Light Activation of Phosphoenolpyruvate Carboxylase in Leaf Discs of C_4 Plant Species in Relation to Alkalinization of Cell Sap

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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To

my beloved parents

Whose sacrifice and prayers
made this possible

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/ express my deepest gratitude to my parents who always wished me the best in life and giving me full support throughout my academic career.

In compiling a list of names I am haunted by the fact that, since my *Research Work* has taken some years to complete, there will be some omissions. For these I offer my apologies. My sincere gratitude is due to all the faculty members of the School, whose ideas influenced me and whose encouragement has sustained me. I wish to make special attention of those whose, each in his or her way, had a particularly important impact on the development of this *work:* Professor VS Rama Das, Professor (late) AS Murthy, Professor Ch. RK Murthy, Professor CK Mitra, Dr (Mrs) Aparna Datta-Gupta, Dr MNV Prasad, Dr V Mohana Chari, Dr P Appa Rao and Dr N Siva Kumar.

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University of Hyderabad *AV Rajagopalan*

DECLARATION

The candidate declares that this work has been carried out by him under the supervision of **Professor AS Raghavendra,** Dept of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India and that this work has not been submitted for any degree or diploma of any other University.

Professor AS Raghavendra
(Supervisor)

Mr A V Rajagopalan.
(Candidate)
Enrol. No. 91LSPH02

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CERTIFICATE

This is to certify that the thesis entitled **"Light Activation of Phosphoenlpyruvate Carboxylase in Leaf Discs of C_4 Plant Species in Relation to Alkalinization of Cell Sap"** is based on the results of the work done by **MR A V Rajagopalan** 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.

Professor RP Sharma
Head

Professor AS Raghavendra
(Supervisor)

Professor AR Reddy
Dean

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University of Hyderabad ***AV Rajagopalan***

Abbreviations

AP	Alkaline phosphatase
Chl	Chlorophyll
DCDP	3,3-dichloro-2-hydroxyphosphinoyl-methyl-2-propenoate
Glc-6-P	Glucose-6-phosphate
L/D ratio	Light/dark activity ratio
MDH	Malate dehydrogenase
ME	Malic enzyme
OAA	Oxaloacetic acid
PEPC	PEP Carboxylase
PEPC-PK	PEPC-protein serine/threonine kinase
PEPC-PP	PEPC-protein phosphatase

*A11 remaining abbreviations are according to those found in *Plant Physiology*, January 1997, Vol 113, No. 1. pp. xiii-xv.

Contents

Chapter One

Introduction

* **Review of literature**

* **Areas for future research and scope of the present study**

Review of Literature

Phosphoelpyruvate Carboxylase (PEPC; Orthophosphate: oxaloacetate Carboxylase [phosphorylating], EC 4.. 1.1.31) is a ubiquitous enzyme and occurs in mesophyll cytosol of plant cells. The widespread occurrence of the enzyme is complemented by its diverse functions. However, the most important function of PEPC is its essential role in primary carbon fixation during C4 and Crassulacean acid metabolism (CAM) plants (Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

The literature on the properties and functions of PEPC from C_4 plants, C_3 species and prokaryotes has been reviewed frequently (Utter and Kolenbrander 1972, O'Leary 1982, Latzko and Kelly 1983, Andreo *et al* 1987, Deroche and Carrayol 1988, Stiborova 1988, Gonzalez and Andreo 1989, Jiao and Chollet 1991, Vance and Gantt 1992, Nimmo 1993, Lepiniec *et al.* 1994, Rajagopalan *et al* 1994, Toh *et al* 1994, Chollet *et al* 1996). In view of the extensive literature available on PEPC, reference is mostly made to references which appeared in 1986 or later.

Occurrence and Activity

To our knowledge, all plant tissues (from both lower and higher plants) examined so far have been found to contain PEPC. The enzyme is reported in also several species of bacteria, cyanobacteria and green algae. However, PEPC could not be found in fungi, marine diatoms and dinoflagellates (examined so far), which appear to use PEP carboxykinase (PEPCK) to carboxylate PEP and form oxalacetate (OAA) and ATP.

PEPC is localized in the cytosol of mesophyll cells in C_4 plants, in the photosynthetic cells of CAM plants. In C3 plants, the enzyme may be localized in both the cytosol and the chloroplasts of the leaves (O'Leary 1982, Latzko and Kelly 1983, Andreo *et al.* 1987, Lepiniec *et al.* 1994, Rajagopalan *et al* 1994).

PEPC catalyzes the irreversible β-carboxylation of PEP in the presence of HCO_3^- and Me^{2+} to yield OAA and Pi. PEPC is considered as a marker enzyme for C4 mesophyll cells as the activity of PEPC in bundle sheath preparations was only about 3 to 7% of that in mesophyll cells (Meister *et al.* 1996). Thus, PEPC is the key enzymes of C_4-dicarboxylic acid metabolism in plants (Andreo *et al.* 1987, Rajagopalan *et al.* 1994, Chollet *et al.* 1996). In general, the activity-levels of this enzyme in C3 leaves is about 2 to 5 % of that found in the mesophyll cells of C4 leaves (Kluge 1983, Latzko and Kelly 1983). Extremely high activities of PEPC are reported from the thermogenic tissues of Araceae members (ap Rees *et al.* 1981) and root nodules of Fabaceae (Vance and Heichell 1991, Vance and Gantt 1992, Woo and Xu 1996).

Purification and Stability

Homogenous preparations of PEPC have been made from leaves of plants belonging to different photosynthetic types. The specific activities of the enzyme range from 20 to 50 jimol mg^{-1} protein min^{-1} . The specific activities of the C_4 and CAM plant PEPCs are of the same magnitude as those of the C3 plant-enzyme.

The native leaf and recombinant forms of PEPC are highly susceptible to limited proteolysis near the N-terminus during extraction and subsequent purification of the enzyme (Nimmo *et al.* 1986, McNaughton *et al.* 1989, Arrio-Dupont *et al.* 1992, Baur *et al.* 1992, Wang *et al.* 1992, Weigend and Hincha 1992, Duff *et al.* 1995). The presence of protease inhibitors, particularly chymostatin, throughout the enzyme preparation helps to preserve the N-terminus of PEPC (McNaughton *et al.* 1989, Carter *et al.* 1991, Jiao and Chollet 1991, Baur *et al.* 1992, Rajagopalan *et al.* 1994, Chollet *et al.* 1996). The loss of N-terminus region has no major influence on

the enzyme's electrophoretic mobility, V_{max}, and carbon isotope effects, but decreases markedly the *in vitro* phosphorylability of PEPC and sensitivity of the enzyme to its allosteric effector, L-malate (Chollet *et al.* 1996).

The omission of chymostatin during purification of PEPC resulted in a truncated protein. The relative molecular mass of the enzyme from maize was only 105 kD in the absence of chymostatin in contrast to 109 kD in its presence (McNaughton *et al.* 1989). When the truncated PEPC was phosphorylated in presence of PEPC-protein kinase (PEPC-PK) and [γ-^{32}P]ATP, the enzyme was not phosphorylated, and the properties of the enzyme (except malate sensitivity) were similar to the dark-form (McNaughton *et al.* 1989, Arrio-Dupont *et al.* 1992).

The integrity of PEPC can be preserved during isolation by the inclusion of glycerol, L-malate, and protease inhibitors (such as chymostatin or phenylmethylsulfonyl fluoride or pepstatin or benzamidine) and by the use of rapid purification protocols employing fast-protein liquid chromatography (FPLC), high-pressure liquid chromatography (HPLC), or immunoadsorbent column chromatography (Vidal 1980, Nimmo *et al.* 1989, McNaughton *et al.* 1989, Arrio-Dupont *et al* 1992, Baur *et al.* 1992, Wang *et al* 1992, Wang and Chollet 1993a, Duff *et al* 1995, Zhang *et al* 1995). By using necessary precautions and protocols of rapid purification, preparation of highly active, (with an intact N-terminal) PEPC can be made from leaves (C_4, CAM and C_3) or root nodules.

Isoforms

Up to four isoforms of PEPC are identified in higher plants: C4 photosynthetic form, C3 photosynthetic one, CAM-form and dark- or non-autotrophic PEPC. These

isoforms can be distinguished by their chromatographic, immunological and kinetic properties (Rajagopalan *et al.* 1994).

In sorghum leaves, two isoforms of PEPC are present, namely, the E (etiolated)- form and the G (green)-form (Thomas *et al.* 1987). The E-form occurs in etiolated leaves and exhibits characteristics of C_3-form, while the G-form is present in green leaves and shows typical kinetic and regulatory properties of C4-form. No interconversion occurs indicating that these two forms are encoded by two different mRNAs.

At least a minimum of three isoforms are detected in green leaves, etiolated leaves and roots of maize (Hudspeth and Grula 1989, Schaffner and Sheen 1992). These isoforms are encoded by different genes (Thomas *et al.* 1987, 1990, Hudspeth and Grula 1989, Hermans and Westhoff 1990). In *Vicia faba,* immunologically three distinct isoforms, *viz.,* mesophyll-PEPC, epidermal-PEPC, and guard cell-PEPC forms are identified. Each PEPC represents an isoform specific for a determined physiological function in a given cell type (Schulz *et al.* 1992).

The occurrence of multiple forms of PEPC suggests that an important event during the evolution of C_4 plants has been the development of a regulatory mechanism for preferential expression of the C4 specific gene (Nelson and Langdale 1992, Ku et al. 1996).

Physiological Role

C_4 and CAM plants

PEPC catalyzes the β-carboxylation of PEP to yield OAA and Pi, and, thus plays a key role in the metabolism of C4 and CAM plants. In C4 plants, the initial carbon fixation through PEPC occurs in mesophyll cells, while the subsequent

decarboxylation of the C4-acids takes place in the bundle sheath cells where the ribulose 1,5-*bis*phosphate carboxylase/oxygenase (Rubisco) (and Calvin cycle) mediates the refixation of released CO_2 . The decarboxylation of the C4 acids results in a marked rise in the internal CO_2 of bundle sheath tissue (Hatch 1987, Furbank and Taylor 1995). PEPC, thus, forms an essential base of primary carbon fixation in the CO_2 concentrating mechanism, in view of the low K_M for HCO_3 (<10 μM) of PEPC (Edwards and Walker 1983, Raghavendra and Das 1993). The substantially high concentration of CO_2 in the bundle sheath achieved by carboxylation-decarboxylation has two important consequences; (i) optimization of carboxylating activity of Rubisco on, and (ii) suppression of the oxygenase activity of Rubisco (due to the high CO2 concentration) and restriction of photorespiratory mechanism.

Like C4, CAM represents an ecological adaptation to water deficiency in the environment. The capability of CAM to fix CO_2 primarily by PEPC (independent of light) and extended storage of the C4 acids in vacuoles enable CAM plants to harvest external CO_2 during the light. The C4 acids are decarboxylated during subsequent day and CO_2 is refixed through Calvin cycle. The situation in CAM resembles that of C4 photosynthesis, where >90% of the net CO_2 fixation is via PEPC (Ting 1985, Leegood and Osmond 1990, Leegood 1993, Raghavendra and Das 1993).

C3 **plants**

The C_3 plants also contain PEPC, although the activities are several times lower than those in C4 leaves and CAM tissues (Latzko and Kelly 19083, Rajagpopalan *et al.* 1994). However, these levels are significant, when compared with the activitie limiting enzymes in plants. Several possible function of PEPC in C3 plants have been proposed: (a) replenishment of tricarboxylic acid cycle (TCA) intermediates, (b)

NADPH generation, (c) recapture of respired CO_2, (d) carbon metabolism of aquatic plants, (e) 'malate fermentation', (0 energy overflow through 'cyanide-resistant' pathway, (g) nitrogen assimilation and amino acid biosynthesis, (h) pH maintenance, (i) electroneutrality, (j) response to light quality, and (k) low temperature sensitivity (Latzko and Kelly 1983).

Root nodules

PEPC plays an important role in supporting symbiotic dinitrogen fixation in legume root nodules (Day and Copeland 1991, Vance and Heichel 1991, Vance and Gantt 1992). Anaplerotic CO_2 fixation (by PEPC) provides respiratory substrate (for example, malate or succinate) for the bacterioid (Rosendahl *et al.* 1990), carbon skeletons for the biosynthesis of amino acids (King *et al.* 1986) and organic acids to maintain intracellular pH at neutrality (Israel and Jackson 1983).

The predominance of PEPC in root nodules may vary depending on the type of legume species. In ureide-transporting legumes such as soybean, PEPC may play a minor role in the synthesis of carbon skeletons (for example, OAA and 2-oxoglutarate) for amino acid formation (King *et al.* 1986). On the other hand, a steady supply of OAA is required for the incorporation of ammonia into aspargine and glutamate/glutamine in amide-transporting legumes such as alfalfa and lupin (Anderson *et al.* 1987).

The *in vivo* $^{14}CO_2$-fixation assay and xylem sap analysis show that in *Sesbania rostrata* the transport of fixed N is transferred as aspargine. The close relationship between nodule PEPC and nitrogenase activities suggests that CO_2-fixation contributed directly to N assimilation in stem nodules of this species (Sadasivam *et al.* 1993).

Non-photosynthetic tissues

The atmospheric CO_2 fixation by PEPC is important during fruit maturation (Blanke and Lenz 1989), seed formation and germination (Watson and Duffus 1988, Khayat *et al.* 1992, Sugimoto *et al.* 1992), or in metabolic interactions between the style and elongating pollen tube (Jansesn *et al.* 1992). The activation of PEPC is stimulated during cell division and organogenesis in plants (Coudret and Ducher 1993).

