3} EDTA. 2 mol m^{-3} DTE, 1% (w/v) PVP and 005% (v/v) Triton X 100; assay medium consisted of 50 mol m^{-3} **HEPES KOH** (pH 8.3), 2.5 mol m^{-3} $MgCl_2$, 3 mol m^{-3} phenylhydrazine and 5 mol m^{-3} glycolate. A lag of 3 min was taken. Catalase (EC 1.11.1.6) (Tolberl *et al.*, 1969); NADH-hydroxypyruvate reductase (NADH-HPR, **EC** 1.1.1.29) (Kleczkowski and Randall, 1988): Extraction medium consisted of 50 mol m^{-3} **HEPES** KOI! (pll 7.5), 5 mol m^{-3} MgCl,, 1 mol m^{-3} **KOTA,** 3 mol m^{-3} DIE and 1% (w/v) **PVP;** assay **medium** consisted of 50 mol m^{-3} MES KOH (pH 6.5), 0·2 **mol** m^{-3} NADH and 1 mol m^{-3} hydroxypyruvate. Glycerate kinase **(EC** 2.7.1.31) (Ilsuda and Edwards, 1980); glutamate glyoxylate aminotransferase **(EC** 2.6.1.4) (Kisaki and Tolbert, 1969), using Dowex-1 (acetate) to **remove** unreacted ^{14}C-glyoxylate; Serine glyoxylate aminotransferase (EC 2.6.1 45) (Smith, 1973), using Dowex-50 (H$^+$) to **remove** unrcactcd ^{14}C-serine.

All **enzymes** were assayed at 30 C. except carbonic anhydrase (4 (', but recalculated for 30 ('). Spectrophotometric assays were conducted **in** a Shimadzu UV-Vis Spectrophotometer (Model UV-160A). Radioactivity was monitored using a liquid scintillation counter (Model Beckman **LS** 1800).

The maximum catalytic activities were determined by optimizing conditions during assay for individual **enzymes** in each species. **Further** experiments were **performed** to ensure that low levels of the photorespiratory enzymes were not due to **any** endogenous inhibitory substances. For this, **enzyme activities** were determined in extracts prepared by co-homogenizing the leaves of the **intermediate** species with pea or mixing the leaf extracts. The data **presented** here are the averages (± s.c.) of three to six **independent measurements** made on different days

Chemicals

The **buffers** used were from either Sisco Research Labs or **Spectrochem** (Bombay, India). Substrates, cofactors and ion exchange media were from Sigma **Chemical** Co., (USA)/Boehringer **(Germany),** All other **chemicals** were of analytical grade **from** BDH, Sisco Research Labs or **S** Merck. Bombay, India.

RESULTS

The activity **levels of several** photosynthetic and photorespiratory enzymes were assayed in the three (", C_4 intermediate species of *Alternanthera* and *Parthenium* and were **compared** with C_3 (*A. sessiles*) and C_4 (*A. pungens*) species of *Alternanthera*. The enzyme complement of *Pisum sativum* (C_3), *Zea mays* (C_4) and *Amaranthus hypochondriacus* (C_4) were included as **further references,**

The activity levels **of GO,** catalase, NADH-HPR and glycerate kinase in leaves **of** the intermediates were 28 to 35% less than those in (', species (Fig. 1). The activities of photorespiratory enzymes in C_4 plants were only a small fraction (< 18%) **of** those in C_3 leaves.

The reduction of photorespiratory enzymes was not dramatic, but the reduced levels were consistently **recorded,** during replicated enzymes assayed **on different** days. The reduction was not due to any endogenous inhibitors since either mixing of leaf extracts (data **not** shown) or co-homogenation of leaves **of** pea and C_3 C_4 intermediates did not show any inhibition of expected activity (Table 1).

The activity level of RuBPO was low in *Parthenium*, but this may be due partly to an endogenous inhibitor as co-extraction with *Pisum salivum* tissue recovered only 60% of the expected activity (data not shown).

The activities of C_4 photosynthetic enzymes were sev-

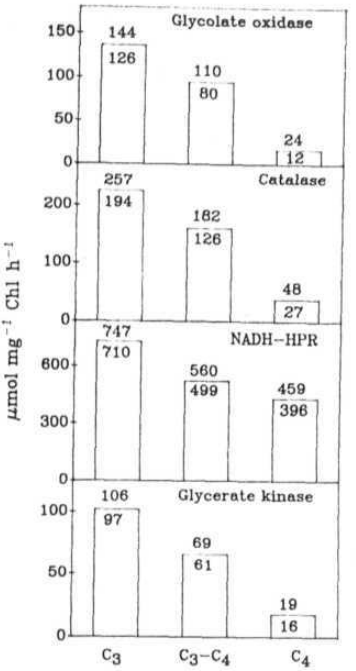

FIG. 1. The averaged activities of four photorespiratory enzymes, glycolate oxidate catalase, NADH-hydroxypyruvate reduclasc and glycerate kinase in C_3, C_4 or C_3 C_4 intermediate plants. The species examined were: *Alternanthera sessilis* and *Pisum sativum* (C_3), *Alternanthera ficoides*, *A. tenella* and *Parthenium hysterophorus* (C_3 C_4 intermediates) and *Alternanthera pungens*, *Amaranthus hypochondriacus* and *Zeamays* (C_4) The values presented in the figure are the range of aclivilict for each photosynthetic type. Activity levels of catalase are expressed in mmol mg $^{-1}$ Chl h $^{-1}$.

crul-l'old higher in C_4 species compared to those in C, species. For example. PPDK was very active in C_4 species, but was barely detectable in C, species. The activity levels of key C_4 photosynthetic enzymes (PEPC, PPDK and NAD-MI') were 2- to 7-fold higher in the C, C_4 inlet-mediate species than those in C, but were not as pronounced as in C_4 species. On the other hand, the level of CA in all the plant species was similar (lig. 2).

The activity levels of photorespiratory aminotransferascs, namely glutamate glyoxylate aminotransferase and serine glyoxylate aminotransferase, were less (23 lo 37%) in intermediates than those in C_3 species. The activity levels of aspartate- and alanine-aminotransferases were slightly increased (60 to 130% over C, species) in C, C_4 intermediate species of *Alternanthera* and *Parthenium* (Fig. 3). However, the ratios of PEPC/RuBPC and PEPC/RuBPO indicated that the intermediates were similar to C_3 species (lig. 4).

The chlorophyll content of leaves was similar among all the plant species studied, but the Chl *a*/*b* ratio increased progressively from C, to (', C_4 intermediates and C_4 species. The C, species had the lowest Chl </> ratio of 275, while the C_4 species had the highest (3·46). The C_3 C_4 intermediate species had the intermediate value of 300 (Fig. 5).

DISCUSSION

This is the first report of the profile of several enzymes involved in photorespiratory carbon/amino acid metabolism in three C, C_4 intermediate species of *Alternanlhera* and *Parthenium*, which have been identified as C_3 C_4 intermediates based on their low /' values and partial Kranz anatomy (Rajendrudu *et al.*, 1986; Moore *et al.*, 1987). At least lour enzymes of the photorespiratory metabolism, namely, glycolate oxidase, catalase, NADH-hydroxypyruvate reductase, and glycerate kinase, were significantly reduced (28 to 35%) in the leaves of these intermediates (Fig. 1). A limitation in photorespiratory

TABLE 1. *Glycolate oxidase and NADH-hydroxypyruvate reductase activities in co-homogenized leaf extracts of pea ami C_3 C_4 intermediate species*

The values in parentheses indicate the % of recovery in the co-homogenized extract. The values are averages of four independent measurements.

Species combination	Glycolate oxidase		NADH-hydroxypyruvate reductase	
	Expected	Measured	Expected	Measured
	(µmol mg $^{-1}$ Chl h $^{-1}$)			
Pisum sativum + *Alternanthera denella*	113	106 (94)	604	575 (95)
Pisum sativum + *Alternanthera ficoides*	108	KM (96)	608	566(93)
Pisum sativum + *Parthenium hysterophorus*	118	115 (97)	635	610 (96)

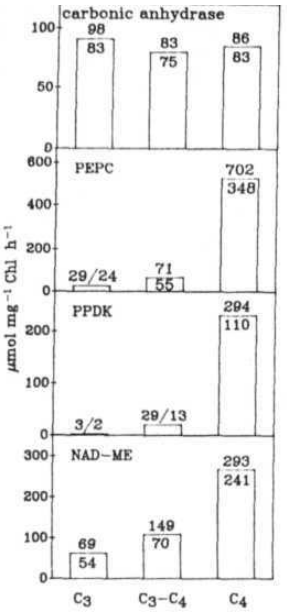

FIG. 2. Averaged activities of four photosynthetic enzymes, carbonic anhydrase, PEP carboxylase, pyruvate phosphate dikinase and NAD-malic enzyme in leaves of C_3, C_4 or C_3 C_4 intermediate species. For the species included refer to legend of Fig. 1. The NAD-malic enzyme activity-levels of C_4 species, the average of *A. pungens* and *Amaranthus hypochondriacus* are taken (since *Zea mays* is a NADP-ME species). The values in the figure indicate the range of activity for each photosynthetic type. The activity levels of carbonic anhydrase are expressed in mmol mg^{-1} Chl h^{-1}.