Finally, PEPC is implicated also in dark CO_2-fixation by a `primitive' form of endoxymbiotic association in a lichen (Kluge *et al.* 1991) and some autotrophic bacteria which directly assimilate CO_2 via PEPC (Buchanan and Arnon 1990).

Protein stucture

Amino acid sequences of plant PEPCs are well conserved and are easy to align (Lepiniec *et al.* 1993, Lepiniec *et al.* 1994, Toh *et al.* 1994). The deduced amino acid sequences of PEPC from higher plants, bacteria, cyanobacteria and recombinant sorghum PEPC protein are available (Lepiniec *et al.* 1993, 1994, Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

The C4-PEPC from maize is composed of 970 amino acid residues (Kagami *et al.* 1986, Hudspeth and Grula 1989) compared to 952 of *Sorghum* (Cretin *et al.* 1990), 966 of *Flaveria trinervia* (Poetsch *et al.* 1991), 956 of potato (Merkelbach *et al.* 1993), 967 of soybean, 964 of tobacco, 976 of *Flaveria pringlei*, 1025 of *Anacystis variabilis*, 1016 of *Anacystis nidulans*, 919 of *Corynebacterium glutamicale* and 883 of *Escherichia coli* (Lepiniec *et al.* 1993, 1994, Rajagopalan *et al* 1994, Chollet *et al* 1996).

The sequence homology PEPC from higher plants ranges from 60 to 90 % (Rajagopalan *et al* 1994). However, there is only 30 to 50 % sequence homology of

maize PEPC with that of *E. coli* and *Anacystis nidulans* (Ishijima *et al.* 1985). The lower degree of similarity between different prokaryotic and higher plant PEPC enzyme is consistent with the observation that these PEPC forms differ from one another in catalytic and regulatory properties (Andreo *et al.* 1987, Jiao and Chollet 1991, Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

Chemical modification of maize PEPC revealed at least four kinds of essential amino acid residues to be at or near the active site of the enzyme: two arginines (Iglesias *et al.* 1984), four lysines (Podesta *et al.* 1986, Wagner *et al* 1988), two histidines (Iglesias and Andreo 1983) and five to seven cysteine residues (Chardot and Wedding 1992a). Some of these essential residues (whose location is clearly documented) of PEPC from maize are given in Table 1.1 (modified from Rajagopalan *et al.* 1994)..

The region surrounding the lysine (K^{606} of maize or K^{600} of *Flaveria trinervia)* is the most conserved residues in the primary structure of plant PEPC. This region seems to be involved in PEP binding/catalytic activity (Jiao *et al.* 1990, Poetsch *et al.* 1991). The cysteine residues may be involved in subunit-interactions to maintain the tetrameric structure (Andreo *et al.* 1987) or redox-regulation (Chardot and Wedding 1992a). A recent finding of complete absence of cysteines in PEPC from *Thermus* sp., a thermophilic bacterium, excludes the direct involvement of these residues in catalysis (Nakamura *et al* 1995).

Site-directed mutagenesis of the active-site domain of PEPC have thus far been attempted with the enzyme from *E. coli* and *Sorghum.* His^{13}* is required for carboxylation in *E. coli* but the mutant H138N can catalyze PEP hydrolysis to

Table 1.1. The location (number from the N-terminal region of the protein) of important amino acid residues and their function on PEPC from maize. Adapted from Rajagopalan *et al.* (1994).

Amino acid residue	Motif/Location	Possible function	Occurrence/ remarks
1. N-terminal Serine	EKHHS[15]IDAQLR	Regulatory phosphorylation	Higher plant-invariant
2. Histidine I	LVLTAH[177]PTQS	Formation of OAA	Both pro- and eukaryotes
3. Lysine[a]	GYSDSGK[606]DAG	PEP binding/catalysis	-do-
4. Histidine II	FH[639]GRGGTVGGGGP	unknown[b]	-do-
5. Cysteine	196; 308; 335; 419; 424; 426, 687	unknown[c]	-do-
6.Valine	EYRSV[731]VK	unknown	C_4-specific
7. C-terminal serine	PWIDS[780]WTQ	unknown	-do-

* Highly conserved sites.
[b] Allosteric regulation in *E. coli.*
[c] Speculative involvement in maintenance of the tetrameric structure/redox regulation.

pyruvate in the presence of HCO_3^- (Terada and Izui 1991, Terada *et al.* 1992). His^{579} is not obligatory for catalysis, although the residue it is species-invariant (Terada *et al.* 1991). Replacement of conserved Arg^{587} by Ser (R587S) also gives an enzyme that catalyzes hydrolysis, but not carboxylation (Terada *et al.* 1992, Yano *et al.* 1995).

Reversible phosphorylation of a Serine (Ser) residue in the N-terminal domain E/DK/RXXSIDAQLR (Ser^8, Ser^{15}, Ser^{11} in *Sorghum,* maize and *Flaveria trinervia,*

respectively) of PEPC is a cardinal event in the regulation by light or darkness of the C_4 and CAM enzymes, respectively (Carter *et al.* 1991, Jiao and Chollet 1991, Bakrim *et al.* 1993, Nimmo 1993, Rajagopalan *et al.* 1994). The posttranslational modification is sufficient to increase several-fold the enzyme's apparent K_I for L-malate and its catalytic activity at suboptimal but physiological levels of pH and PEP, without affecting markedly K_M for PEP or V_{max} at optimal pH (Jiao and Chollet 1991, Wang *et al.* 1992). Changing The target Ser-residue of the recombinant *Sorghum* C4-PEPC to a Cys (S8C), or an aspartate (S8D) by site-directed mutagenesis, demonstrated that the introduction of negative charge into this PEPC domain mimics the regulatory effect of phosphorylation (Wang *et al.* 1992). Confirmation of this result has been obtained by -SH modification (*S*-carboxymethylation) of the mutant S8C *Sorghum* C4-PEPC (Duff *et al.* 1993).

Along with site-directed mutagenesis, X-ray crystallography has become the *sine qua non* of enzymology, though plant PEPC is yet to be subjected to crystallography. Preliminary crystallization and X-ray crystallography of *E. coli* has been reported (Inoue *et al.* 1989).

Reaction mechanism

The carboxylation of PEP by PEPC is essentially irreversible with a AG° of 7 kCal mol^{-1} at 25°C (Rajagopalan *et al.* 1994). PEPC is dependent on bivalent cations and prefers Mg^{2+} *in vivo* , but can use Mn^{2+} or Co^{2+} *in vitro* (O'Leary 1982, 1983). PEPC is apparently the only non-biotin containing Carboxylase that uses bicarbonate, rather than CO_2, as the substrate (O'Leary 1982). Since the carbon atom of HCO_3 is quite unreactive towards nucleophilic attack (compared to CO2), the mode of activation of HCO_3 by the enzyme is an enigma.

A proposed model of reaction mechanism of PEPC and sequence of reaction events is presented in Fig. 1. The order of binding of reactants to PEPC is suggested to be Mg^{2+}, PEP, and bicarbonate. The high level of synergism in the binding of substrates was proposed to be the basis (Janc *et al* 1992 a,b,c) of an earlier report that the Mg^{2+}-PEP complex was the substrate of PEPC (Wedding *et al.* 1988). After the sequential binding of Mg^{2+}, PEP, and bicarbonate to the enzyme, the reaction appears to proceed in two major steps: (i) the formation of carboxyphosphate and the enolate of pyruvate; decomposition of carboxyphosphate to form CO_2 and phosphate within the active site; and then (ii) carboxylation of the enolate by the CO_2 and formation of OAA (Rajagopalan *et al.* 1994). This stepwise mechanism is analogous to that suggested for biotin-dependent carboxylations and for carbonyl phosphate synthetase. Additional evidence for the stepwise mechanism is provided by the observation that PEPC shows synergetic inhibition by oxalate and carbamyl phosphate, which are structural analogues of the carboxyphosphate-enolate intermediate (O'Leary 1983, Chollet *et al.* 1996). The use of (Z)- and (*E*)-3-fluorophospho*enol*pyruvate (F-PEP) as substrate confirmed that carboxylation of PEP occurred by the combination of the enolate of pyruvate (rather than carboxyphosphate) with CO_2 (Janc *et al.* 1992 a,b,c). Both phosphate and carboxy groups are important for substrate binding (Gonzalez and Andreo 1988, 1989). Functional analogues for CO_2 and HCO_3^- are rare in enzymatic reactions. Formate could form an alternative for HCO3" in the reaction catalyzed by PEPC from *Zea mays,* producing formylphosphate and pyruvate, the maximum velocity being 1 % of that with bicarbonate at pH 8 (Janc *et al.* 1992 a,b,c).

Besides the normal carboxylation reaction, PEPC from maize catalyzes a HCO3 -dependent hydrolysis of PEP to pyruvate and Pi (Ausenhus and O'Leary

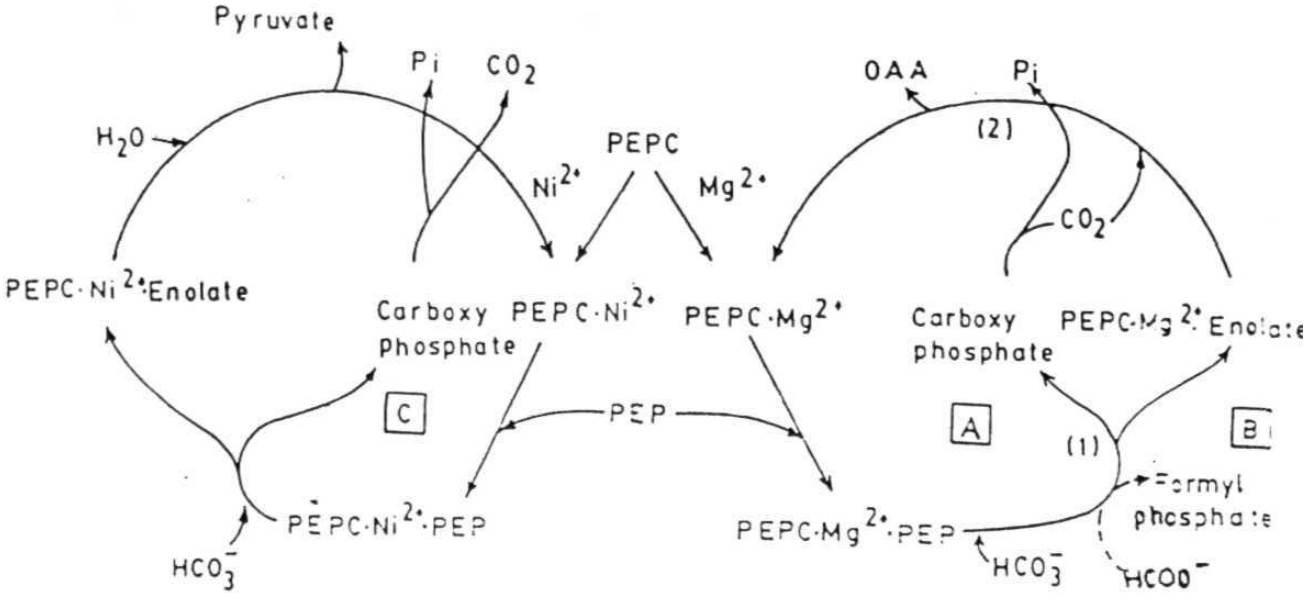

Figure 1.1. A schematic representation of the reaction catalyzed by C_4-photosynthetic phospho*enol*pyruvate carboxylase. The enzyme combines with Mg^{2+} and then with PEP. During the carboxylation of PEPC.Mg^{2+}.PEP (A) two partial reactions are envisaged: (1) Formation of carboxyphosphate and the enolate of pyruvate; Release of CO_2 and Pi from carboxyphosphate. (2) Combination of CO_2 with the enolate to form OAA. Use of F-PEP yields F-OAA. Formate can replace bicarbonate in the carboxylation to produce formylphosphate (B). In the presence of a suital metal like Ni^{2+}, PEPC can function as a bicarbonate-dependent phosphatase to hydrolyze PEP to pyruvate and Pi (C).

1992). This hydrolysis is a minor component of the overall reaction flux with Mg^{2+} under *in vivo* conditions (<5%), but it increases with other metals (cations) and constitutes over 50% of the total reaction flux when Ni^{2+} is used. The hydrolysis rate decreases in the following order: $Ni^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+}$. PEPC catalyzes the hydrolysis of phosphoglycolate or L-phospholactate at extremely slow rates (2000-4000 times less than that with PEP). It is speculated that the binding of metal ion to the enolate oxygen stabilizes the enolate intermediate. The more stable the enolate, the less reactive it is towards carboxylation and greater the opportunity for hydrolysis (Ausenhus and O'Leary 1992).

The pH dependence of V_{max}/K_M (PEP) indicates that only the fully ionized form of PEP binds to the enzyme and that an enzyme group with a pKa near 7.5 must be deprotonated for the enzyme to be active. O'Leary (1983) reported that the dependence of V_{max}/K_M (HCO_3^-) shows that only HCO_3^-, and CO_3^{2-} binds to the enzyme. The size and shape of the active site of PEPC have been studied by the use of a variety of inhibitors. It is hoped that these studies may lead to the design of efficient inhibitors and ultimately to the development of herbicides specifically directed towards C4 species.

Regulation

Many internal (for example, pH or metabolic effectors) and external (for example, light or temperature) factors influence the activity of C4-PEPC. The extent of influence may differ depending on the source of PEPC and other interacting factors.

Light

Like many of the photosynthetic enzymes, the kinetic and regulatory properties of C4-PEPC are modulated by light/dark transitions *in vivo* (Jiao and Chollet 1991, Nimmo

1993, Lepiniec *et al.* 1993, 1994, Huber *et al.* 1994, Rajagopalan *et al.* 1994, Chollet *et al.* 1996). The phenomenon is in addition to the well-known light-induced increase in PEPC-protein in leaves (Sims and Hague 1981). The enzyme extracted from preilluminated leaf tissue exhibits two- to three-fold more activity than the dark form, particularly at suboptimal but physiological levels of pH and PEP. Besides an increase in the activity, there is a marked decrease in the malate sensitivity of the light-form of PEPC but only a marginal change occurs in the sensitivity towards Glc-6-P (Huber and Sugiyama 1986, Doncaster and Leegood 1987, Rajagopalan *et al.* 1993).

Two mechanisms of reversible covalent modification of the enzyme could explain the reversible activation of C4-PEPC in light: oxidation-reduction of thiol groups (Iglesias and Andreo 1984, Andreo *et al.* 1987) or phosphorylation/dephosphorylation of Ser/Thr-residues (Jiao and Chollet 1991, Nimmo 1993, Rajagopalan *et al* 1994, Chollet *et al.* 1996). Although the first mechanism plays an important role in the modulation of some of the Calvin cycle enzymes (Buchanan 1992), thiol groups are not implicated in light activation of PEPC *in situ* or *in vivo* (Jiao and Chollet 1991, Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

Phosphorylation of PEPC in *Sorghum* mesophyll protoplasts under light was modulated by cytosolic Ca^{2+} and pH (Pierre *et al.* 1992). Experiments with isolated protoplasts of *Sorghum* and *Digitaria sanguinalis* further confirmed that the cytosolic pH may play an important role during light activation and phosphorylation of PEPC and a reduction in sensitivity to malate (Giglioli-Guivarc'h *et al.* 1996, Duff

et al. 1996). Light induces marked alkalization of cytosol in mesophyll cells of C_4 plants (Yin *et al,* 1993, Raghavendra *et al.* 1993).

The possible role of cytosolic pH in regulation and light-activation of PEPC is further discussed in the following pages.

Temperature

Temperature regulates the activity of higher plant PEPC. There is a decrease in the apparent K_M (PEP) of the C_4-isoform with a rise in temperature, but this is offset by an increase in V_{max}. At lower temperature (for example, 2°C), the enzyme is stable only when assayed at suboptimal pH (<7). But at higher pH, cold inactivation was observed in *Cynodon dactylon, Atriplex halinus* and *Zea mays* (Angelopoulos *et al.* 1990).

The effect of temperature on the oligomeric status of PEPC *in vitro* is ambiguous. Temperatures above 25°C cause either aggreagation (in the case of C_4) or dissociation (in the case of CAM) of the enzyme (Wu and Wedding 1987). Inactivation due to cold/chilling may result from the deoligomerization of an active tetrameric form of PEPC into inactive (or less active) dimers or monomers (Walker *et al.* 1986, Stamatakis *et al.* 1988, Willeford *et al.* 1990).

The cold lability of the enzyme also depends on the source. Krall and Edwards (1993) showed that the enzyme from *Panicum miliaceum* was very stable under low temperature, whereas the enzyme from *P. maximum* lost 50% of its activity when incubated at 0°C for 60 min. Unlike pyruvate, orthophosphate dikinase (PPDK) from maize, which was stabilized against cold-lability by a number of inorganic ions (Krall *et al.* 1989), PEPC from leaves of *P. miliaceum* and *P. maximum* was

unaffected by the presence or absence of cations (for example, Mg^{2+} or Mn^{2+}) at moderate temperatures (Krall and Edwards 1993).

Both light and dark forms of the enzyme from *P. maximum* are equally susceptible to cold inactivation, suggesting that the cold lability of the enzyme, at least in this species, is independent of its phosphorylation state (Krall and Edwards 1993). Compatible solutes (like proline and betaine) protect PEPC from *P. maximum* against cold inactivation, possibly by increasing the enzyme concentration and favouring the equilibrium towards the tetrameric form (Krall and Edwards 1993).

Temperature may cause conformational/aggregational changes in the C4-PEPC of certain species, but the relevance of these results to all C4 species is doubtful in view of the limited literature (Chardot and Wedding 1992b).

Salt/water stress and nitrogen nutrition

Water stress, salinity and nitrogen nutrition play an important role in modulating the expression and regulation of PEPC in higher plants.

When plants of *Cynodon dactylon* and *Sporobolus pungens* (C4 species of family Poaceae) were exposed to salt stress, the affinity of the enzyme for PEP increased (Manetas *et al.* 1986). The two most common osmoprotectants, proline and betaine, which accumulate under water stress in many of the plants, stimulate the activity of PEPC. In *C. dactylon,* PEPC was activated by betaine while proline acted as a competitive inhibitor. However, the effects of these osmolytes are manifested, *in vitro,* only at low levels of PEP (Manetas *et al.* 1986).

However, the effects of salinity (salt stress) on C4-PEPC are not as dramatic as in facultative CAM plants like ice plant (*Mesembryanthemum crystallinum* L.), a C3-

CAM intermediate, where the transition from C_3 to CAM is accompanied by up to a 50-fold increase in PEPC activity, mainly due to enhanced transcription of the PEPC gene (McElwain *et al.* 1992).

Under conditions of high water stress, rapid wilting of excised leaves occurs in *M. crystallinum.* Limited water loss, of even 5%, causes a strong induction of PEPC mRNA transcripts (Pipenbrock and Schmitt 1991). This is much less than the 15% water loss that occurs daily from intact, well-watered plants during the light period (Winter and Gademann 1991). Even well-watered plants may therefore exhibit marked induction of *ppc* transcript should undergo PEPC transcript induction during the day and these trasncripts are reduced during rehydration at night (Vernon *et al.* 1989, Pipenbrock and Schmitt 1991, Winter and Gademann 1991). In addition to water stress, other control signals, emanating from the root, modulate PEPC gene expression in ice plant leaf-PEPC levels. For example, the bulk of cytokinin, which is thought to be the putative root-derived signal (Davies and Zhang 1991), is synthesized in the roots. Studies of Pipenbrock and Schmitt (1992) showed cytokinin as the possible regulator of PEPC mRNA transcription during water stress. Exogenous cytokinin suppresses PEPC transcript induction in detached, wilting leaves.

Water stress increases the level of abscisic acid (ABA) in cells. There are reports that exogenous application of ABA to leaves enhances the activity of PEPC (Chu *et al.* 1990). Induction of *ppc* transcripts during water stress is also reported in *Sedum acre*, *S. telephium* and *Portulacaria afra* (Groenhof *et al.* 1988, 1989, Borland and Griffiths 1992). Thus the induction of *ppc* transcripts during water stress in CAM plants is also under the control of ABA and cytokinin.

Nitrogen regulates markedly the biosynthesis of PEPC in leaves of from C3, C_4 and CAM plants (Champigny and Foyer 1992, Foyer *et al.* 1994, Gadal *et al.* 1995, Lara *et al.* 1995). Sugiharto *et al.* (1990, 1992b) observed a selective accumulation of PEPC protein in N-starved leaves of maize, when supplied with NO_3^- or glutamine (Gln). The rise in the level of PEPC mRNA and protein was more pronounced in maize plants supplemented with NH_4^+ or Gln than those with NO_3 (Sugiharto *et al.* 1990, 1992a, b, Sugiharto and Sugiyama 1992). The induction of particularly the C_4-isoform of PEPC by NO_3^-, Gln or NH_4^+ corresponds well with the high efficiency of these plants to use N, which channel most of the leaf N into PEPC (Hatch 1987, Raghavendra and Das 1993). In contrast, Rubisco, which comprises up to 50% of leaf soluble protein in C_3 plants, accounts for only 10 to 20% of N in C_4 plants. Nitrate could therefore function as a signal metabolite activating the cytosolic protein kinase, thereby modulating the activity of PEPC, in wheat (Champigny and Foyer 1992).

pH

Being a cytosolic enzyme the activity of PEPC depends naturally on cytosolic pH (Rajagopalan *et al.* 1993,1994). At a pH of around 8, the enzyme is very active, when the pH becomes acidic, the enzyme becomes inactive, slowing down the rate of carboxylation (Davies 1986, Rajagopalan *et al.* 1993, 1994). At a pH >7, there is an increase in the affinity of the enzyme for PEP and Mg^{2+} and a decrease in the inhibition by L- malate. L- malate inhibition is competitive at pH 8, but non-competitive at pH 7 (Gonzalez *et al.* 1984, Andreo *et al.* 1987).

The regulation of intracellular pH in plant cells has attracted interest during the last few years as reliable methods to measure cytosolic and vacuolar pH have been developed (Guern *et al.* 1991). Several metabolic activities of plant cells lead to

significant production or consumption of H^+ (Raven 1985, 1995, Kurkdjian and Guern 1989, Guern *et al*. 1991).

Recent data favours the view that changes in cytoplasmic pH can strongly influence activity of PEPC *in vivo* (Pierre *et al.* 1992, Rajagopalan *et al.* 1993, Echevarria *et al.* 1994, Giglioli-Guivarc'h *et al.* 1995, 1996, Pacquit *et al.* 1995, Duff *et al.* 1996).

In mesophyll cells of C_4 plants, there is a marked cytosolic alkalinization upon illumination (Yin *et al.* 1990, 1993, 1996, Raghavendra *et al.* 1993). The changes in pH could modulate the intracellular ions (for example, Ca^{2+}; Ward *et al.* 1995). Nevertheless, changes in pH may modulate the catalytic activity of PEPC either directly or indirectly through regulation of PEPC-protein kinase (PEPC-PK) or PEPC-protein phosphatase (PEPC-PP) or both (Rajagopalan *et al.* 1993, 1994, Wang and Chollet 1993).

However, most of the information gathered on the pH sensitivity of PEPC comes from experiments *in vitro.* The pH sensitivity is dependent on the conditions of the assay (O'Leary 1982). For example, the pH sensitivity of PEPC is relatively high when the enzyme activity is limited by PEP and inhibited by L- malate. It is essential to reexamine the pH sensitivity of PEPC under limiting conditions.

Guern *et al.* (1983) while reviewing the role of intracellular pH on the enzyme suggested that PEPC activity measured *in vivo* is much lower than that measured *in vitro.* This suggests that PEPC activity is limited *in vivo,* the limitation being much higher than that observed *in vitro.* The activity of PEPC may be limited by the presence of feedback inhibitors such as L- malate. Further, the pH sensitivity of PEPC is usually estimated from experiments the pH of medium is modified, while other

conditions of the assay are kept constant. This is likely to be not a true representation of the *in vivo* situation, as concentrations of H^+, Mg^{2+} (and other ions), L- malate, Glc-6-P, and other known effectors of PEPC could vary simultaneously. Thus, PEPC could be sensitized to small pH modifications by variation of the concentration of other effectors. Our knowledge of the pH sensitivity of PEPC is therefore, not precise enough and needs to be reevaluated, particularly under *in vivo* conditions and the presence of other effectors.

Metabolic effectors: Activators/inhibitors

The activity of PEPC is subjected to feedback inhibition by dicarboxylic acids such as OAA, L-malate, *etc.* (O'Leary 1982, Andreo *et al.* 1987). The pattern and extent of L-malate inhibition, however, depends on (i) phosphorylation status of the enzyme, (ii) assay conditions, and (iii) intactness of N-terminal (McNaughton *et al.* 1989, 1991, Jiao and Chollet 1991, Ausenhus and O'Leary 1992, Wang *et al.* 1992). In addition to several metabolic inhibitors, organic acids and analogues of PEP/pyruvate are powerful inhibitors of the enzyme. These analaogues of PEP are employed also to analyze the reaction mechanism of the enzyme (Gonzalez *et al.* 1987, Gonzalez and Andreo 1989, Janc *et al.* 1992, Chollet *et al.* 1996). Another selective inhibitor of PEPC, 3,3-dichloro-2-dihydroxyphosphinoyl-methyl-2-propenoate (DCDP) (Jenkins *et al.* 1987), was used to evaluate the extent of the C_4 pathway in C3-C4 intermediates (Brown *et al.* 1991) and submerged aquatic macrophyte *Hydrilla verticillata* (Spencer *et al.* 1996). Chemical-modifying agents like pyridoxal phosphate are used to probe the active/catalytic site of the enzyme (Jiao *et al.* 1990). Other inhibitors of PEPC are listed in Table 1.2.

Table 1.2. Inhibition of C_4 PEPC activity by different metabolites/chemicals. Within each group, the compounds are arranged in increasing order of their inhibition constant (K_I). Adapted from Rajagopalan *et al.* (1994).

Inhibitor/metabolite	$K_{I\ (mM)}$
End products	
L-Malate	0.02
Oxalacetate	1.0
PEP analogues	
1-Hydroxycyclopropane carboxylic acid phosphate	0.007
Phospho*enol*-3-bromopyruvate	0.007
Z-methylphspho*enol*pyruvate	0.017
DCDP	0.035
L-phospholactate	0.1
E-Methylphospho*enol* pyruvate	0.1
Dimethyl phospho*enol* pyruvate	0.4
Phospho*enol* pyruvate phosphonate	0.4
E-3-Cyanophosho*enol* pyruvate	1.4
Phosphonolactate	2.0
Phospho*enol* thiopyruvate	2.0
3-bromopyruvate	2.3
Phosphomalate	2.7
Phosphoglycolate	2.9
Phosphonopropionate	10.0
Amino acids/ organic acids	
Epoxymaleate	0.04
Aspartate	0.9
α-hydroxypridinemethanesulfonate	1.0
Malonate	2.5
Oxalate	23.5
Bicarbonate analogues/ inorganic compounds	
Inorganic phosphate	5.0
Sulphite[a]	5.0
Bisulfite	5.0
Formate[a]	25.0
Miscellaneous	
Glyphosine (N,N-bis[phopshomethylglycine]	**2.5**

[a]Competitive with bicarbonate.