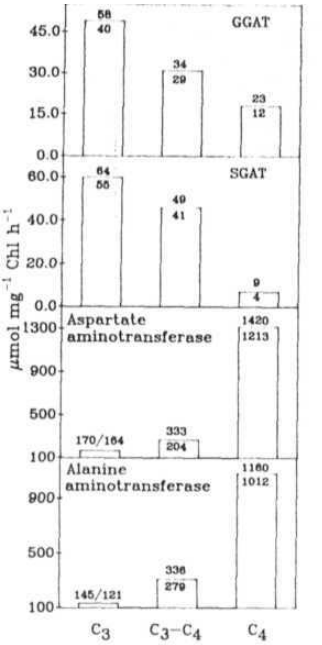

FIG. 3. Average activities of four aminotransferases glutamate glyoxylate aminotransferase, serine glyoxylate aminotransferase, aspartate aminotransferase and alanine aminotransferase. See legend of Fig. 1 for the C_3, C_4 or C_3 C_4 intermediate species studied. The values in the figure represent the range of activity for each photosynthetic type.

turnover of glycine was associated with C_3-C_4 intermediacy in *Moricandia arvensis* (Kumar and Abrol, 1990). Also in our intermediates, the enzymes of photorespiratory glycine metabolism were partly reduced (23 to 37% less than those in C_3 species) (Fig. 3). Our results therefore suggest that there is a partial reduction in photorespiratory enzymic capacity of C_3 C_4 internicdiale species.

Another indication of photorespiratory capacity is the activity ratio of PGA-/phosphoglycolalc phosphatase (Randall *et al.*, 1971). This ratio is about 2 4 in C_4 plants, but is less than one in C, species indicating that the metabolism of phosphoglycerate is far more active than that of phosphoglycolate in leaves of C_4 plants. The ratios of phosphoglycerate to phosphoglycolate phosphatase (Fig. 4) in C_3 C_4 intermediates suggest a decreased potential of metabolizing phosphoglycolate.

Our data may not be able to explain readily the gas exchange phenotype of intermediates and their ability to grow in normal air. Mutants deficient in photorcspiratory enzymes (Oliver and Kim, 1990; Lea and Blackwell, 1990; Somerville, 1986) can not grow in air, but survive in elevated CO_2. Therefore, the reduction in photorespiratory metabolism is normally detrimental to plants. The reasons for the ability of the intermediates of *Alternanthera* and *Parthenium* to survive in air are nol clear at present.

Our observation that the leaves of C_3 C_4 intermediates had slightly higher activities of al least three C_4 enzymes: PEPC, PPDK and NAD-ME than C_3 species (fig. 2), is at variance with those of Rajendrudu *et al.* (1986) and Moore *et al.* (1987), who reported low activities of C_4 enzymes in *Alternanthera* and *Parthenium*, respectively These enzyme activities could vary due to several factors including growth conditions, but we arc sure of the high levels of C4 cycle enzymes in these intermediates in view of our sufficiently replicated assays. However, we doubt the operation of a typical C_4 pathway in our intermediates, since the ratios of PEPC/RuBPC in leaves ol *A. tenella*, *A. ficoides* and *P. hysterophorus* are close to those of C_3 species (1ig. 4). Similarly, the leaves ol *P. hysterophorus* did not incorporate much of ^{14}C into

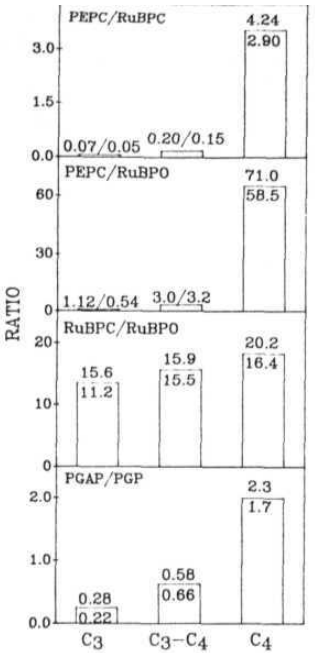

FIG. 4. The average ratios of PEPC/RuBPC and PEPC/RuBPO, RuBPC/RUBPO and PGA-phosphoglycolate phosphatase in leaves of the C_3, C_4 or C_3-C_4 intermediate plant species. The species included are indicated in the legend of Fig. 1. The values of RuBPO in *Parthenium hysterophorus* were not considered. The values presented in the figure represent the range for each photosynthetic type.

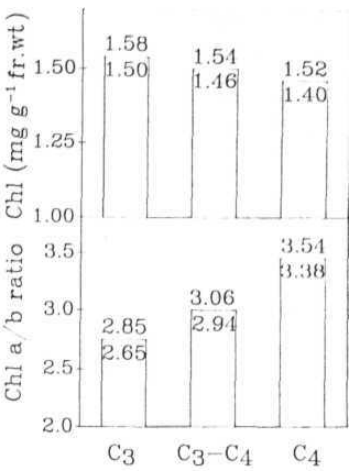

FIG. 5. The chlorophyll Content and Chl *a/b* ratios for the three photosynthetic types as described in the legend of Fig. 1. The values presented in the figure represent the range for each photosynthetic type.

C_4 acids during short-term labelling experiments (Moore *et al.*, 1987).

In C_4 plants, the Γ value does not change even at low light intensity, while a marked increase in Γ under low light intensities is expected in C3-C_4 intermediate species having a light-dependent CO_2 refixation mechanism (Monson *et al.*, 1984). However, the decrease in Γ of *Alternanthera* intermediates under low light (Rajendrudu *et al.*, 1986) was not as drastic as that of C_3-C_4 intermediates of *Panicum* (Brown and Morgan, 1980).

The amount of chlorophyll was similar in all the species, whereas the Chl *a/b* ratio increased progressively from C_3 to C_3-C_4 intermediates and C_4 species (Fig. 5). In C, plants, the light-harvesting chlorophyll of PS I contains mostly Chl *a* and the light-harvesting chlorophyll of PS II is thought to contain similar amounts of Chl *a* and *b* (Ku *et al.*, 1991). The higher ratios of Chl *a/b* reflects the higher energy requirements of C_4 plants.

The reduction in photorespiration in C, C_4 intermediates is proposed to be due either to an efficient refixation of photorespired CO_2, supported by either anatomical features or the operation of a limited (or near complete) C_4 cycle (Edwards and Ku, 1987; Raghavendra and Das, 1993). Based on this criterion, the C_3-C_4 intermediates were classified into two categories: the first one with an operational C_4 cycle (e.g. *Flaveria brownii*) or those without a C_4 pathway (e.g. *Moricandia arvensis*). However, Rawsthorne (1992) proposed that the confinement of glycine decarboxylase to mitochondria in the bundle sheath cells forms the physiological base of all C3-C4 intermediates including those of *Morkandia* and *Flaveria*. We suggest a partial reduction in enzymic capacity could be an additional factor for the reduction in the photorespiratory pathway at least in the intermediates of *Alternanthera* and *Parthenium*.

A partial 'Kranz' anatomy occurs in all the present C_3-C_4 intermediates (Rajendrudu *et al.*, 1986; Moore *et al.*, 1987). Our attempts to separate mesophyll and bundle sheath tissues so far have been unsuccessful. In *Morkandia arvensis*, the reduced photorespiration is due to the predominant localization of glycine decarboxylase in bundle sheath cells, leading to an efficient recycling of photorespiratory CO_2 (Rawsthorne *et al.*, 1988). Further work is necessary to determine the intercellular distribution of photorespiratory enzymes in the intermediate species of *Alternanthera* and *Parthenium*. Nevertheless, we wish to draw attention to the significant reduction in photorespiratory enzymic capacity, along with a small increase in PEP carboxylase/NAD-ME system in the intermediates of *Alternanthera* and *Parthenium*.

ACKNOWLEDGEMENTS

This work was supported by a Ciranl from the **Department** of Science and **Technology,** New Delhi (No. SP/SO/A43/88). MTD was a recipient of a Senior **Research** Fellowship from the Council of Scientific and Industrial Research, New Delhi. We also wish to thank the anonymous referee for extremely **useful** comments.

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Photosynthesis Research 00 (000) (000), 1993

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Regular paper

Photorespiration in C_3–C_4 intermediate species of *Alternanthera* and *Parthenium*: Reduced ammonia production and increased capacity of CO_2 refixation in the light

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Received 25 June 1993; accepted 8 September 1993

Key words: bicarbonate, glycine, α-hydroxypyridine melhanc-sulfonatc, isonicotinyl hydrazidc, methionine sulfoximinc, photorcspiratory ammonia

Abstract

The pattern of photorespiratory ammonia (PR-NH_3) formation and its modulation by exogenous bicarbonate or glycine were investigated in C_3–C_4 intermediates of *Alternanthera (A. ficoides* and *A. tenella) and Parthenium hysterophorus* in comparison to those of C_3 or C_4 species. The average rates of PR-NH_3 accumulation in leaves of the intermediates were slightly less than (about 25% reduced) those in C, species, and were further low in C_4 plants (40% of that in C,). The levels of I'R-NH_3, in leaf discs decreased markedly when exogenous bicarbonate was present in the incubation medium. The inhibitory effect of bicarbonate on PR-NH_3 accumulation was pronounced in C, plants, very low in C_4 species and was moderate in the C_3–C4 intermediates. Glycine, an intermediate of photorespiratory metabolism, raised the levels of PR-NH_3 in leaves of not only C_4 but also C_3–C_4 intermediates, bringing the rates close to those of C, species. The rale of mitochondrial glycine decarboxylation in darkness in C_3–C_4 intermediates was partially reduced (about 80% of that in C, species), corresponding to the activity-levels of glycine decarboxylasc and serine hydroxymethyltransferase in leaves. The intermediates had a remarkable capacity of reassimilating photorespiratory CO_2 in vivo, as indicated by the apparent refixation of about 85% of the CO_2 released from exogenous glycine in the light. We suggest that the reduced photorespiration in the C_3–C_4 intermediate species of *Alternanthera* and *Parthenium* is due to both a limitation in the extent of glycine production/decarboxylation and an efficient refixation/recycling of internal CO_2.