PEPC from root nodules is also inhibited by L-malate and phosphorylation protects the enzyme from such inhibition in a way similar to the enzyme from C_4 or CAM plants (Jiao and Chollet 1991, Schuller and Werner 1993, Zhang *et al.* 1995).

The most widely used allosteric activator of PEPC is Glc-6-P (Andreo *et al.* 1987, Rajagopalan *et al.* 1994). PEPC is activated by many other phosphate esters (Rajagoplan *et al.* 1994). Some of these phosphate-esters are dephosphorylated, due to phosphatase reaction of the enzyme. Walker *et al.* (1988) reported that such phosphatase activity may be mechanically related to the activation process. However, a recent examination of maize PEPC indicated that the activation of the enzyme occurred without dephosphorylation of activator molecule (Bandarian *et al.* 1992).

Activation of PEPC by glycine has so far been observed with enzyme from C4 monocots (Nishikido and Takanashi 1973, Doncaster and Leegood 1987, Bandarian *et al.* 1992, Gillinta and Grover 1995, Gao and Woo 1996). This observation correlates well with the structural information on PEPC which suggests that C4 enzyme of monocot (for example, maize, sorghum) evolved separately from that of other C4 dicots, CAM and C_3 plants (Lepiniec *et al.* 1993, 1994, Chollet *et al.* 1996).

The activity of nodule PEPC is also regulated by Glc-6-P and Fru-6-P. These metabolites broaden the pH optimum thereby moderating the effect of small fluctuations in intracellular pH on PEPC activity *in vivo.* The effectors stimulate PEPC activity at physiological PEP concentration and protect the enzyme against inhibition by L-malate and other dicarboxylates (for example, aspartate, succinate, 2-oxoglutarate, malonate) (Woo and Xu 1996).

Posttranslational regulation

Regulatory phosphorylation is an important mode of post-translational regulation of PEPC, besides the allosteric control of enzyme activity (Jiao and Chollet 1991, Nimmo 1993, Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

Two types of posttranslational modification of enzymes are known. One is regulatory phosphorylation mediated by protein kinases (MacDonald and Buchanan 1990, Macintosh and Macintosh 1993, Nimmo 1993, Huber *et al.* 1994), while the other is a change in the oligomeric state of the enzyme induced *in vitro* (Frieden *et al.* 1987, Kruger and Kluge 1987, MacDonald and Buchanan 1990). Both processes are reversible. The change in properties of C4-PEPC due to phosphorylation/ dephosphorylation are well-acknowledged (Jiao and Chollet 1991, Rajagopalan *et al.* 1994, Chollet *et al.* 1996), but the physiological significance of association/ dissociation of the enzyme is yet to be established (Chollet *et al.* 1996).

The regulatory phosphorylation of photosynthetic PEPC has been studied extensively and reviewed frequently (Jiao and Chollet 1991, Nimmo 1993, Huber *et al.* 1994, Lepiniec *et al.* 1994, Rajagopalan *et al.* 1994, Chollet *et al.* 1996). A protein kinase catalyzes the phosphorylation of PEPC, while a type 2A protein phosphatase dephosphorylates the enzyme. Upon phosphorylation, there is a marked decrease in the sensitivity of the enzyme to L-malate with only minor changes in V_{max}. These changes in regulatory properties of the enzyme are reversed by dephosphorylation. Both phosphorylation and dephosphorylation of PEPC have been demonstrated *in vivo* as well as *in vitro* (Jiao and Chollet 1991, Nimmo 1993, Rajagopalan *et al.* 1994).

The CAM-PEPC is upregulated at night and down-regulated during day, thereby reflecting the classical diurnal changes in the pattern of CO_2 assimilation in

CAM plants (Nimmo *et al.* 1987, Carter *et al.* 1991, Cook *et al.* 1995). Studies on CAM plants under continuous night or day conditions indicated that CAM physiology, as well as the L-malate sensitivity of PEPC, was controlled by an endogenous circadian rhythm rather than by light or dark signal *per se* (Nimmo *et al.* 1987, Griffiths 1988). In marked contrast, C_4 PEPC is reversibly light activated *in vivo* principally by light-dependent synthesis and activation of PEPC-PK (Jiao *et al.* 1991, Wang and Chollet 1993a, Li and Chollet 1994).

Phosphorylation of PEPC occurs at a single serine residue near the N-terminus. This is further precisely identified as Ser^{8}, Ser^{11}, Ser^{15} in *Sorghum*, *Flaveria trinervia*, and maize C_4 enzymes, respectively, and Ser^{11} in PEPC from the facultative CAM plant *Mesembryanhemum crystallinum* (Rajagopalan *et al.* 1994). This target Ser-residue, (Lys/Arg-X-X-Ser-) typical of higher plant PEPC is absent in bacterial and cyanobacterial primary structure deduced so far (Lepiniec *et al.* 1993, 1994, Toh *et al.* 1993, Relle and Wild 1994, Nakamura *et al.* 1995). The phosphorylation site is only two amino acid residues removed from a basic amino acid group (Lys-12 in the case of maize PEPC) in the primary sequence, a feature similar to that of various protein serine/threonine kinase substrates (Fallon and Trewavas 1993, Macintosh and Macintosh 1993, Hunter 1995). Thus, a specific phosphrylation site is accessible to both homologous and heterologous protein kinases, for example, for mammalian protein-kinase A (Jiao and Chollet 1990, Terada *et al.* 1990).

The molecular mechanism by which protein phosphorylation regulates C_4-PEPC has been addressed by site-directed mutagenesis and chemical modification. The introduction of a monomeric aspartate residue at position 8 in the *Sorghum* PEPC

by site-directed mutagenesis (S8D) or sequential mutagenesis (S8C) and *S*-carboxymethylation functionally mimics the effects of regulatory phosphorylation on the target enzyme. In contrast, various substitutions (S8T, S8Y, S8C, *S*-carboxymethylated S8C) are without any major influence (Wang *et al.* 1992, Duff *et al.* 1993, 1995). Consequently, addition of negative charge to this N-terminal domain by reversible phosphorylation appears to be the base of PEPC-regulation.

The extremely low abundance PEPC-PK has been partially purified 4000-fold. It is likely to be a monomer of about 37/30 kD (maize: Li and Chollet 1993, tobacco: Wang and Chollet 1993b) or about 39/32 kD (CAM; Li and Chollet 1994) polypeptide. The partially purified PEPC-PK is not able to perform either autophosphorylation or the phosphorylation of heterologous substrates (for example, casein, histone III-S, bovine serum albumin, leaf sucrose-phosphate synthase [SPS]). Similarly, position-8 *Sorghum* C_4-PEPC mutants (S8Y, S8D, S8C) are not phosphorylated except for threonine substitution (Wang *et al.* 1992, Wang and Chollet 1993a, Li and Chollet 1994). In contrast, all PEPC isoforms examined so far (C_4, CAM, C_3-leaf, root nodule) serve as substrates *in vitro* (Wang and Chollet 1993a, 1993b, Li and Chollet 1994, Zhang *et al.* 1995), but with a distinct preference for their corresponding PEPC-PKs (Chollet *et al.* 1996).

PEPC-PK in C_4 leaves is activated by light and inactivated in the dark (Echevarria *et al.* 1990, Jiao *et al.* 1991a, McNaughton *et al.* 1991, Bakrim *et al.* 1992, Wang and Chollet 1993b). The activity of PEPC-PK was pH-dependent in maize (Wang and Chollet 1993b) and ice plant (Li and Chollet 1994), the optimal pH being 7.8 to 8. Inhibition by cycloheximide, a cytosolic protein synthesis inhibitor, of light-activation of PEPC indicates that the biosynthesis of C4-PEPC-PK (or some related

regulatory protein) is necessary for the light-activation/seryl-phosphorylation of PEPC in C_4 plants (Jiao and Chollet 1991, Bakrim *et al.* 1992, Rajagopalan *et al.* 1994).

The activity of PEPC-PK was Ca^{2+}-independent and was not affected by Ca^{2+}/calmodulin or other possible cytoplasmic compounds like Fru-1,6-*bis*P, pyrophospshate (PPi) and thioredoxin *h* (Jiao and Chollet 1991, Wang and Chollet 1993a). However, Ogawa *et al.* (1992) and Izui *et al.* (1995) while reporting the occurrence of a Ca^{2+}-dependent PEPC-PK, suggested that there may be multiple forms of protein kinases in cytosol of maize leaves. Two protein-serine kinases, CK and BK, capable of *in vitro* phosphorylation of PEPC have been partially purified from light-adapted *Sorghum* leaves (Vidal *et al.* 1990, Bakrim *et al.* 1992). The CK protein kinase is Ca^{2+}-dependent but does not require calmodulin for its activity, while the BK protein kinase requires Mg^{2+} for activity and is proposed to be the one involved in phosphorylation *in vivo* since it decreases the extent of L-malate inhibition in a reconstituted phosphorylation system. Further detailed studies are necessary to elucidate the regulation of PEPC-PK, particularly in relation to cytosolic Ca^{2+} and pH, in leaves of C_4 plants.

The phosphorylated PEPC is dephosphorylated by a protein phosphatase of type 2A (Carter *et al.* 1990, McNaughton *et al.* 1991). The role of type 2A protein phosphatase has been reviewed recently (Chen *et al.* 1992, Cicirelli 1992, Hubbard and Cohen 1993, Macintosh and Macintosh 1994, Mayer-Jackel and Hemmings 1994, Smith *et al.* 1995, Smith and Walker 1996). However, the available information on the properties of PEPC-PP from leaves of C_4 plants is scant. Incubation of phosphorylated form of CAM-PEPC with purified rabbit PP 2A caused a drastic decrease in the apparent K_I (malate) of the enzyme from 3 to 4 mM to 0.05 to 1.0 mM

(Carter *et al.* 1990). Similarly, the treatment of light-form of C_4 PEPC with alkaline phosphatase (Jiao and Chollet 1988, Arrio-Dupont *et al.* 1992) increased L-malate of PEPC (at pH 7.3), to a level similar to that observed for the dark-adapted form of PEPC. Presence of 10 to 25 nM of okadaic acid, a classic inhibitor of type 2A phosphatase, conserves the phosphorylation status of the enzyme in CAM plants (Carter *et al.* 1990). Similar results were also obtained by using other inhibitors of type PP 2A, for example, microcystin-LR, nodularin, tautomycin, cantharidin (Jiao *et al.* 1991a, Smith and Walker 1996).

Dlumination did not bring about any change in the activity of the PP 2A in maize (McNaughton *et al.* 1991). However, the possibility that a short-term control of PEPC-PP could regulate the phosphorylation status of PEPC is not ruled out completely (Carter *et al.* 1991). The balance between the activities of PPEC-PK and PEPC-PP may determine the phosphorylation state of PEPC and its sensitivity to L-malate (Rajagopalan *et al.* 1994).

The studies on leaf CO_2-exchange have highlighted the impact of the PEPC-regulatory phosphorylation cycle on overall carbon fixation during C_4 photosynthesis or CAM (Carter *et al* 1991, 1995, Bakrim *et al.* 1993). For example, when the activity states of PEPC-PK and, thus PEPC, were down regulated *in vivo* by short-term pretreatment with cytosolic protein synthesis inhibitors in the light (C_4) or prior to the night period (CAM), net leaf CO_2 uptake was diminished markedly. In contrast, no effects were observed on the activation state of other nuclear-encoded, photosynthesis-related enzymes, stomatal conductance, or CO2 uptake by a C3 leaf (Jiao *et* al. 1991a, Bakrim *et al.* 1992, 1993). Thus, the phosphorylation of photosynthetic PEPC is a cardinal regulatory event that influences the primary

Carboxylase to function in the leaf cytosol even in the presence of millimolar levels of C_4 acids (for example, L-malate) required for C4 photosynthesis and CAM.

Oligomerization

The active form of PEPC is a homotetramer (Andreo *et al.* 1987, Rajagopalan *et al.* 1994). The enzyme can exist also as a dimer or monomer depending on pH, ionic strength (Walker *et al.* 1986, Wagner *et al.* 1987), temperature (Wu and Wedding 1987) and PEPC concentration (Willeford and Wedding 1992).

Enzyme dissociation is promoted by acidic pH (<7), NaCl, absence of dithiol-reductants or high temperature. At the optimal pH of 8, the enzyme existed in tetrameric form and its response to PEP was hyperbolic. The *in vitro* dissociation of the enzyme induces sigmoid kinetics of PEP binding and increases K_M (PEP) (Wagner *et al.* 1987). In the presence of NaCl, the C_4 enzyme dissociates into dimers (at pH 7) but the presence of PEP, Mg^{2+} or Glc6P can prevent this dissociation. The dark and light forms of C4-PEPC show different degree of sensitivity to NaCl (Rajagopalan *et al.* 1994). Ligands (for example, Glc-6-P or L-malate) can alter the aggregation state of the enzyme, thereby changing its activity (Willeford *et al.* 1990, Willeford and Wedding 1992). Glycerol or high protein concentration shifted the equilibrium to the larger and active tetrameric form (Podesta and Andreo 1989).