Abbreviations: GDC - glycine decarboxylase; GS - glutamine synthetase; GOGAT - glutamate-oxoglutarate aminiotransferase; α-HPMS - α-hydroxy-2-pyridinemethanesulfonic acid; INH - isonieotinyl hydrazidc; MSO - L-methionine sulfoximinc; PR-NH_3 - photorespiratory-ammonia; SHMT - serine hydroxymethyltransferase

Introduction

Leaves of C, plants exhibit high rates of photo respiration whereas C_4 plants show little or no apparent photorespiratory activity (Ogren 1984, Sharkey 1988, Canvin 1990). During photorespiration, ammonia is formed due to decarboxylation of glycine in mitochondria (Oliver et al. 1990). Photorespiratory ammonia (PR-NH_3) plays a significant role in the nitrogen metabolism of C_3 plants (Keys et al. 1978, Singh et al. 1985, Lea et al. 1990).

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PR-Nil, is reassimilated in vivo mainly via the GS-GOGAT pathway (Keys et al. 1978). MSO is an irreversible inhibitor of GS, the enzyme that catalyzes the primary incorporation of NH_4 into amino acids. Addition of MSO results in NH_4 accumulation in leaves of higher plants (Platt and Rand 1982, Berger and Fock 1983, Martin et al 1983, Ikeda et al. 1984, Rhodes et al. 1986) However, the extent of NH_4 accumulation in the presence of MSO is two to threefold higher in C_3 than in C_4 leaves (Martin et al. 1983). Phosphinothricin (glufosinate), another irreversible inhibitor of GS, also caused the accumulation of ammonia in several C_3 and C_4 plants (Lacuesta et al. 1989, Wendler et al. 1990, 1992, Shelp et al. 1992).

A CO_2 compensation point in the range of 5–39 $\mu l\ l^{-1}$ indicated that photorespiratory metabolism was partially reduced in C_3–C_4 intermediate species (Ogren and Chollet 1982, Edwards and Ku 1987, Rawsthorne 1992, Raghavendra and Das 1993, Raghavendra and Devi 1993). Direct estimates of photorespiratory metabolism in C_3–C_4 intermediates are very few (cf. Holbrook et al. 1985, Ku et al. 1991), since most of the studies have been on photosynthetic carbon metabolism.

This paper summarizes our study of PR-ammonia in C_3–C_4 intermediate species We examined the extent of PR–NH_4 accumulation in C_3–C_4 intermediates of *Alternanthera* (*A ficoides. A. tenella*) and *Parthenium hysterophorus* and compared these results with those from C, (*A. sessiles*, *Pisum saiivum)* and C_4 (*A. pungens*. *Amaranlhus hypochondriacus*, *Zea mays)* species. Our findings indicate that photorespiratory ammonia metabolism is partially reduced in the C_3–C_4 intermediates of *Alternanthera* and *Parthenium*. We also report the marked modulation of PR NH_4 in leaf discs by bicarbonate or glycine.

Matt-rials and methods

Plant material

Plants of *Amaranlhus hypochundriacus* (cv. AG-67), *Pisum sativum* (cv. Arkel) and *Zea mays* (cv. Ganga 5) were raised from seed. *Alternanthcra ficoides, A. pungens, A. sessiles, A. lenella* and *Parthenium hysterophorus* were propagated by transplantation of cuttings. The seedlings/plants were grown in soil supplemented with farmyard manure in 30 cm diameter plastic tubs. The plants were grown outdoor, under a natural photoperiod, of about 12 h and mean temperatures of 30 °C/20 °C day/night. Fully expanded third and fourth leaves were harvested from 3- to 4-week-old plants between 8(H and 9(M a.m. for the experiments.

Extraction and estimation of ammonia. Leaf discs of ca. 20 mm^2 were prepared from freshly collected leaves under water. Twenty leaf discs (ca. 80 mg) were placed in petridishes containing 5 ml of water or MSO (2.5 mM), IN1I (35 mM), α-HPMS (20 mM), glycine (15 mM) or other test combinations. The discs were illuminated (1000 $\mu E\ m^{-2} s^{-1}$) for 2 h at 30 ± 1 °C.

At the end of the illumination period, the leaf discs were washed quickly with water, blotted dry and extracted in 2% (w/v) boric acid (Kumar et al. 1984). The extract was centrilugcd at 12000 *g* for 20 min at 4 °C. Ammonia was estimated in the supernatant fluid by phenol-hypochlorite (Solorzano 1969). The blue complex formed in the presence of sodium nitroprusside was measured at MO nm in a spectrophotometer (Shimadzu UV-vis 160 A).

Photorespiratory ammonia

Leaf discs accumulate ammonia upon illumination, particularly in the presence of MSO, an inhibitor of GS However, a variety of sources contribute to ammonia accumulation in the presence of MSO (Singh et al. 1985). Photorespiratory inhibitors like INH (inhibits (il)C') and α HPMS (inhibits glycolate oxidase) are used to distinguish the extent of non-photorespiratory ammonia accumulation (Kumar and Abrol 1989, 1990). The difference in the NH_4 levels of samples with MSO and those with (INH + MSO) or (α HPMS + MSO) represents the PR NH_4 (see Table 1)

Glycine decarboxylation

Leaf discs of 20 mm^2 were punched from freshly collected leaves under water. Randomly selected leaf discs were blotted dry and floated with the

Table 1. Accumulation of PR-ammonia in leaf discs of C_3, C_4 and C_3–C_4 intermediate species, determined in presence of MSO (2.5 mM) and INH (35 mM) or α-HPMS (20 mM)

Species	Water	MSO	PR-ammonia determined with	
			INH[a]	α-HPMS[b]
			μmol NH_3 mg Chl^{-1} h^{-1}	
C_3 Species				
Alternanthera sessiles	2.80	6.44	2.64 ± 0.20	3.12 ± 0.28
Pisum sativum	3.10	7.21	2.96 ± 0.18	3.40 ± 0.20
C_3–C_4 Intermediates				
Alternanthera tenella	2.00	6.11	2.18 ± 0.20	2.34 ± 0.22
Alternanthera ficoides	1.98	6.02	2.31 ± 0.23	2.28 ± 0.20
Parthenium hysterophorus	1.95	5.87	2.27 ± 0.21	2.16 ± 0.23
C_4 Species				
Alternanthera pungens	0.98	2.85	1.08 ± 0.07	1.14 ± 0.12
Amaranthus hypochondriacus	0.91	2.79	1.02 ± 0.10	1.12 ± 0.10
Zea mays	1.03	2.94	1.12 ± 0.09	1.22 ± 0.11

[a] NH_3 levels in **presence** or **absence** of INH, i.e |MSO - (MSO + INH))
[b] NH_3 levels in presence or absence of α-HPMS, i.e. |MSO - (MSO + HPMS)|

adaxial surface upwards in flat injection vials containing center wells (5 discs, equivalent to 30–90 μg Chl **in** each vial). The vials contained 2.5 ml of a buffered medium (0.3 M sorbitol, 0.3 M Mes–KOH pH 5.5, 1 mM KH_2PO_4 and 1 mM $MgCl_2$). The centre wells contained filter paper wicks wetted **with** hyaminc hydroxide. The vials were sealed with rubber septa and [1-C]glycine was injected at zero time to give a final **concentration** of 50 mM (0.5 mCi). The reaction was terminated after 1 h of illumination (100 μE m^{-2} s^{-1}) or darkness at 25 °C by injecting 3 N HCl **(Holbrook** et al. 1985). After 30 to 60 min, the filter paper wicks in the center wells were removed and ^{14}C-radioactivity determined using a liquid scintillation counter (Beckman Model LS 1800).

Enzyme assays

A crude mitochondrial fraction was prepared from leaves as described by Bergman et al (1980). Small leaf pieces (ca. 10–20 mm^2) were **ground** vigorously (to break bundle sheath strands) in a mortar and pestle at 4 °C using 4 vol of prechilled extraction buffer **(25** mM Hepes–KOH, **pH7.8,** 0.3 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 4 mM cystcinc, 0.1% (w/v) BSA and 0.6% (w/v) PVP). The homogenate was filtered through cheese-cloth and centrifuged at 5000 g for 3 min and the supernatant fraction was re-centrifuged at 20000 g for 7 min. The pellet constituted the crude mitochondrial preparation.