The significance of the oligomeric transition of PEPC during light-activation/dark-deactivation is debatable since oligomerization/deoligomerization have been observed only *in vitro* (Kruger and Kluge 1987, Wu and Wedding 1987, 1992, Grammatikopoulos and Manetas 1990, Meyer *et al.* 1991). There is no convincing evidence to support the involvement of these aggregation-state changes in the diurnal regulation of the CAM- and C4-PEPC isoforms *in vivo* (Rajagopalan *et al.*

1994, Chollet *et al.* 1996). Reports by McNaughton *et al.* (1989) and Weigend and Hincha (1992) suggest that the reversible oligomeric conversion of PEPC may not be a mechanism that regulates the light/dark transition of PEPC in higher plants. When extracted, PEPC existed in the form of tetramer and dimer in the leaves of maize and *Crassula argentea,* respectively, and malate sensitivity of the enzyme was not correlated to the oligomeric state (Weigend and Hincha 1992).

The riddle of enzyme oligomerization can not be resolved easily due to experimental/technical limitations on such experiments (Rajagopalan *et al.* 1994). Ottaway (1988) reported that NAD-reductase and mammalian phosphofructokinase (PFK) are inactivated by oligomerization regulated by ligand binding, while enzymes such as pyruvate Carboxylase and threonine deaminase undergo irreversible inactivation on dilution. Similarly, dilution of PEPC can affect the equilibrium of dimer/tetramer state and thereby the enzyme's activity *in vitro* (Wu *et al.* 1990). A way to overcome this problem is to use compatible solvents to keep enzyme dilution to minimum (Krall and Edwards 1993).

Further, the dark- and light-forms of C4-PEPC may change their oligomeric status in response to ionic strength or pH of the medium. For example, NaCl induces dissociation of dark-form of PEPC into dimers (McNaughton *et al.* 1989), while having no effect on disaggregation of the light-form (Arrio-Dupont *et al.* 1992). Wu and Wedding (1992) reported that the interaction of PEPC with chloroplast membrane *in vitro* inactivates the enzyme by promoting dissociation of the active tetramer. Protein phosphorylation can induce oligomeric interconversion of enzymes (Frieden 1989). It is therefore possible that phosphorylation and oligomerization of PEPC

interact with each other, but this phenomenon is yet to be evaluated critically and remains speculative at present.

Structure and expression of PEPC gene (ppc)

The nature of *ppc* gene had been studied extensively in several plants: C_4 species (maize, sorghum, ***Flaveria trinervia***), C3 plants (tobacco, soybean, potato, *Flaveria pringlei),* a facultative CAM species (***Mesembryanthemum crystallinum***) and prokaryotes *(E. coli*, *Anacystis nidulans*, *Anabaena variabilis, Corynebacterium glutamicum*). The structure and interrelationships of *ppc* genes are reviewed in Lepiniec *et al.* (1994), Rajagopalan *et al.* (1994) and Toh *et al.* (1994).

Consistent with enzyme's functional diversity, small multigene families code for *ppc.* For example, three *ppc* nuclear genes - *SvC3, SvC3RI,* and *SvC4* - have been characterized in *Sorghum* that encode the C_3-like housekeeping and root forms and the C_4 photosynthetic isoform, respectively (Lepiniec 1993, Lepiniec *et al.* 1993, 1994). The maize family consists of at least five genes (Grula and Hudspeth 1987), which can be classified into three distinct groups (Schaffner and Sheen 1992). The C4 *ppc* is unique and is located near the centromere of chromosome 9, while three other genes have been mapped to different loci on chromosomes 4L, 5, and 7 (Grula and Hudspeth 1987, Izui *et al.* 1992, Kawamura *et al.* 1992). Both C_3 and C_4 species in the dicot genus *Flaveria* contain very similar families of distinct *ppc* subgroups (Poetsch *et al.* 1991, Hermans and Westhoff 1992). The C_4 isoform in *Flaveria trinervia* is encoded by the *ppcA* subgroup of the family. Homologous *ppcA* genes are identified in the C_3 species, *F. pringlei,* which are weakly expressed, and their transcripts do not exhibit the strict leaf-specific accumulation as in the related C4 species (Hermans and Westhoff 1992).

In the facultative CAM plant *Mesembryanthemumcrystallinum*, two isogenes - *ppcJ, ppc2* - have been described (Cushman *et al.* 1989, 1993), and another distinct member might exist; the transcriptional activity of *Ppc1* is strongly and selectively enhanced during C_3 to CAM transition induced by salt stress (Cushman *et al.* 1989). The *Brassica napus* (C3 species) genome contains more than four highly similar *ppc* genes, but some of them lack specific introns (Yanai *et al.* 1994). *Ppc* gene families have been either found or suggested to exist in sugarcane, *Amaranthus*, tobacco, alfalfa, rice, wheat (Lepiniec *et al.* 1994), and *Arabidopsis* (Newman *et al.* 1994).

The plant *ppc* contains nine introns (except in *Brassica napus,* Yanai *et al.* 1994) of variable length but with identical location with respect to the coding regions. Consensus intron/exon splice sites (aG*GT*aag-tgc*AG*g) are conserved. Generally, a classical gene organization is observed, although in some C4- and C_3-type *ppc* there is no typical TATA box, and multiple polyadenylation sites are found in the 3'-untranslated region (Lepiniec *et al.* 1994, Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

Short repetitive 5'-flanking sequences are characteristic of the light-regulated genes such as the one encoding the small subunit of Rubisco *(rbcS).* The *ppc* contains several such sequences. The maize nuclear factor (MNF) and *SV40* Sp1 binding sites - (AAGG) and (CCGCCC), respectively - are also found in C4 *ppc*-promoters. In addition, the presence of CpG islands is consistent with the possible regulation of specific sites in the promoters of both C4 and C3 *ppc* by changes in DNA methylation status (Lepiniec *et al.* 1993, 1994, Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

Differential expression

C_4 photosynthesis results from the integrated metabolic activities of two distinct cell types: mesophyll and bundle sheath cells. The specialization of mesophyll and bundle sheath cells required for C4 photosynthesis is achieved by selective cell/tissue specific expression of genes encoding typical C4 pathway enzymes through transcriptional/translational regulation. In mature leaves of C4 plants, PEPC protein and its transcript are expressed in only the mesophyll cells (Hatch 1987, Nelson and Langdale 1992, Langdale *et al.* 1995, Ku *et al.* 1996).

The C_4-form of PEPC protein is located in mesophyll cells of C4 plants and is formed only on illumination of seedlings.. Thus, the expression of *ppc* depends not only on the organ/cell, developmental stage of the leaf, but also illumination. The differential expression of *ppc* is proposed to be due to specialized nuclear factors, methylation of DNA, and the presence of distinct promoters (Sheen and Bogorad 1987, Kano-Murakami and Matsuoka 1992, Schaffner and Sheen 1992).

One mode of tissue specific gene expression is through transcriptional regulation by nuclear regulator factors. Three kinds of nuclear factors - MNF1, MNF2a, and MNF2b) - interact with the 5'-flanking promoter region of the maize *ppc* and regulate the expression (Yanagisawa and Izui 1990, 1992). Another mode for selective expression of *ppc* only mesophyll is DNA methylation (Ngernprasirtsiri *et al.* 1989). DNA methylation at the promoter region prevents the binding of nuclear activator proteins, restricting gene expression (Iguchi-Araga and Schaffner 1989). Methylation of *ppc* occurred in the bundle sheath - particularly at the final stages of tissue differentiation - but not in the mesophyll (Yanagisawa and Izui 1990).

Schaffner and Sheen (1992) suggests that during the evolution of C_4 photosynthesis, the *ppc* achieves a specific expression pattern by the acquisition of unique promoter and TATA box-like TAT TT sequences probably by genomic rearrangement. Transient expression studies showed that the 5'-flanking sequences were able to modulate the cell-specific expression of the *ppc* in C4 plants (Schaffner and Sheen 1992).

The increase in PEPC activity during greening or leaf development in C4 plants is due to the synthesis/accumulation of both mRNA and protein (Sheen and Bogorad 1987). Phytochrome might regulate C_4 *ppc* expression during development in *Sorghum* (Thomas *et al.* 1987, 1990). The involvement of another photoreceptor besides phytochrome is possible (Gamble and Mullet 1989), but is not yet demonstrated.

Genetic engineering and transgenic plants

Along with the characterization of the cDNA encoding the C4-type PEPC, the gene was cloned and expressed in bacteria *(E. coli)* as well as in C3 plants (Rajagopalan *et al.* 1994, Chollet *et al*. 1996). In transgenic tobacco transformed with maize C_4-*ppc1* containing the upstream regulatory region (about 2 kb), a low level of PEPC transcripts was produced; although their size was abnormally large, accumulation still required light (Hudspeth *et al.* 1992). These transformants possessed a two-fold increase in PEPC activity that was correlated with the appearance of a high K_M (PEP) for C4 form of the enzyme and an elevated level of malate. However, these biochemical changes did not result in any detectable physiological effects with respect to the rate of leaf net photosynthesis in air and to the CO_2 compensation concentration.

In a related study, the maize C_4 *ppc* was placed under the control of a CaMV 35S promoter (Kogami *et al.* 1994). Although the transgenic tobacco plants contain *ppc* transcripts of the correct size and above twice as much PEPC protein, their growth rate was retarded to that of the non-transformed plants. Transgenic tobacco plants transformed with either the C_4 *ppc* from *Sorghum* or chimeric constructs containing the promoter of the C4 gene from maize fused to the *gusA* reporter gene showed high expression of transcripts as well as leaf mesophyll-cell specificity (Matsuoka and Sanada 1991, Tagu *et al.* 1991). Similar results have been reported recently (Matsuoka *et al.* 1994) in transgenic rice using the above experimental strategy.

Molecular evolution of PEPC

The operation of C_4 photosynthesis requires the strict compartmentation of relevant enzymes in either mesophyll or bundle sheath cells. Such intercellular is a distinguishing feature of C4 plants: several of key enzymes utilized in C4 photosynthesis are present in leaves of also C3 plants. However, their expression patterns are distinct from those in C4 plants. During the evolution of C4 photosynthesis, the expression of some of the proteins is modified to provide the level and cell specificity required for efficient functioning of the CC>2-concentrating mechanism leading to C_4 pathway (Ehleringer *et al.* 1991, Berry 1993, Raghavendra and Das 1993, Apel 1994)..

Phylogenetic trees have been constructed using unambiguously aligned sites from the PEPC amino acid sequences as well as on the basis of parsimony or distance analyses (Albert *et al.* 1992, Hermans and Westhoff 1992, Izui *et al* 1992, Lepiniec 1993, Lepiniec *et al.* 1993, 1994, Toh *et al.* 1994). Two different hypotheses were

proposed regarding the origin of C_4-PEPC in *Sorghum* and maize by Lepiniec *et al.* (1993, 1994).

First assuming the validity of the deduced phylogenetic trees, C4-PEPC could have araised from a duplication event that occurred before monocot and dicot plants were diverged. As a result, a homologous protein should be present. To our knowledge, no molecular data have verified this hypothesis till today. The only explanation could be a loss of the corresponding gene in all dicot lineages after the monocot emergence.

The second hypothesis assumes that the position of the *Sorghum* and maize C4-PEPC is not correct in the phylogenetic tree. Indeed, a general feature of the evolution of duplicated genes is an accumulation of amino acid substitutions relative to synonymous nucleotide changes. The C4-PEPC of *Sorghum* and maize could have simply resulted from a weak selective pressure. A faster evolutionary rate and a positive pressure towards XXC/G codon usage have been observed in the C_4-type *ppc* in *Sorghum* and maize (Lepiniec *et al.* 1993, 1994). Non-consistency of the evolutionary rate of one branch can hide true relationships in a evolutionary tree. Even maximum parsimony methods might be unrelative estimates of free topology under conditions of unequal rates of substitutions between lineages. The preexistence of the basic forms of PEPC regulation in the C3-enzyme, allosteric control by dicarboxylic acid (inhibition) and Glc-6-P and Fru-6-P (activation), N-terminal phosphrylation supports this hypothesis.

According to Lepiniec *et al.* (1993), C4-PEPCS from monocots *{Sorghum* and maize) are clearly distinguishable from the various C3 and CAM isoforms and also from their indigenous C_3-counterparts. In contrast, the photosynthetic enzyme in the

C_4-dicot *Flaveria trinervia* is more closely related to the various isoforms in C_3 and CAM dicots (Hermans and Westhoff 1992, Poetsch *et al.* 1991) than to the two monocot C4-PEPC. Further, because the promoters of the C4- *ppc* in *F. trinervia* and the orthologous gene in *F. pringlei* (C3) are very similar. It has therefore been suggested that a C3 promoter could have been 'turned' to meet the special demands of C_4 photosynthetic pathway (Hermans and Westhoff 1992).

Further investigations involving PEPC sequences from different genera are required to refine the phylogenetic relationships of the microbial and plant enzymes, including sequence analysis of PEPC from green algae and gymnosperm species *(Picea abies,* Relle and Wild 1994).

Areas for future research and scope of the present study

Areas for future research

While the past decade has presented considerable progress in the information on PEPC, further research is necessary in several emerging areas. Among the promising aspects are the possibilities in studying the three-dimensional structure of PEPC. In addition, several components of signal transduction during responses and regulation of both PEPC and PEPC-PK to factors, such as light or nitrogen, are to be identified. Further studies are needed to understand the properties and regulation of type 2A protein phosphatase that dephosphorylates plant PEPC in the cytosol. With the successful generation of the C_4-PEPC-deficient mutant in the dicot *Amaranthus edulis* (Dever *et al.* 1995) and the development of an efficient, *Agrobacterium*-mediated transformation system for C4-dicots (Chitty *et al.* 1994), an exciting stage is set for the genetic manipulation of C4 phosotynthesis *in vivo* by engineering the regulatory properties and the amount of PEPC in the leaf cytosol.