The mitochondrial fraction was examined for the activity of GDC (EC **2.1.2.10)** by using [1-^{14}C]glycine (specific activity of 14.5 Ci/mol). The reaction was performed in sealed flat vials with center wells containing paper wicks wetted with hyamine hydroxide (for trapping the released $^{14}CO_2$). The assay medium of 1.0ml, modified from that of Oliver (1979), consisted of 50 mM Tris–HCl, **pH** 7.8, **2 mM NaNO$_3$,** 0.3 M sorbitol, 2 mM **EDTA. 1 mM** $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM KH_2PO_4, 2mM DTT, 0.1 mM **pyridoxal** 5′-phosphate, 1 mM NAD, 10 mM glycine, and **mitochondria** corresponding to 2.5-5.0 μg **protein.** The reaction was initiated by injecting radioactive glycine. allowed to continue for 30 min in a shaking water both at 30 °C, and was stopped by adding 0.2 ml of 40% (v/v) perchloric acid. After continued shaking for 30 to 60 min, **the filter** paper wicks in the center wells were analysed for **C-radioactivity.**

The mitochondrial **preparation** was **further** processed (Woo 1979) for assaying SUM I (EC **2.1.2.1)** at 25 °C by using [U-^{14}C]serine (specific radioactivity of 135 Ci/mol). The crude mitochondrial pellet was resuspended in 20 mM phosphate buffer, pH 7.5, and 1 mM **DTT.** Then the fraction was solubilized with 0.05% (v/v) Triton-X 1200 and incubated at 0 °C for 10 min before use. The assay medium consisted of 20 mM

phosphate buffer, pH 7.4, 1 mM 2-mercaptocthanol, 1 mM EDTA, 0.1 mM pyridoxal-5-phosphate, 2 mM tetrahydrofolate, 2 mM DTT, 5 mM [U-^{14}C]serine (0.5 μCi) and solubilized mitochondria equivalent to 2.5-5.0 μg protein ml^{-1} (Taylor and Weissbach 1965).

Maximum catalytic activities were determined by optimizing the rection conditions during the assay of the individual enzymes in each species.

Chlorophyll / protein estimation and replication

Chlorophyll was estimated as per Arnon (1949) and protein by using Coomassie Brilliant Blue G and BSA as standard (Bradford 1976).

The experiments were repeated on different days. The data presented here are averaged (±SE) of three to six experiments made on different days.

Chemicals

α-HPMS was from Aldrich Chemical Co, USA; MSO and hyaminc hydroxide from Sigma Chemical Co, USA; Dimedon from Fluka, Switzerland; and INH was from Citadel Fine Pharmaceuticals, Tamil Nadu. [^{14}C]glycine and [^{14}C]serine were from the Board of Radiation and Isotope Technology, Bombay, India. All other chemicals were of analytical grade from cither Sisco Research Laboratories or Spectrochem, Bombay, India.

Results

A steady increase in PR-Nil, occurred upon illumination of leaf discs for up to 90–120 min, but there was no such increase when leaves were kept in darkness (Fig. 1). PR-NH_3 accumulation was maximum in *Pisum sativum* and *Alternanthera sessiles* (C,). low in *A pungens* (C_4) and moderate in *A. ficoides* (C_3–C_4 intermediate). The average rates of PR-NH, accumulation, estimated using MSO and either INH or α-HPMS (Table 1), were reduced in the C_3–C_4 intermediates by 20-30%, compared to C, species, while PR-NH, in C_4 plants was further lowered (≤40% of that in C,). The values of SE

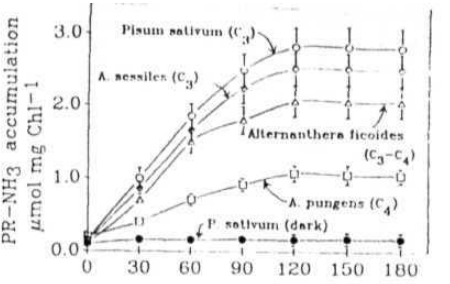

Fig 1 Photorespiratory ammonia (PR-NH_3) accumulation upon illumination of leaf discs of C_3, C_4 and C_3–C_4 intermediate species (open symbols). Ammonia accumulation in darkness (closed symbols) was negligible The values are averages of at least three independent experiments (±SE) performed on different days

tend to be high but the average values of PR-NH, in C_3–C_4 intermediates were always less than those of C, plants.

The presence of bicarbonate in the incubation medium reduced the extent of PR-Nil, accumulation in pea leaf discs, with the saturation concentration of bicarbonate being 5 mM (data not shown). The level of PR-NH_3 accumulation (measured with MSO and INH) was markedly inhibited in the presence of 5 mM bicarbonate in both C, and C_3–C_4 intermediate species (Table 2). However, the inhibition by bicarbonate of PR-NH, formation was less in C_3–C_4 intermediate species (about 40% decrease over control) than that in C, species (nearly 55%). The effect of bicarbonate on PR-NH, was almost negligible (<10% decrease) in C_4 species.

Glycine enhanced ammonia accumulation in all the species examined (Table 2). The extent of stimulation by glycine was maximum (>200%) in C_4 species, low in C, plants (about 60%) and increased in C_3–C_4 intermediates (nearly 100%).

The activity-levels of two photorespiratory mitochondrial enzymes, GDC and SHMT, were partially reduced (75% of C_3 activity) in C_3–C_4 intermediate species but were very low (<30% of C,) in C_4 plants (Table 3).

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Table 2. Effect of bicarbonate and glycine on accumulation of PR-NH_3 (measured in presence of 35 mM NH_4Cl, as in Table 1) in C_3, C_4 and C_3–C_4 intermediate species. Values in parentheses indicate the effect of test compounds as % control (i.e water 100%)

Species	Control (water)	Bicarbonate (5 mM)	Glycine (15 mM)
		μmol mg Chl^{-1} h^{-1}	
C_3 Species			
Alternanthera sessiles	2.3 ± 0.18	1.0 ± 0.03 (43)	4.0 ± 0.41 (174)
Pisum sativum	2.8 ± 0.22	1.2 ± 0.05 (43)	4.2 ± 0.42 (150)
C_3–C_4 *Intermediates*			
Alternanthera tenella	1.9 ± 0.08	1.1 ± 0.05 (58)	4.2 ± 0.38 (221)
Alternanthera ficoides	2.0 ± 0.07	1.2 ± 0.06 (60)	4.1 ± 0.44 (205)
Parthenium hysterophorus	2.0 ± 0.09	1.3 ± 0.07 (65)	3.9 ± 0.35 (195)
C_4 Species			
Alternanthera pungens	1.1 ± 0.06	1.0 ± 0.05 (91)	3.4 ± 0.32 (309)
Amaranthus hypochondriacus	1.1 ± 0.05	1.0 ± 0.03 (91)	3.3 ± 0.31 (300)
Zea mays	1.1 ± 0.06	1.0 ± 0.04 (91)	3.4 ± 0.37 (309)

Table 3. Activity-levels of mitochondrial glycine decarboxylase and serine hydroxymethyl transferase in C_3, C_4 and C_3–C_4 intermediate species

Species	Glycine decarboxylase	Serine hydroxymethyl transferase
	μmol mg Chl^{-1} h^{-1}	
C_3 Species		
Alternanthera sessiles	3.74 ± 0.21	64 ± 5.8
Pisum sativum	4.3 ± 0.28	71 ± 6.1
C_3–C_4 *Intermediates*		
Alternanthera tenella	2.78 ± 0.19	48 ± 4.4
Alternanthera ficoides	2.91 ± 0.21	52 ± 4.6
Parthenium hysterophorus	3.05 ± 0.26	55 ± 5.0
C_4 Species		
Alternanthera pungens	0.98 ± 0.05	19 ± 1.6
Amaranthus hypochondriacus	0.93 ± 0.56	18 ± 1.5
Zea mays	0.99 ± 0.06	24 ± 1.4

The rates of $^{14}CO_2$ evolution from [1-C]glycine in darkness were low in C_4 leaf discs, intermediate in C_3–C_4 intermediate species and higher in C_3 species (Table 4). However, the rales of glycinc decarboxylation in the light by C_4 species were only a fraction of those in C_3 species, while the rates in C_3–C_4 intermediates were about 20% of those in C_3 species. Evidently, the extent of **refutation** of $^{14}CO_2$ evolved from ^{14}C-glycine in the light was very high in C_4, low in C_3 but quite considerable in C_3–C_4 intermediates.

Discussion

Photorespiration occurs only in the light, when **phosphoglycolatc,** the substrate for **photorespiration,** is available (Ogrcn and Chollet 1982, Ogren 1984, Sharkey 1988). Accordingly, PR-

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Table 4. Decarboxylation of [1 – ^{14}C]glycine in dark or light and apparent refixation by leaf discs of C_3, C_4 and C_3–C_4 intermediate species

Species	Glycine decarboxylation			
	Dark	Light	Dark/Light	Apparent refixation (%)*
		μmol $^{14}CO_2$ mg Chl^{-1} h^{-1}		
C_3 Species				
Alternanthera sessilis	5.42	3.11	1.74 ± 0.13	43
Pisum sativum	4.54	2.46	1.85 ± 0.16	46
C_3–C_4 *Intermediates*				
Alternanthera tenella	4.38	0.67	6.54 ± 0.61	85
Alternanthera ficoides	4.14	0.46	9.00 ± 0.87	89
Parthenium hysterophorus	4.20	0.53	7.93 ± 0.76	87
C_4 Species				
Alternanthera pugens	2.50	0.05	50.00 ± 7.60	98
Amaranthus hypochondriacus	2.52	0.04	63.00 ± 6.20	98
Zea mays	2.40	0.04	60.00 ± 5.40	98

* % of photorespiratory CO, refixed in light.

NH, accumulated during illumination of leaf discs (Fig. 1), demonstrating the dependence of PR-NH, on photosynthetic metabolism. On the other hand, PR-NH, accumulation in the dark was essentially negligible due to the absence of photorespiration. The lowered levels of PR-NH, accumulation in the C_3–C_4 intermediates (Table 1) suggest that **photorespiratory** metabolism is **significantly** reduced in these species. Earlier reports also suggested a reduction in PR-NH, metabolism in C_3–C_4 **intermediates** of *Moricandia arvensis* and *Parthenium hysterophorus* (Kumar and Abrol 1989. 1990).

The decrease in PR-NH, accumulation **(Table** 2) reflects the inhibitory effect of CO, on photorespiration. The primary reaction of **photorespiration** is the oxvgcnasc activity of rubisco, which is **modulated b>** the availability of CO, vs. (), **(Ogrcn** 1984). An increase in the **CO,** concentration is expected to increase carboxylase activity. while reducing the oxygenation and thereby photorespiratory metabolism. **However,** exogenous bicarbonate did not completely inhibit photorespiratory ammonia accumulation. even in C_3 plants. The reason for this is not clear. but could be due mainly **to** photosynthetic O, evolution in light. The reduced sensitivity of PR-NH, to bicarbonate in the C_3–C_4 intermediates is an indication of efficient CO_2 recycling/concentration leading to reduced photorespiratory metabolism.

Photorespiratory carbon metabolism in leaves of higher plants involves the coordinated functioning of three organelles (viz., **chloroplast,** peroxisome and mitochondrion) and is closely related to the C, photosynthetic carbon reduction cycle (Schnarrenberger and Fock 1976, Ogrcn and Chollet 1982, Ogrcn 1984, Sharkey 1988, Canvin 1990). Therefore, the reduction **in photorespiration of** C_3–C_4 intermediates could be due to a limitation in any of the steps/reactions. Work from our laboratory indicated a partial reduction in levels of key photorespiratory enzymes in intermediates of *Alternanthera* and ***Parthenium*** (Devi and Raghavendra 1993, Devi et al. 1993). The present results reveal that the C_3–C_4 intermediates have a reduced capacity of not only production but also decarboxylation of glycine (Table 4). This may be due in part to the reduction in the related **enzymes** (Table 3). Kumar and **Abrol** (1990) and Ku et al. (1991) also reported a reduction in GDC and SUM I **activities** in C_3, C_4 intermediates of ***Mum andia*** and *Flaveria*, **respectively.**

The reduced apparent **photorespiration** in *Moricandia arvensis*, a C_3–C_4 intermediate, has been attributed to be due mostly to the exclusive confinement of **GDC** in bundle sheath cells (Rawsthorne et al. 1988, Rawsthorne 1992). Nevertheless, there are reports of reduced turnover (synthesis and metabolism) of glycinc in ***Parthenium hysterophorus*** and ***Moricandia arvensis*** (Kumar and Abrol 1989, 1990).

The decarboxylation of glycinc constitutes a

major source of NH_3 during photorespiration in barley (Lea el al. 1990) and maize leaves (Merger and Fock 1983) The enhancement of NH_3 accumulation by exogenous glyeine was earlier observed in C, (tnungbean, Kumar et al. 1984) and C_4 species (maize, Berger and Fock 1983; *Amaranthus*, Kumar et al. 1984). The enhancement of ammonia accumulation by glycine in not only C_4 species but also in the intermediates (Table 2) suggests that a primary reason for reduced photorespiration in these plants is a marked limitation in glycine production.

The decrease in CO_2 evolution from exogenous glycine in the light in interpreted to be due to the internal refixation of released CO_2 by photosynthesis. Therefore, the ratio of glycine decarboxylation in dark to light is taken as an apparent measure of the extent of recycling of photorespiratory CO, (Holbrook et al. 1985, Kumar and Abrol 1990). This ratio in C_3-C_4 intermediates is nearly four times greater than that in C_3 species, indicating a significant refixation of CO_2 in vivo. In C_4 species, the ratio is very high (Table 4) due basically lo the efficient carbon fixation mechanism of c_4 photosynthesis

We suggest that reduced photorespiration in C_3-C_4 intermediates of *Aliernanlhera* and *Parthenium* is due both to a decrease in glyeine formation/decarboxylation and an increase in the capacity for internal refixation of photorespired CO, in the light. Significant levels of PEP carboxylase in leaves of these intermediates (Devi and Raghavcndra 1993) may lead to CO, refixation and subsequent recycling through NAD-ME and rubisco. The reduced sensitivity of PR-NH_3 to exogenous bicarbonate is an indication of an efficient CO, recycling mechanism in these C_3-C_4 intermediates (Raghavendra and Das 1993).

Acknowledgements

This work was supported by Grant (No. SP/SO/A43/88) from Department of Science and Technology, New Delhi. MTD was recipient of a Senior Research Fellowship from Council of Scientific and Industrial Research, New Delhi. We thank Dr TVR Nair, Nuclear Research Laboratory. Indian Agricultural Research Institute, New Delhi, for a kind gilt of α-HPMS.

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Photosynthesis Research 00; (XX)-(XX), 19..
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Regular paper

Patterns of phospho*enol*pyruvate carboxylase activity and cytosolic pH during light activation and dark deactivation in C_3 and C_4 plants

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Received 11 February 1993; accepted in revised form 16 July 1993

Key words: C_3 and C_4 plants, cytosolic alkalization, light activation, PEP carboxylase, pH effect

Abstract

The rate and extent of light activation of PEPC may be used as another criterion to distinguish C_3 and C_4 plants. Light stimulated phospho*enol*ypyruvate carboxylase (PEPC) in leaf discs of C_4 plants more than three times greater but stimulation of PEPC was limited about 30% over the dark-control in C_3 Species. The light activation of PEPC in leaves of C_3 plants was complete within 10 min. while maximum activation in C_4 plants required illumination for more than 20 min, indicating that the relative pace of PEPC activation was slower in C_4 plants than in C_3 plants. Similarly, the dark-deactivation of the enzyme was also slower in leaves of C_4 than in C_3 species. The extent of PEPC stimulation in the alkaline pH range indicated that the dark-adapted form of the C_4 enzyme is very sensitive to changes in pH. The pH of cytosol-enriched cell sap extracted from illuminated leaves of C_4 plants was more alkaline that that of dark-adapted leaves. The extent of such light-dependent alkalization of cell sap was three times higher in C_4 leaves than in C_3 plants. The course of light-induced alkalization and dark-acidification of cytosol-enriched cell sap was markedly similar to the pattern of light activation and dark-deactivation of PEPC in *Alternanthera pungens*, a C_4 plant. Our report provides preliminary evidence that the photoactivation of PEPC in C_4 plants may be mediated at least partially by the modulation of cytosolic pH.

Abbreviations: CAM – Crassulacean acid metabolism: G-6-P – glucose-6-phosphate: PMSF – phenylmethyl sulfonyl fluoride: PEPC – phospho*enol*pyruvate carboxylase; PEPC-PK - phospho*enol*pyruvate carboxylase-protein kinase

Introduction

Phospho*enol*pyruvate carboxylase (PEPC, Orthophosphate: oxaloacetate carboxylase (phosphorylating), EC 4.1.1.31) catalyzes the β-carboxylation of PEP to yield oxalacetate and Pi. PEPC provides the primary route of carbon assimilation in C_4 and crassulacean acid metabolism (CAM) plants (O'Leary 1982, Andreo et al. 1987), whereas in C_3 plants and algae the enzyme plays an auxiliary role (Latzko and Kelly 1983).

Like many of the C, and C_4 photosynthetic enzymes, the kinetic and regulatory properties of PEPC undergo marked changes during light/dark transitions, particularly in C_4 and CAM plants (e.g. Doncaster and Leegood 1987). Extracted from pre-illuminated C_4 leaf tissue, the enzyme exhibits two to three times more activity than the dark form when assayed under suboptimal but physiological assay conditions. The light-form also exhibits less sensitivity to malate inhibition than the dark form (Karabourniotis et al. 1983, 1985, Huber and Sugiyama 1986. Don-

caster and Leegood 1987, Jiao and Chollet 1988). The reversible phosphorylation of the enzyme in light is a major mechanism of post-translational regulation in C_4 plants (Review: Jiao and Chollet 1991). The effect of light on PEPC is hardly discernible in leaves of C_3 species (Chastin and Chollet 1989). Limited photoactivation of the enzyme was demonstrated in maize mesophyll protoplasts (Devi and Raghavendra 1992), suggesting a high degree of coordination between mesophyll and bundle sheath cells required for the activation of the enzyme (e.g. Jiao and Chollet 1992). A marked activation of PEPC was reported in a reconstituted system with an artificial photosensitive dye (Maheswari and Bharadwaj 1991). Phosphorylation of PEPC in sorghum mesophyll protoplasts under light was modulated by calcium and pH (Pierre et al. 1992). Light induced phosphorylation of PEPC was observed in wheat leaves (Von Quy et al. 1991) and guard cell protoplasts of *Vicia faba* (Schnabl et al. 1992), although no light activation of PEPC could be recorded in guard cell protoplasts of *Commelina communis* (Willmer et al. 1992).

The present work was initiated to reevaluate the effect of light on PEPC in plant species belonging to different photosynthetic groups and to assess whether the light activation pattern can be used as a criterion to establish the C_4 form of PEPC. Since activity of PEPC is maximal in an alkaline pH (O'Leary 1982) and illumination causes an alkalization of the cytosol in leaves of both C_3 and C_4 plants - with the degree of alkalization being quite high in C_4 plants (Yin et al. 1990, 1993, Raghavendra et al. 1993) - we also evaluated the possible role of cytosolic pH in regulating the activity of PEPC in leaves of C_4 plants. Thus as a cytosolic enzyme, the light-induced cytosolic alkalization may increase PEPC activity, particularly in C_4 plants.