Site-directed mutagenesis has been useful to engineer/express PEPC in transgenic C_3 plants and *E. coli*, and to assess the role of important amino acid residues in structural/functional relationships of the enzyme. Further detailed analyses of cDNA and the PEPC from C3-C4 intermediate plant species would help to trace the evolution of the enzyme in different photosynthetic types (Lepiniec *et al.* 1993, 1994, Rajagopalan *et al.* 1994).

It is necessary to study further the mechanism of modulation of C4-PEPC by light or temperature and to identify the secondary messengers of these environmental signals (for example, cytosolic Ca^{2+} and pH). The role of Ca^{2+} should be reexamined in detail, in view of conflicting reports on Ca^{2+}-dependent PEPC-PKs in sorghum and maize leaves (Bakrim *et al.* 1992, Ogawa and Izui 1992, Ogawa *et al.* 1992, Izui *et al.* 1995) and the suppression of PEPC-phosphorylation *in situ* in Ca^{2+}-depleted *Sorghum* and *Digitaria sanguinalis* protoplasts (Pierre *et al.* 1992, Giglioli-Guivarc'h *et al.* 1995, 1996). Similarly the exact role of cytosolic pH should be identified (for example, Pierre *et al.* 1992, Rajagopalan *et al.* 1993, Giglioli-Guivarc'h *et al.* 1996). The interrelationship between oligomerization and phosphorylation status of PEPC should be evaluated *in vivo* as well as *in vitro* (Rajagopalan *et al.* 1994),

Another area of interest is the hormonal regulation of C_4-*ppc* expression. The available information indicates that PEPC is activated by ABA (Huber and Sankhla 1976, Schmitt and Pipenbrock 1992), but this should be reevaluated in view of the reports on regulation of *ppc* expression in CAM plants by ABA/cytokinin or salt/water stress (Schmitt and Pipenbrock 1992, Sugiharto *et al.* 1992a). Further research on these aspects of PEPC would improve our knowledge of this '*multifaceted*' enzyme in plants.

Scope of the present study

The major objective of the present work is to study and analyze the regulation of PEPC in leaves of C_4 plants with particular emphasis on light activation of the enzyme. The following aspects are examined:

1 Patterns of PEPC activity and cytosolic pH during light-activation and dark-deactivation in C3, C3-C4 intermediate and C_4 plant species

2 Acid-/base-modulation of PEPC in leaf discs of the three photosynthetic type plant species.

3 The assessment of indirect phosphorylation/dephosphorylation and phosphate sequestration on the activity and phosphorylation status of PEPC from *Alternanthera pungens* (NAD-ME type C4 dicot).

4 Effect of *in vivo* feeding of inorganic salts on the activity of leaf PEPC from *A. pungens* (C4) and *A. sessilis* (C3).

5 Buffering capacities of leaf extracts from C3, C3-C4 intermediate and C4 species compared to those of stem and root.

The results are discussed with reference to relevant literature.

Chapter Two

Materials and Methods

Plant Material

Plants of *Arachis hypogea* L. (cv. ICGS 44), *Amaranthus hypochondriacus* L. (cv. AG-67), *Lycopersicon esculentum* Mill, *Pisum sativum* L. (cv. Arkel), *Sorghum bicolor* (L.) Moench (cv. CS 591) and *Zea mays* L. (cv. Ganga-5) were raised from seeds. Four species of *Alternanthera* genus: *A. sessilis* (L.) R. Br. *ex* DC (C_3 species), *A. ficoides* (L.) R. Br. *ex* Roem & Schult), *A. tenella* Colla (C_3-C_4 intermediates) and *A. pungens* H.B.K (C_4 species) were propagated by transplantation of cuttings. Other plants (Table 2.1) were collected from University campus.

The plants were grown outdoors under a natural photoperiod of *ca.* 12 h and the average temperatures between 40 °/27 °C, day/night (in summer) and 28 °/13 °C, day/night (in winter). The plants were grown in 30-cm plastic tubs or earthen pots in soil supplemented with farm-yard manure (in a ratio of 1:3).

Preparation of Leaf Discs

Third to fifth leaves (counting from the fully developed youngest one) were excised under water from 3-to-4-week-old plants between 08 00 and 09 00 a.m. (about two to three h after sun-rise) and were used for the experiments. Immediately after harvesting, the leaves were thoroughly washed and discs of *ca.* 20 mm^2 each were punched (under water) using a sharp paper punch. The discs were floated (in darkness) on 20 mL of distilled water in 9 cm-diameter Petri dishes at 30 °C, for 2 h.

After completion of the pre-dark treatment, the leaf discs were illuminated (white light; Philips Comptalux R95 lamps; 4 x 150 W), at a photosynthetic photon flux density (PPFD) of 1000 $\mu mol\ m^{-2}\ s^{-1}$ for 20 min (unless otherwise mentioned).

Table 2.1. Plant species used in the present study.

Photosynthetic type/ species (and cultivar)	Family
C_3 species	
Alternanthera sessilis (L.) R. Br. *ex* DC	Amaranthaceae
Arachis hypogea L. (cv. ICGS 44)	Fabaceae
Commelina communis L.	Commelinaceae
Lycopersicon esculentum Mill	Solanaceae
Pisum sativum L. (cv. Arkel)	Fabaceae
Tagetes erecta L.	Asteraceae
Tridax procumbens L.	Asteraceae
C3-C4 **Intermediate Species**	
Alternanthera ficoides (L.) R. Br. *ex* Roem & Schult	Amaranthaceae
Alternanthera tenella Colla	Amaranthaceae
Mollugo nudicaulis L.	Aizoaceae
Parthenium hysterophorus L.	Asteraceae
C4 Species	
Alternanthera pungens H.B.K	Amaranthaceae
Amaranthus hypochondriacus L. (cv. AG-67)	Amaranthaceae
Amaranthus viridis Derf	Amaranthaceae
Gomphrena globosa L.	Amaranthaceae
Portulaca oleracea L.	Portulaceae
Sorghum bicolor (L.) Moench (cv. CS 591)	Poaceae
Zea mays L. (cv. Ganga 5)	Poaceae

A 15-cm water filter between the tungsten lamps and the Petri dishes helped to minimize and avoid any rise in temperature. Leaf discs left in darkness for the same duration were used as controls. Both pre-dark incubation and illumination of leaf discs were done in double distilled water (or test solution). Usually 20 leaf discs (*ca.* 80 mg), unless specified otherwise, were used in each experiment.

Preparation of Cell Sap and pH Measurement

The leaf discs (pre-darkened or illuminated) were ground with 4 vol of 0.3 M unbuffered sorbitol (pH 7.0, unless otherwise mentioned) in a prechilled mortar using a pestle. Grinding was done gently, so as to avoid the disruption of bundle sheath cells.

The homogenate was immediately filtered through four layers of cheesecloth and centrifuged at 15 000 *g* (Hermle Z 320 K Refrigerated Microcentrifuge, Germany) for one min, so as to remove the intact organelles and the debris. The supernatant (which represents cytosol pH) was used to measure the pH using a digital pH meter (Systronics Digital pH meter, Model 335) with a microelectrode. In some of the experiments, the same extract, prepared in unbuffered 0.3 M sorbitol, was used for both estimating cell sap pH and determining PEPC activity.

Acid-/Base-Loading of Leaf Discs

After 2-h preincubation in darkness, the leaf discs were blotted dry and transferred to Petri dishes containing 10 mL of test solution (weak acid or base in respective buffers) and left in darkness for 2 h. Leaf discs left in respective buffers alone for 2 h served as controls.

The test solutions (cytosolic pH modifying agents) were prepared in respective buffers: 5 mM Tricine-KOH, pH 8.5 for NH_4Cl, procaine; 5 mM sodium acetate buffer, pH 4.5 for propionic or other weak acids. The pH of the external medium, measured after the completion of the preincubation period, showed very little variation (≤7% from initial value).

Cycloheximide (CHX) Feeding

A stock solution of 2 mM CHX was prepared in 1 % (v/v) ethanol and diluted to 5 µM CHX with distilled water. The control solution was prepared by dilution of 1 % (v/v) ethanol to appropriate concentration (as used for final CHX concentration). Leaf discs were incubated in darkness in Petri dishes containing either 10 mL of diluted ethanol (control) or 5 µM CHX for 6 h at 30 ± 1 °C.

After the completion of the incubation period, the leaf discs were washed thoroughly with distilled water, then either left in darkness or illuminated for 30 min at 1000 µmol m^{-2} s^{-1}, and used for extraction/assay of PEPC.

Mannose Feeding

α-D-mannose (dissolved in 5 mM Hepes-KOH, pH 7.0) was fed to the leaf discs for 2 h in darkness. After completion of the incubation, leaf discs were prepared and either illuminated or kept in darkness for 30 min, and then the leaf extracts were prepared for PEPC assay, as described below.

Feeding of Inorganic Salts

A stock solution of 1 M of each inorganic salt (KNO_3, $NaNO_3$, NH_4NO_3, KCl, NaCl and K2SO4) was prepared and the pH was adjusted to 7. The salts (at the indicated concentrations) were fed to the excised leaves (after 2 h predark treatment) through transpirational stream for 2 h in darkness, unless otherwise specified. Twenty mL of each solution was used for feeding.

Extraction of PEPC

Twenty leaf discs *(ca.* 80 mg) were homogenized with 320 µL of prechilled extraction buffer containing 100 mM Hepes-KOH, pH 7.3, 10 mM $MgCl_2$, 2 mM K_2HPO_4, 5% (v/v) glycerol, 1 mM EDTA, 1 mM chymostatin, 2 mM PMSF, 2 mM benzamidine, 5 mM DTT, 10 mM sodium fluoride, 0.3 M sorbitol, 1% (w/v) BSA and 2% (w/v) insoluble PVP. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15 000 *g* at 4 °C for one min in a microcentrifuge. An aliquot was kept aside prior to centrifugation, for chlorophyll estimation. The supernatant was used for assaying the enzyme. The enzyme preparation was left at 30 °C during the experimentation.

Assay of PEPC

PEPC activity was measured spectrophotometrically by coupling the carboxylation reaction to NAD-malate dehydrogenase and monitoring P-NADH oxidation at 340 nm at 30 °C in a dual beam UV-vis spectrophotometer (Shimadzu UV-160 A or Hitachi 150-20, Japan). The assay medium (1 mL) contained : 50 mM Hepes-KOH, pH 7.8 (unless otherwise specified), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 4 U NAD-MDH, 0.25 mM NADH and crude leaf extract equivalent to 1 µg Chl.

The reaction was started by the addition of PEP to make a final concentration of 2.5 mM (unless otherwise mentioned) and the change in absorbance was monitored at 340 nm. PEPC activity was calculated by using the molar extinction coefficient of NADH (6.2 $mM^{-1} cm^{-1}$ at 340 nm).

Fresh stock solutions of PEP (50 mM) were prepared weekly in 50 mM Hepes-KOH buffer and pH of the solution was adjusted to the assay pH.

PEPC activity was expressed in µmol β-NADH oxidized mg^{-1} Chl h^{-1} at 30 °C.

Malate Sensitivity of PEPC

L-malate sensitivity of PEPC was assayed at suboptimal, but physiological, levels of PEP and pH. The assay medium (1 mL) consisted of: 50 mM Hepes-KOH (pH 7.3), 5 mM $MgCl_2$, 1 mM $NaHCO_3$, 4 U NAD-MDH, 0.25 mM NADH, 1.5 mM PEP (pH adjusted), enzyme extract equivalent to 1 µg Chl and with or without 1 mM L-malate (pH adjusted).

Glc-6-P Stimulation of PEPC

Stimulation by Glc-6-P of PEPC activity was estimated at pH 8.0 (50 mM Hepes-KOH) in presence of 1 mM $MgCl_2$, 2 U NAD-MDH, 0.25 mM NADH, 1 mM PEP, and with or without 5 mM Glc-6-P (pH adjusted).

Estimation of Apparent K_M (PEP), K_I (malate) and K_A(Glc-6-P)

The apparent K_M(PEP) was measured at pH 8.0, with 50 mM Tricine-KOH, 0.04 to 5 mM PEP, and 10 mM $MgCl_2$.

The K_I (malate) was estimated at pH 7.3 with 50 mM Hepes-KOH, 0.01 to 5 mM malate, 5 mM $MgCl_2$ and 1.5 mM PEP.

Activation constant for Glc-6-P was estimated at pH 8 with 50 mM Tricine-KOH, 0.01 to 5 mM Glc-6-P, 1 mM $MgCl_2$ and 1 mM PEP.

ATP-Dependent Phosphorylation of PEPC *In Vitro*

Crude extracts of PEPC were prepared from leaf discs of *Alternanthera pungens* after preincubation in various test solutions in darkness and/or illumination. Forty leaf discs were homogenized with a prechilled mortar and pestle using 650 µL of prechilled extraction medium. The homogenate was cleared by centrifugation at 15 000 *g* for one min at 4 °C.

ATP-dependent *in vitro* phosphorylation of PEPC was carried out by preincubating 500 µL of enzyme extract in 500 µL of incubation medium (final concentration: 20 mM Hepes-KOH, 10% (v/v) glycerol, 5 mM $MgCl_2$ and 1 mM ATP - unless otherwise mentioned - and the pH was adjusted to 8 with 0.1 M KOH). The control enzyme was incubated in the above mentioned incubation mixture, except ATP. At the indicated times, 10 µL aliquots were removed for assay of carboxylase activity and malate sensitivity of PEPC at pH 7.3. Carryover of Mg^{2+}-ATP from the incubation mixture into the assay medium had no effect on PEPC activity.

Alkaline Phosphatase-Dependent Dephosphorylation of PEPC *In Vitro*

Alkaline phosphatase (EC 3.1.3.1, AP; from calf intestine) was diluted to 500 U mL^{-1} with 20 mM Hepes-KOH, pH 8.0. AP-dependent dephosphorylation incubation mixture contained (final concentrations) : 20 mM Hepes-KOH (pH 8), 5 U mL^{-1} AP (or as indicated) and 5 mM $MgCl_2$ and the dephosphorylation was carried out at 30 °C. Ten µL aliquots were taken at different times and assayed for PEPC activity and L-malate sensitivity.

Estimation of Buffer Capacity

After the 2 h dark-preincubation, 100 leaf discs *(ca.* 450 mg) were blotted dry and then either kept in darkness (dark control) or illuminated (light sample) for 30 min at an intensity of 1000 $\mu mol m^{-2} s^{-1}$ at 30 °C. The leaf discs were then homogenized in a prechilled mortar and pestle, using 5-mL of either distilled water or unbuffered 0.3 M sorbitol.

Excised stem and root segments (1 g) were washed thoroughly with distilled water and preincubated in darkness for 2 h at 30 °C in distilled water. After the completion of the predark-period, the segments were either left in darkness or illuminated (for 30 min at 1000 $\mu mol\ m^{-2}\ s^{-1}$ at 30 °C) and then extracted by homogenizing in a mortar and pestle using 5-mL of either distilled water or 0.3 M unbuffered sorbitol.

The homogenate was passed through four layers of cheesecloth and the filtrate was rapidly centrifuged at 15 000 *g* for 1 min. The supernatant was used for pH titration. The pH of the extract was adjusted initially to pH 10, using 1 M NaOH. Then freshly diluted 0.1 M HCl was used to titrate, while monitoring the pH.

The volume of 0.1 M HCl needed for bring the pH of water or 0.3 M unbuffered sorbitol (used for extraction) to bring down the pH to 10 to 3 (total buffer capacity) or 8 to 7 (physiological buffer capacity) was subtracted from the volumes of HCl needed to change the pH of the extracts from 10 to 3 or 8 to 7, respecitvely.

Calibration and pH titration were carried out at 30 °C using a digital pH meter (Systrorics Digital pH meter, Model 335).

SDS-PAGE and Western Blotting

Solubilized polypeptides from crude leaf extracts of *Alternanthera pungens* were separated by SDS-PAGE using Mini-Gels (8 cm x 8 cm) of 10% polyacrylamide, as per the principle of Laemmli (1970).

Electrophoresis was performed using 15% stacking gel (2 cm x 8 cm) and 5% resolving gel (6 cm x 8 cm) according to the procedure of Piccioni *et al.* (1982). The stacking gel contained 500 mM Tris-HCl (pH 6.7), 4% (w/v) acrylamide (from stock solution of 30:0.8 of acrylamide and fcwacrylamide) and 0.1% (w/v) SDS. The resolving gel contained 1.5 M Tris-HCl (pH 8.8), 10% (w/v) acrylamide and 0.1% (w/v) SDS.

Leaf extracts or protein samples were dissolved in a sample buffer consisting of 50 mM Tris-HCl (pH 8.8), 8% (w/v) SDS, 50% (v/v) glycerol, 10% (v/v) 2-mercapto-ethanol and 0.04% (w/v) bromophenol blue, boiled for 2 min and loaded (5 $\mu g\ lane^{-1}$) onto 10% SDS-polyacrylamide gel. Electrophoresis was carried out by applying a constant current of 15 mA (Digi-Power Model SJ-1081, Atto Corporation, Japan), for 90 min at 25 °C. The electrode buffer consisted of 25 mM Tris-HCl (pH 8.8), 192 mM glycine and 1% (w/v) SDS.

The proteins were electrophoretically transferred onto a polyvinylidine difluoride (PVDF) membrane (Immobilon-PC from Millipore, procured from Sigma Chemical Co., St. Louis, MO, USA) according to Towbin *et al* (1979). The gel, PVDF membrane, and chromatography papers were soaked in transfer buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% (v/v) methanol for 30 min. The gel and membranes were sandwiched between the filter papers, three on

each side, saturated with buffer and blotted using a semi-dry blotter (LKB 2117 Multiphor) for 2 h at 25 °C, using a constant current of 15 mA.

The transfer of proteins was confirmed by staining with Ponceau S (0.2% [w/v] Ponceau S stain in 3% [w/v] trichloroacetic acid). The stain was removed by repeated washing with distilled water. The membranes were blocked by incubating with 5% (w/v) defatted milk powder in Tris-buffered saline (TBS), containing 25 mM Tris-HCl (pH 7.3) and 150 mM NaCl. Blocking was necessary to saturate the non-specific binding sites. The blocking was allowed for 1 h at 25 °C (Johnson *et al.* 1984) with constant shaking.

The blocked membranes were probed for 1 h with anti-PEPC rabbit serum (raised against PEPC from *Amaranthus hypochondriacus),* diluted 1:500 in blocking solution. The blocked membranes were washed thrice (15 min each wash) with TBS and incubated with the anti-IgG-alkaline peroxidase conjugate (1:7500) for 1 h. The blot was washed thrice (15 min each wash). The washed blot was developed with 35 µL 5-bromo-5-chloro-3-indolyl phosphate (from 50 ng ml^{-1} stock solution) and 70 µL *p*-nitroblue tetrazolium (50 mg ml^{-1} stock) in 10 mL of 16 mM Tris-HCl (pH 6.5), 5 mM NaCl and 0.2 mM $MgCl_2$.

Estimation of Chl and Protein

Chlorophyll was estimated by extracting into 80% (v/v) acetone as per Arnon (1949).

An aliquot of crude leaf extract (12.5 µL) was added to 5 mL of 80% (v/v) acetone. The mixture was shaken, and the absorbance was measured at 652 nm (for chlorophyll) and at 710 (for assessing turbidity). Total Chl content was estimated by using the following formula:

$$\text{Chl (mg mL}^{1}) = \Delta A_{(652\text{-}710)} \times 11.1$$

Total soluble protein was assayed according to Sedmak and Grossberg (1977). Protein samples were prepared in 0.3 M unbuffered saline. The assay consisted of adding 500 µL 0.06% (w/v) CBB-250 solution to 500 uL of protein solution or leaf extract, mixing immediately. The absorbance at 620 nm was determined against a 1:1 mixture of saline and dye. The absorbance of the solution was stable for 60 to 90 min at 25 °C.

Preparation of CBB G-250 solution : 0.06% (w/v) CBB G-250 solution in 3% (w/v) perchloroacetic acid (0.3 M) and was filtered through Whatman No. 1 filter paper to remove any undissolved material.

BSA (defatted, fraction V) was used as the standard.

Experimental Variation and Statistical Analysis

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

Chemicals

Alkaline phosphatase (from calf intestinal mucosa), ATP (Na2-salt), BSA (defatted, fraction V), CBB G-250, bromophenol blue, chymostatin, cycloheximide, α-D-mannose, 2-deoxy-D-glucose, EDTA (disodium salt), Hepes-KOH, NAD-malic dehydrogenase (from bovine heart), L-malate, Ponceau S solution, PMSF, SDS, D-sorbitol, salicylic acid, Tricine-KOH, Tris, *p*-nitroblue tetrazolium, anti-IgG-AP conjugate, D-Glc-6-P (Na_2 salt), and Immobilon-P (PVDF membrane) were procured

from Sigma Chemical Co., St. Louis, MO, USA. Dithiothreitol, PEP (monocyclohexylammonium salt) and Glc-6-P (Na_2 salt) were from Boehringer Mannheim GmbH, Germany. All other chemicals and reagents were of analytical quality purchased from either Sisco Research Laboratories or Spectrochem or E. Merck (India), all three in Bombay, India.

Procaine was a kind gift from Sarabhai Chemicals, Baroda, India.

Chapter Three

Patterns of Phosphoenolpyruvate Carboxylase Activity and Cytosolic pH during Light-Activation and Dark-Deactivation in C3-, C3-C4 Intermediate and C_4 Species

Introduction

Several enzymes of photosynthetic carbon reduction (PCR) cycle are activated on exposure to light (Macdonald and Buchanan 1990, Lawlor 1993, Leegood 1993, Huber *et al.* 1994). C_4-PEPC is also subjected to light/dark modulation that affects its sensitivity towards positive and negative effectors (Rajagopalan *et al.* 1994).

A two-fold light activation of PEPC was first reported in leaves of an NAD-ME type C_4 species *Amaranthus palmeri* (Slack 1968). Preliminary evidence concerning such effect of light was confirmed in *Atriplex tatarica* (Gavalas *et al.* 1981). Karabourniotis *et al.* (1983, 1985) reported that light activation of PEPC is widespread in C_4 species. When extracted from preilluminated C4 leaf tissue, activity of PEPC is two- to three-fold more than that of the dark-form, when assayed under suboptimal (but physiological) assay conditions. The mechanism of modulation of PEPC by light involves protein-serine kinase mediated N-terminal seryl phosphorylation and changes in sensitivity to L-malate (Huber *et* al. 1994, Rajagopalan *et al* 1994, Chollet *et al.* 1996).

An earlier comparative study by Chastain and Chollet (1989) has shown that PEPC in C3 species is not activated by light. However, Van Quy *et al.* (1991) reported that in wheat leaves light activates PEPC by several-fold and the presence of nitrate further enhances the extent of activation by light. Recently Leport *et al.* (1996) found no indication for a stable modulation of PEPC from pea in spite of intensive variation of assay conditions (pH, substrate and effectors concentrations). In their study, pretreatment of leaves with various protein kinase or protein phosphatase inhibitors gave no clear indication for the involvement of protein phosphorylation in the regulation of PEPC in pea plants. They have concluded that protein phosphorylation

plays only a minor role, if any, in the regulation of PEPC in C_3 plants, and suggested that PEPC is allosterically modulated by changes in concentrations of malate and/or Glc-6-P and/or PEP. No light activation of PEPC could be recorded in guard cell protoplasts of *Commelina communis* (Willmer *et al.* 1992). Thus, the light/dark modulation *in vivo* of PEPC from C3 species, which could alters the allosteric properties of enzyme, is not completely clear, and remains ambiguous.

This chapter describes the results of experiments designed to evaluate the effect of light on PEPC belonging to three different photosynthetic groups (C3-, C_4- or C3-C4 intermediate species) and to assess whether the light activation pattern can be used as a criterion to establish the C_4 form of PEPC.

Illumination causes an alkalinization of the cytosol in leaves of both C_3 and C4 plants - with the degree of alkalinization being quite in C_4 plants (Yin *et al.*. 1993, Raghavendra *et al.* 1993). Since PEPC is a cytosolic enzyme, and the light-induced cytosolic alkalinization in mesophyll cells may therefore be used in increasing PEPC activity. The activity of PEPC is maximal in alkaline pH (Gonzalez *et al.* 1984, Andreo *et* al. 1987, Echevarria *et al.* 1994). The possible role of cytosolic pH in regulating the activity of PEPC in leaves of C_4 plants was also evaluated.

Results

Extent and Pace of Light-Activation/Dark-Deactivation

When the predarkened leaf discs were illuminated, the activity of PEPC increased steadily. The increase in PEPC activity in leaf discs of *Alternanthera pungens* (Fig. 3.1 A), an NAD-malic enzyme type C4-dicot species, continued up to 20-25 min of illumination. Limited activation of PEPC in *Alternanthera sessilis* (a C3 species)

and *Alternanthera ficoides* (a C3-C4 intermediate species) was achieved by 10-15 min of illumination (Figs. 1B, C).

Leaves of several species belonging to different photosynthetic types were therefore examined for the extent of PEPC light activation. The activity of PEPC was stimulated by nearly three-fold by light in leaves of C_4 species. On the other hand, illumination enhanced the PEPC activity only by about 40% above the dark level in C_3 species (Table 3.1; Figs. 3.2A, 3.2B, 3.2C)). The extent and pace of light activation in C3-C4 intermediate species of *Alternanthera* (*A. ficoides* and *A. tenella), Parthenium hysterophorus* and *Mollugo nudicaulis* were similar to that of the C3 species, with light/dark PEPC activity ratios ranging from 1.3 to 1.5, *i.e.,* 30 to 50% activation by light over the dark-controls (Table 3.1).

When the extracts from preilluminated leaf discs were left in the dark at room temperature, the activity of PEPC declined gradually with time, reaching a steady state similar to the level of the extracts prepared from dark control leaf discs. The dark-deactivation of PEPC activity was rapid in C_3 species but was slow in C4 species (Fig. 3.3). A survey of several plants confirmed that the pace of dark-deactivation was indeed much slower (requiring 90 to 120 min to reach the dark control level) in C_4 species than that in extracts of C3-C4 intermediate and C_3 species (requires 30 to 60 min) (Table 3.2).

The inclusion of 2 mM PMSF and/or 2 mM benzamidine in the extraction medium did not alter the course of dark-deactivation of C4 PEPC in extracts of *A. pungens,* indicating that dark-deactivation of light-activated PEPC was not due to proteolytic degradation of the enzyme.