Materials and methods

Plant material

Plants of *Amaranthus hypochondriacus* (cv. AG 67), *Amaranthus viridis*, *Arachis hypogea* (cv. ICGS 44), *Cajanus cajan*, *Pisum sativum* (cv. Arkel), *Sorghum vulgare* (cv. CS 59) and *Zea mays* (cv. Ganga 5), were raised from seeds. Plants of *Alternanthera ficoides*, *A. Pungens*. *A. Sessiles*, *A. Tenella*, *Mollugo pentaphylla*. *Parthenium hysterophorus* and *Portulaca oleracea* were collected from the campus and were propagated by transplantation of seedlings or cuttings. The plants were grown outdoors under a natural photoperiod of approximately 12 h and 30 °C/20 °C day/night temperatures, in earthen pots in soil supplemented with farmyard manure. The second to fourth leaves (counting from the youngest fully matured leaf) were harvested between 8.00 a.m. and 9.00 a.m. from 3-4 week old seedlings for the experiments.

Illumination of leaf samples

Discs of 20 mm^2 were punched from freshly collected leaves (under water) and incubated in darkness for 2 h at 30 ± 1 °C. After the pre-dark incubation, the leaf discs were illuminated (white light; Philips comptalux R95 flood bulbs) at an intensity of 1000 $\mu E\, m^{-2} s^{-1}$ (measured after passing the light through water filter) for 20 min unless otherwise mentioned. Both pre-dark incubation and illumination of leaf discs were done in distilled water. The light was passed through a 10-cm water filter to prevent temperature fluctuations due to illumination. Usually 20 leaf discs (ca. 80 mg) were used in each experiment.

Feeding of cycloheximide (protein synthesis inhibitor)

The feeding of cyclohcximide was done by incubating the leaf discs of *Alternanthera pungens* in 5 μM cycloheximide in the dark overnight. After the incubation, the leaf discs were washed thoroughly with distilled water and either left in the dark or illuminated for 20 min at 1000 $\mu E\, m^{-2} s^{-1}$. Stock solution of 10 mM cyclohcximide was prepared in absolute cthanol.

Extraction and assay of PEPC

PEPC was extracted from 1.6 g darkened (or illuminated) leaf discs by quickly homogenizing the tissue at 4 °C in a mortar with 6.4 ml of prechilled extraction buffer (100 mM HEPES

pH 7.2, 10 mM $MgCl_2$, 2 mM K_2HPO_4, 20% (v/v) glycerol, 1 mM EDTA, 2 mM Phenylmethyl sulfonylfluoridc (PMSF) and 10 mM β-mercaptoethanol). The extract was filtered through 4 layers of cheese cloth and rapidly centrifuged at 10 000 *g* for 3 min (Hermle Z 320 K centrifuge). An aliquot was kept aside prior to centrifugation for chlorophyll estimation.

PEPC activity was assayed spectrophotometrically at 30 °C by monitoring NADH oxidation at 340 nm in a dual beam UV–VIS Spectrophotometer (Shimadzu UV-160A). The 1 ml reaction mixture contained 50 mM Tricine–KOH (pH 7.5), 5 mM $MgCl_2$, 10 mM $NaHCO_3$ (prepared in CO_2 free distilled water), 2 Units NAD-malic dehydrogenase, 2.5 mM NADH and crude extract (equivalent to 1.0 to 2.0 μg chlorophyll). The extract was incubated in the reaction mixture for 30 s and the reaction was started by adding 20 μl of 50 mM PEP. (Deviations from this standard procedure are specified in the text.) The reaction was normally linear up to 5 min. Although there are reports that light activation is pronounced if PEPC is assayed at sub-optimal pH and substrate levels or in the presence of effectors like malate or G-6-P (Huber and Sugiyama 1986, Doncaster and Leegood, 1987), we chose to assay the enzyme at standard optimal conditions to make a direct interpretation of the data.

Extraction of cell sap and measurement of pH

Leaf discs were left in darkness for 2 h after which 20 discs (ca. 80 mg) were illuminated for 20 min at 1000 μEm^{-2}s^{-1} at 30 ± 1 °C. The leaf discs (dark-adapted or illuminated) were ground in a mortar (Gently, so as to avoid disruption of bundle sheath cells) with 4 volumes of 0.3 M sorbitol (or water, in some cases). The extract was filtered through a cheese cloth and centrifuged at 10 000 *g* for 3 min so as to remove the intact organelles. The extent of vacuolar breakage due to grinding in 0.3 M sorbitol was estimated by observing the extract under the microscope and was found to be very low. The pH of the supernatant (representing mostly cytosol-enriched cell sap) was monitored with a digital pH meter (Systronic Model 335). In some experiments, the same extract in unbuffered 0.3 M sorbitol was used for determining both the pH and the enzyme activity.

Chlorophyll estimation and replication

Chlorophyll was estimated as per Arnon (1949). The data presented here are the averages (±SE) of at least three independent experiments done on different days.

Materials

PEP (monocyclohexylammonium salt), cycloheximide, malate (monosodium salt), phenylmethyl sulphonylfluoride and malic dehydrogenase were obtained from Sigma Chemical Co, USA; glucose-6-phosphate from Boehringer-Mannheim, Germany; and NADH from Spectrochem, Bombay. All other reagents were of analytical grade.

Results

Extent and pace of light activation/dark-deactivation

When the predarkened leaf discs were illuminated, the activity of PEPC increased steadily. Limited activation of PEPC in *Alternanthera sessiles*, a C_3 species was achieved by 10 min of illumination. The very steep increase of PEPC in leaves of *A. pungens*, a C_4 species, continued up to 20 min of illumination (Fig. 1).

Leaves of several plant species were therefore examined for the extent of activation by light of PEPC (Table 1). Illumination enhanced the PEPC activity in C_3 species an average of 30% above the dark level, while stimulating PEPC activity nearly three times as much in C_4 plants.

If the extract from illuminated leaves was left in the dark at room temperature, the activity of PEPC declined gradually with time, reaching a stead state similar to the level of the dark-adapted sample. Such dark-deactivation of the enzyme was quick in C_3 species (Fig. 2), but was quite slow in C_4 species (Fig. 2). A survey of several plants (Table 2) confirmed that the pace of dark-deactivation was indeed much slower (requiring 90-120 min to reach dark-level activi-

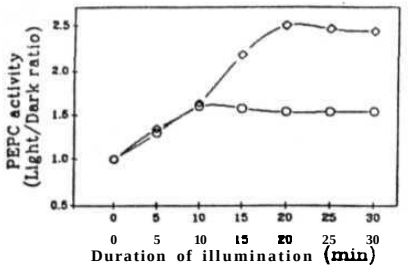

Fig. 1. The activation of PEPC in relation to the duration of illumination in *Altemanlhera.* The leaf discs were kept in darkness for *th* and were exposed to light of 1000 $\mu E m^{-2} s^{-1}$ at 30*C. The details of extraction and enzyme assay are described in 'Materials and methods'. Maximal activation of PEPC required 10 min of illumination in the *Alternanthera sessiles* (C_3; O) compared with 20 min in *A. pungent* (C_4; ◇). The data shown are the mean =SE of three experiments conducted on different days.

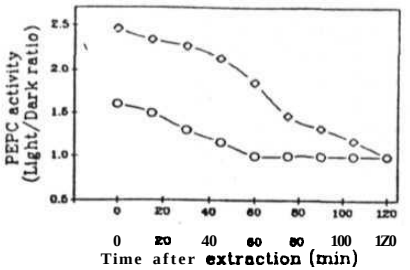

Fig. 2. The rale of decline in PEPC activity of extracts prepared from either illuminated (20 min at 1000 $\mu E m^{-2} s^{-1}$) or dark-adapted leaf discs of *Alternanthera* species. The activity of PEPC in the extracts of pre-illuminated leaves declined gradually and reached the level of the dark-sample by 60 min in *A* sessiles (C,; O) but took 2 h in *A. pungens* (C_4; ◇) Other details were as in Fig 1.

Table 1. Light activation of PEPC from some C_3 and C_4 species

Photosynthetic type/Species	PEPC activity		Light/dark ratio
	Dark	Light	
	μmol mg $Chl^{-1} h^{-1}$		
C_3 SPECIES			
Alternanthera sessiles	33 ± 3*	53 ± 4	1.6
Alternanthera ficoides[b]	45 ± 3	68 ± 5	1.5
Alternanthera tenella[b]	31 ± 3	47 ± 4	1.5
Arachis hypogea	32 ± 3	47 ± 4	1.5
Commelina communis	16 ± 1	17 ± 1	1.1
Lycopersicon esculentum	31 ± 2	46 ± 4	1.5
Mollugo pentaphylla[b]	35 ± 3	50 ± 4	1.4
Parthenium hysterophorus[b]	20 ± 2	25 ± 2	1.3
Pisum sativum	24 ± 2	31 ± 3	1.3
Tagetes erecta	23 ± 2	30 ± 3	1.3
Tridax procumbens	18 ± 1	19 ± 2	1.1
Average			1.4
C_4 SPECIES			
Alternanthera pungens	702 ± 68	1708 ± 154	2.4
Amaranthus hypochondriacus	1448 ± 131	3194 ± 91	2.2
Amaranthus viridis	548 ± 52	1219 ± 112	2.2
Gomphrena globosa	1028 ± 78	2970 ± 254	2.9
Portulaca oleracea	875 ± 79	2482 ± 231	2.8
Sorghum vulgare	924 ± 74	3201 ± 275	3.4
Zea mays	348 ± 27	1151 ± 98	3.3
Average			2.7

'The values are the averages (±SE) of at least three independent experiments. The experimental conditions arc described in Materials and methods.

* Based on their photosynthetic and photorespiratory characteristics. these species were identified as C_3–C_4 intermediates.

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Table 2. Dark-deactivation of light-activated PEPC in leaf extracts from some C_3 and C_4 species.

Photosynthetic type/Species	Elapsed time in darkness after extraction (min)			
	0	30	60	90
	(% of dark-adapted control)[a]			
C_3 SPECIES				
Alternanthera sessiles	160	124	100	100
Alternanthera ficoides[b]	152	123	116	100
Alternanthera tenella	149	119	100	100
Arachis hypogea	149	122	100	100
Commelina benghalensis	106	103	100	100
Lycopersicon esculentum	145	131	101	100
Mollugo pentaphylla[b]	144	127	108	100
Parthenium hysterophorus[b]	126	108	100	100
Pisum sativum	129	107	101	100
Tagetes erecta	129	119	101	100
Tridax procumbens	110	100	100	100
Average	136	117	102	100
C_4 SPECIES				
Alternanthera pungens	243	192	148	121
Amaranthus hypochondriacus	221	184	146	121
Amaranthus virdis	222	157	117	100
Gomphrena globosa	289	235	141	106
Portulaca oleracea	283	197	155	111
Sorghum vulgare	340	227	187	121
Zea mays	330	243	162	115
Average	273	205	151	114

[a] Activity of these extracts are indicated in Table 1. The activity of the dark-control before illumination was taken as 100%.
[b] Based on the photosynthetic and photorespiratorty characteristids, these species were identified as C_3–C_4 intermediates.

ty) in C_4 plants than that of C_3 plants (complete within 30–60 min).

The inclusion of PMSF in the extraction medium did not alter the course of dark-deactivation of C_4 PEPC in extracts of *A. pungens* (data not shown), indicating that dark-deactivation of light activated PEPC was not due to protcolytic degradation of the enzyme.

Sensitivity to malate and C-6-P

Upon light activation, the sensitive of PEPC from *A. pungens* (a C_4 species) to malate decreased and to G-6-P increased (Table 3). For example, 2.5 mM malate inhibited about 90% of enzyme activity in extracts from dark-adapted samples, but inhibited only 40% of its activity in the light form. During dark-deactivation, the sensitivity of the enzyme to malate increased gradually along with a steady decrease in catalytic activity of the PEPC (data not shown).

Table 3. Sensitivity of PEPC to malate and G-6-P. as indicated by their apparent KI and KA, respectively[a]

Species/(Photosynthetic type)	$K_{I(malate)}$		$K_{A(malate)}$	
	Dark	Light	Dark	Light
	mM			
Alternanthera sessiles (C_3)	1.2	1.3	2.0	2.0
Alternanthera ficoides[b]	1.0	1.4	2.3	2.2
Alternanthera pungens (C_4)	0.5	2.0	2.5	1.3

[a] PEPC activity was assayed in the presence or absence of the effector, in extracts prepared from leaf discs adapted to darkness for 2 h or illuminated for 20 min.
[b] C_3–C_4 intermediate species.

There was no change in the sensitivity of PEPC in C_3 species (*A. sessiles*) or C_3–C_4 intermediate (*A. ficoides*) to malate or G-6-P upon illumination.

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Effect of medium pH on PEPC activity

To establish whether pH was the cause or the effect of changes in PEPC activity, we checked the activity of the dark-form of the enzyme by varying the medium pH between 7.0 and 8.2. The activity of the dark-form increased steadily from pH 7.0 to 7.8 (Fig. 3. The dark-form was also more sensitive to pH changes when the light-form (data not shown).

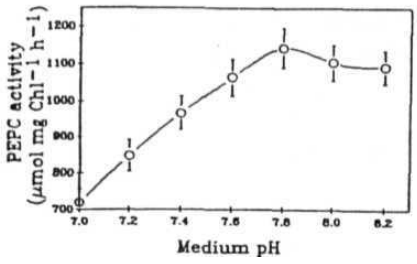

Fig. 3. The effect of medium pH on PEPC activity extracted from pre-darkened leaf discs of *Alternantherapungens*. The leaf discs were incubated in darkness for 2 h prior to extraction. Maximum activity was observed at a medium pH of 7.8. The data are averages (±SE) of at least three independent experiments.

Relationship of cytosolic pH change to light activation of PEPC

Mechanical extraction of cell sap poses problems, since grinding damages cellular organelles including vacuoles; however 0.3 M sorbitol appeared to minimize breakage of vacuoles (Table 4). The cell solution prepared in water tended to be acidic, presumably due to vacuolar breakdown, as more vacuolar breakage was observed under the microscope (data not shown). On the other hand, the use of 0.3 M sorbitol resulted in a slightly alkaline cell sap, presumably due to the enrichment of the cytosol and reduction in the contribution from cell organelles including vacuoles.

Illumination caused a measurable increase in pH of cytosol-enriched cell sap prepared from the leaves of *Alternanthera pungens*, a C_4 species. The pH of cytosol-enriched cell sap

Table 4. Light-induced alkalization of cytosol-enriched cell sap in C_3. *V*, and C_3–C_4 intermediate species, extracted in either distilled water or 0.3 M sorbitol (adjusted to pH 7.0 before extraction)

Photosynthetic type/ species	*pH of cell sap*					
	Water			*0.3 M Sorbitol**		
	Dark	*Light*	*ΔpH*	*Dark*	*Light*	*ΔpH*
C_3 SPECIES						
Alternanthera sessiles	6.78 ± 0.02	6.84 ± 0.03	0.06	7.06 ± 0.02	7.13 ± 0.02	0.07
Alternanthera ficoides[*]	6.84 ± 0.01	6.90 ± 0.03	0.06	7.14 ± 0.02	7.23 ± 0.02	0.09
Alternanthera tenella[*]	6.86 ± 0.02	6.93 ± 0.01	0.07	7.11 ± 0.03	7.20 ± 0.02	0.09
Cajanus cajan	6.88 ± 0.02	6.93 ± 0.03	0.05	7.14 ± 0.03	7.20 ± 0.03	0.06
Mollugo pentaphylla[*]	6.91 ± 0.03	6.95 ± 0.01	0.04	7.16 ± 0.03	7.24 ± 0.02	0.08
Parthenium hysterophorus[*]	6.92 ± 0.01	6.98 ± 0.03	0.06	7.13 ± 0.01	7.21 ± 0.03	0.08
Pisum sativum	6.84 ± 0.03	6.88 ± 0.03	0.04	7.10 ± 0.03	7.16 ± 0.03	0.06
Tridax procumbens	6.84 ± 0.02	6.87 ± 0.02	0.03	6.14 ± 0.03	7.20 ± 0.02	0.06
Average			0.05			0.07
C_4 SPECIES						
Alternanthera pungens	6.88 ± 0.03	6.94 ± 0.01	0.06	7.26 ± 0.02	7.45 ± 0.03	0.19
Amaranthus hypochondriacus	6.90 ± 0.01	7.02 ± 0.03	0.12	7.24 ± 0.03	7.43 ± 0.02	0.19
Portulaca oleracea	6.86 ± 0.02	7.00 ± 0.01	0.14	7.15 ± 0.03	7.33 ± 0.03	0.18
Gomphrena procumbens	6.94 ± 0.03	7.04 ± 0.03	0.10	7.22 ± 0.02	7.40 ± 0.03	0.18
Average			0.10			0.18

* Represents cytosol-enriched cell sap.

[*] Identified as C_3–C_4 intermediate species

increased during illumination and decreased after transfer to darkness. The extent of light-dependent alkalization of cytosol-enriched cell sap was three-fold higher in leaves of the four C_4 species evaluated than in the four C_3 plants (Table 4). The degree of cell sap alkalization in C_3–C_4 intermediates was more similar to C_3 plants.

The buffering capacity of cell sap, extracted from dark-adapted or illuminated leaves, was checked by titrating with 0.1 N NaOH. There was no change in the buffering capacity as indicated by the magnitude of pH shifts due to the addition of NaOH (data not shown). The activity of PEPC and the changes in pH were followed simultaneously in the same cell sap prepared in unbuffered 0.3 M sorbitol prepared from illuminated leaves of *A. pungens* (Fig. 4. The PEPC activity, as well as the pH of the cytosol-enriched cell sap, increased on a similar course during illumination and decreased during subsequent darkness. The course of deactivation of PEPC and acidification of the cell sap in darkness were again strikingly similar (Fig. 5).

Effect of cycloheximide on light activation of PEPC

When leaf discs of *Altemanthera pungens* (C_4 species), incubated in darkness overnight in 5 μM cycloheximide (eukaryotic protein synthesis inhibitor) were illuminated, the activity of PEPC increased, but to an extent lower than that in the inhibitor in the absence of preincubation (Table 5).

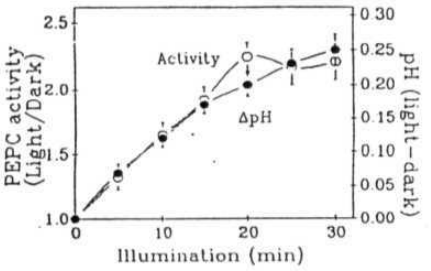

Fig. 4. The effect of light on pH of cell sap (•) and PEPC activity (O) in leaf discs of *Alternanthera pungens* (a C_4 plant) measured simultaneously in cytosol-enriched cell sap. Samples of cell sap were prepared at different periods of illumination. The pH values of dark-adapted samples were subtracted to calculate ApH. PEPC activity is represented as the ratio to that of the corresponding dark-sample. The data are the averages of at least three independent measurements.

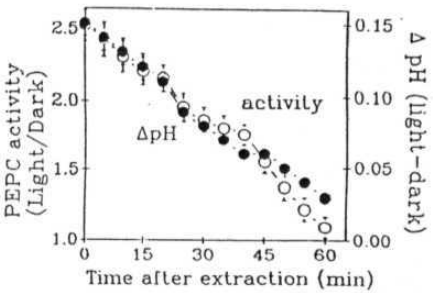

Fig. 5. The course of PEPC deaclivation (O) and decrease in pH (•) of cytosol-enriched cell sap prepared from illuminated leaf discs of *Alternantherapungens* and incubated in darkness. The ΔpH of the cell sap (light - dark value) and light/dark ratios of PEPC activity were simultaneously measured as indicated in Fig 3 and 'Materials and methods'. The data arc the averages from three replicate experiments

Table 5. Effect of cycloheximide. protein synthesis inhibitor. on light activation of PEPC in *Allernanlhera pungens*

	PEPC activity			Cell sap pH		
	Dark	Light	Light/Dark	Dark	Light	ApH
	μmol mg Chl^{-1} h^{-1}					
Control	701 ± 59	1750 ± 162	2.5	7.25 ± 0.03	**7.44 ± 0.03**	**0.19**
Cycloheximide treated	694 ± 61	1308 ± 124	1.9	7.23*0.02	**7.41 ±0.02**	**0.18**

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Discussion

The present article describes the pattern of light activation of PEPC from C_3 and C_4 species. The extent of activation by light varied considerably within each photosynthetic group (Table 1). We demonstrated that the light activation of PEPC in C_4 plants is distinct from that in C, species, in both the extent and rate of activation/dark-deactivation.

Upon illumination, there was a three-fold enhancement in PEPC activity in leaf extracts from C_4 species, compared to a more limited activation in leaves of C_3 species (Table 1). Marginal activation by light of PEPC in C_3 or C_3–C_4 intermediate species of *Flaveria* has been reported (Chastin and Chollet 1989). The present study confirms the marginal activation by light of PEPC in C_3 plants.

Although the species of *Alternanthera ficoides*, *A. tenella*, *Mollugo pentaphylla* and *Parthenium hysterophorus* (Devi and Raghavendra 1993, Raghavendra and Das 1993) were identified as C_3–C_4 intermediates, based on their photosynthetic and photorespiratory characteristics, PEPC from these species had similar characteristics to those of C_3 species.

There is a great deal of literature on the activation time of PEPC in leaves of C_4 plants. Most of the studies used intact leaves and indicated a requirement of 40-60 min to reach maximum activation (Jiao and Chollet 1989, Van Quy and Champigny 1992, Van Quy et al. 1991). A few studies used leaf slices and reported that 15 min was long enough to activate PEPC (Doncaster and Leegood 1987). Variation in the duration of illumination needed for PEPC activation in C_4 plants appears due to difference in the experimental system: intact leaves or leaf slices. Our experiments with leaf discs floated on water confirm that 20 min of illumination are necessary to activate PEPC in leaves of C_4 plants.

The slow pace of light activation/dark-deactivation of PEPC in C_4 species (Fig. 1 and Table 2) is strikingly similar to the slow but steep rate of light-dependent cytosolic alkalization/dark-acidification in leaves of C_4 plants, compared to the quick but small cytosolic pH changes in C_3 plants (Yin et al. 1990, 1993, Raghavendra et al. 1993). We feel that the pattern of light activation of PEPC can be taken as an additional criterion to distinguish C_3 and C_4 plants. Comparative studies on C_3 and C_4 species of the same genera have indicated a distinct isofonn of PEPC in the leaves of C_4 species (Nakamoto et al. 1983, Adams et al. 1986, Bauwe and Chollet 1986).

Light-dependent cytosolic alkalization in vivo has been reported in leaves of C_3 and C_4 plants, using pH-dependent fluorescent dyes (Yin et al. 1990, 1993, Raghavendra et al. 1993). An indication that cytosolic pH could be an important factor regulating the activity of PEPC came from the observation that illumination raised the pH of cytosol-enriched cell sap (Table 4). Since the dark-form of the enzyme is very sensitive to medium pH (in vitro) (Fig. 3), we feel that light-induced cytosolic alkalization will have a marked effect on the dark-form of the enzyme (in vivo). The importance of an alkaline cytosolic pH in activating PEPC was further confirmed by simultaneous measurements of cell sap pH and PEPC activity in leaves (Figs. 4 and 5). Despite the technical limitations, our data suggest that illumination causes alkalization of cell sap. Upon illumination, the degree of cytosolic alkalization was much greater than vacuolar acidification in both C_3 and C_4 plants evaluated by Raghavendra et al. (1993) and Yin et al. (1993). In their studies, changes in cytosolic pH was enormous, particularly in mesophyll cells of C_4 leaves, and unlike the weak acidification of the vacuole. This indicates that most of the light-induced change in pH of the 'cell sap' in this study is probably the result of cytosolic alkalization.

The reduction in the extent of light activation of PEPC when leaf discs were pre-incubated in the presence of protein synthesis inhibitor cycloheximide clearly indicates that the turnover of protein-serine kinase is also needed for the activation of PEPC, as suggested by Jiao et al. (1991). It was also shown that pH effects the phosphorylation status of PEPC from sorghum mesophyll protoplasts (Pierre et al. 1992). Our results provide evidence for the regulation of PEPC in leaf discs of *Alternanthera pungens* by pH.

The major mechanism of light regulation of C_4 PEPC is the reversible phosphorylation o(the

enzyme during light/dark transitions (Nimmo et al. 1987, Ranjeva and Boudet 1987, Jiao and Chollet 1988, 1989, Echevarria et al. 1990). A soluble PEPC-protein serine kinase catalyzes the phosphorylation of the protein (Review: Jiao and Chollet 1989, 1990, 1991), while a type 2A phosphatase brings out the dephosphorylation of the enzyme (Carter et al. 1990). Although the activity of PEPC-PK is stimulated in light (Echévarria et al. 1990, Jiao and Chollet 1992, Jiao et al. 1991), the modulation of PEPC-PK or protein phosphatase by cytosolic pH is yet to be established.

We confirm that a marked light activation of PEPC (greater than 3 times the activity over dark-control) occurs mainly in C_4 plants. The extent of activation of PEPC on illumination was limited (about 30% over the dark-control) but detectable in C_3 species. We demonstrate the distinctly slow rate of activation (in light) and deactivation (in darkness) of PEPC in leaves of C_4 plants.

PEPC is a classic example of marked modulation of enzyme activity by pH (Davies 1979, O'Leary 1982). Apart from its extreme sensitivity to even small pH variations, PEPC has a tendency to aggregate or dissociate depending on microenvironment and effectors (e.g. Wu et al. 1990). Experiments with isolated protoplasts of maize (Devi and Raghavendra 1992) or sorghum (Pierre et al. 1992) indicated that the cytosolic pH may play an important role during the light activation of PEPC and in the reduction in sensitivity to malate (due to phosphorylation of the enzyme). Our experiments provide preliminary evidence of a marked correlation between a light-induced increase in pH of cell sap and PEPC activation due to illumination. We suggest that the cytosolic pH could affect the activity of PEPC either directly or indirectly (By regulating protein kinase or protein phosphatase, or both). The concept of PEPC regulation by cytosolic pH appears to be a distinct possibility, even if it involves modulation of PEPC-PK or protein phosphatase, besides marked regulation of the PEPC itself. Further experiments are necessary to trace the exact sequence of events during modulation of PEPC activity in response to light-dependent alkalization of cytosol.

Concluding remarks

The extent and pattern of light activation of PEPC in the leaves of C_4 plants are quite distinct from those of C_3 plants. Illumination causes cytosolic alkalization as indicated by the rise in pH of cytosol-enriched cell sap in the leaves of C_4 plants. The increase in cytosolic pH could enhance PEPC activity in one or more of the following ways: (a) increase in PEPC-PK activity and phosphorylation of PEPC, (b) change in the oligomeric state of the enzyme, and /or (c) an unknown change in conformation of the enzyme. All three factors could be interrelated. For example, the change in oligomeric/conformational state of the enzyme, due to cytosolic alkalization, may increase its activity or promote the extent of phosphorylation of the enzyme, or both.

Acknowledgements

This work was supported by a Grant from the Department of Science and Technology. New Delhi (No. SP.SO/A43/88). AVR and MTD were recipients of Junior and Senior Research Fellowships from the University Grants Commission and Council of Scientific and Industrial Research, New Delhi, Respectively. We also wish to thank the anonymous referees for extremely useful comments on the original manuscript.

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