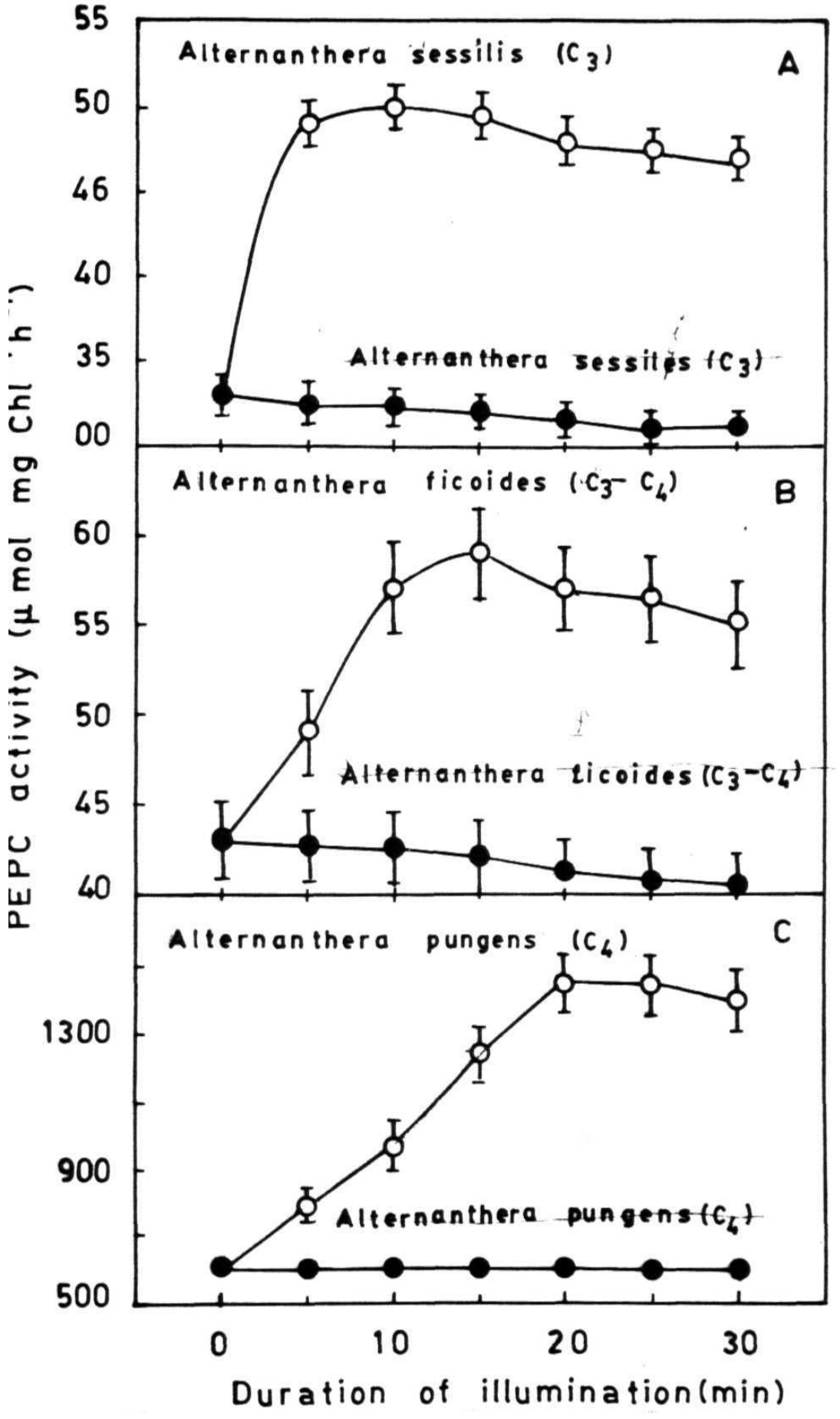

Figure 3.1. The activation of PEPC in relation to the duration of illumination in leaf discs of C3, C4, and C3-C4 intermediate species of *Alternanthera*. PEPC assay was carried out at pH 7.3 with 1.5 mM PEP. Maximal activation of the enzyme required 10, 15, and 30 min in C_3 (A), C3-C4 intermediate (B), and C_4 (C) species, respectively. The activity of light-form of PEPC was shown by open symbols (O) while the dark-control was shown by closed symbols (•).

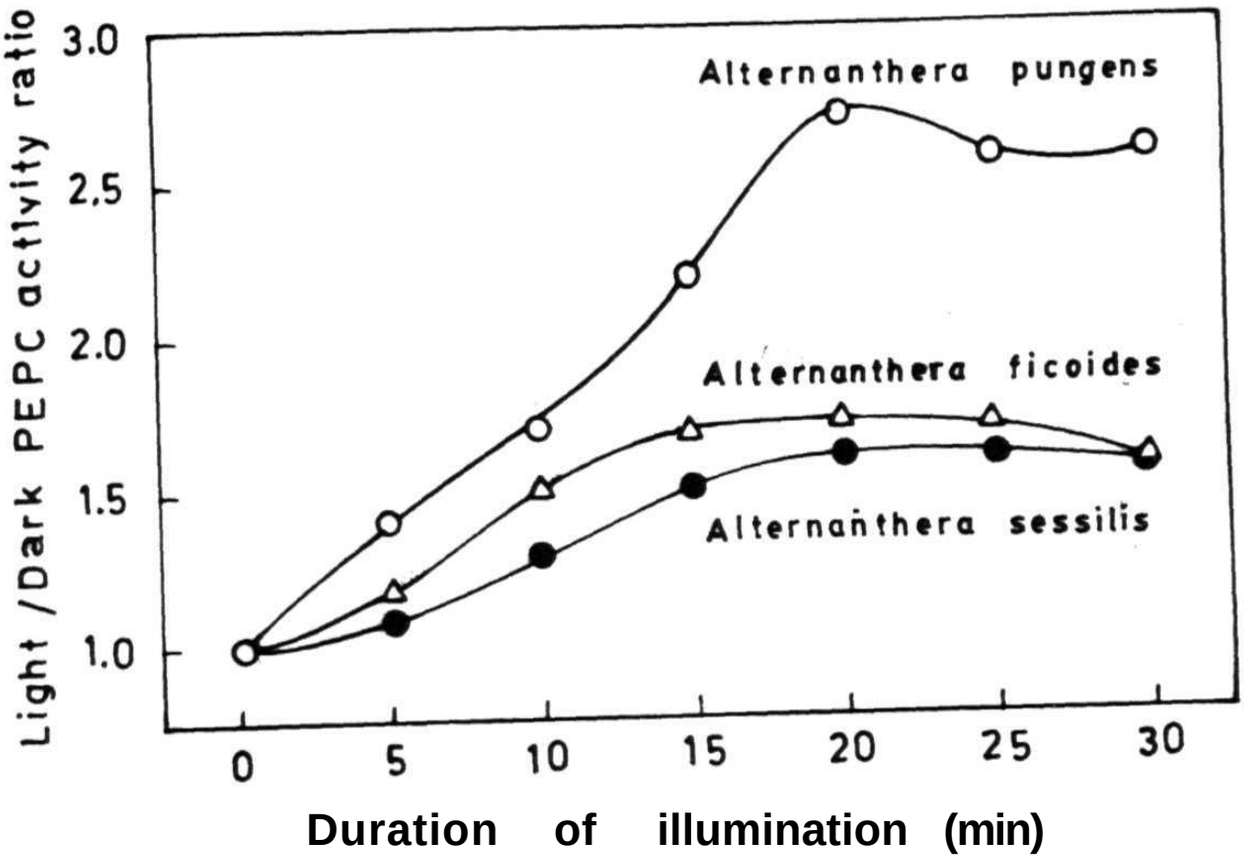

Figure 3.2. Effect of illumination on PEPC activity as indicated by the ratio of enzyme activity in leaf discs which are illuminated to that in dark-adapted ones. Dlumination of leaf discs from *Alternanthera sessilis* (C3; •) increased PEPC activi ty of *A. ficoides* (C3-C4 intermediate; A) and ***A. pungens*** (C4; O). Illumination of leaf discs from *A. sessilis* increased PEPC activity by 1.6-fold, that of *A. ficoides* by 1.5-fold. However, PEPC activity from the C4 species *A. pungens* enhanced by 2.4-fold upon illumination of leaf discs at a light intensity of 1000 μmol m^{-2} s^{-1} at 30 °C.

Table 3.1 Light-activation of PEPC from leaf discs of C_3, C3-C4 intermediate and C_4 species

Photosynthetic type/ Species	PEPC activity		Light/Dark Ratio
	Dark	Light	
	$\mu mol\ mg^{-1}\ Chl\ h^{-1}$		
C_3 Species			
Alternanthera sessilis	33±2	53±4	1.6
Arachis hypogea	32 ± 3	47 ± 4	1.5
Commelina benghalensis	16±1	17±1	1.1
Lycopersicon esculentum	31 ±2	46 ±4	1.5
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	25	35	1.4
C3-C4 Intermediate Species			
Alternanthera ficoides	45 ± 3	68 ± 5	1.5
A. tenella	31 ±3	47 ±4	1.5
Mollugo nudicaulis	35±3	50±4	1.4
Parthenium hysterophorus	20 ± 2	25 ± 2	1.3
Average	33	48	1.4
C_4 Species			
Alternanthera pungens	702 ±68	1708 ± 154	2.4
Amaranthus hypochondriacus	1448 ±85	3194 ± 91	2.2
A. viridis	548 ±52	1219 ±112	2.2
Gomphrena globosa	1028 ±78	2970 ±254	2.9
Portulaca oleracea	875 ±79	2482 ± 231	2.8
Sorghum bicolor	924 ± 74	3201 ±275	3.4
Zea mays	348 ±27	1151 ±98	3.3
Average	839	2275	2.7

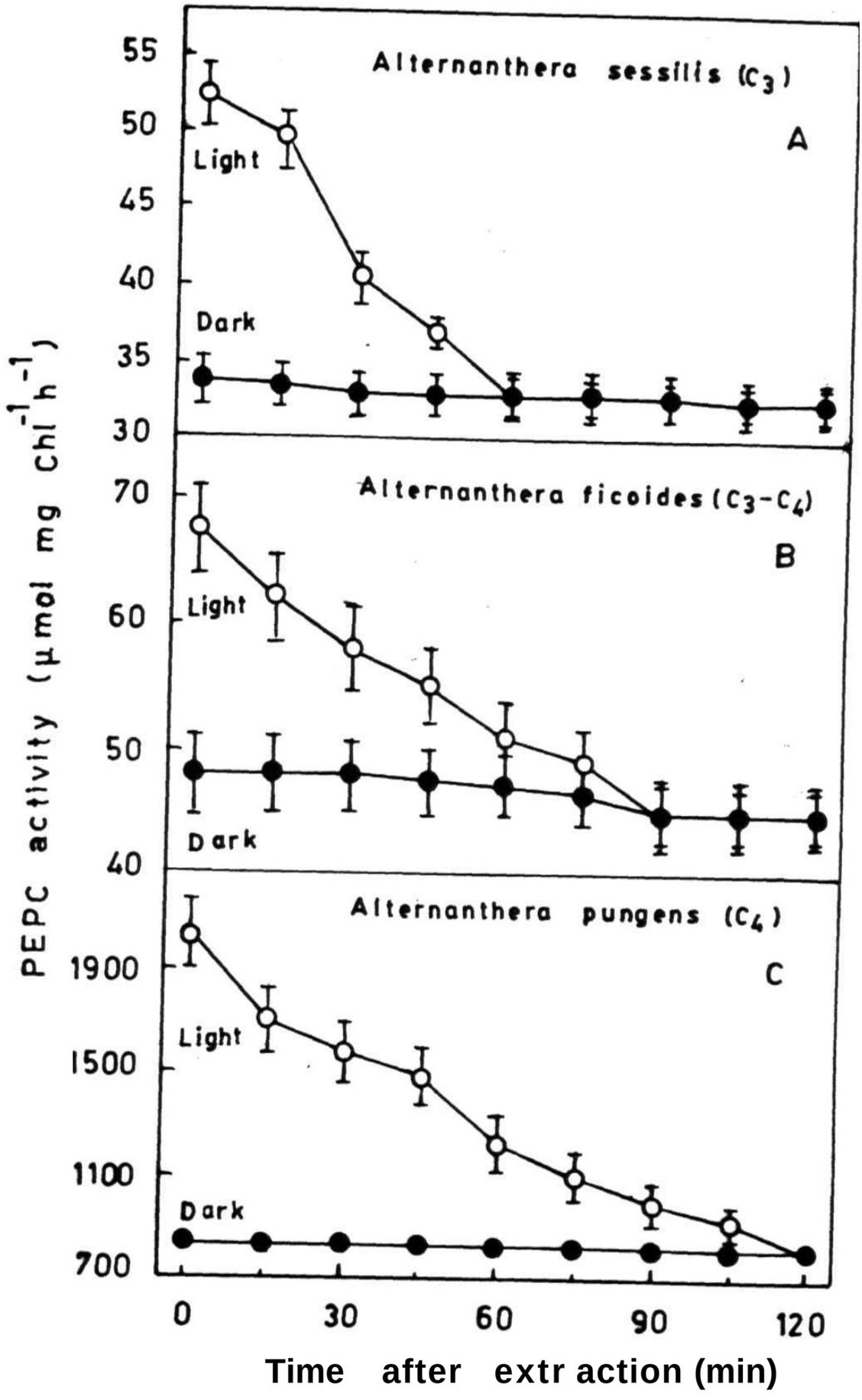

Figure 3.3. The rate of decline in PEPC activity of extracts prepared from illuminated or dark-adapted leaf discs of *Alternanthera* species. The activity of PEPC in the extracts of preilluminated leaf discs (O) gradually declined and reached the dark-control (represented by closed [•] symbols) by about 60 min in A. *sessilis* (C_3; A), 80 to 90 min in *A. ficoides* (C_3-C_4 intermediate; B) and 120 min in A. *pungens* (C_4; C). Other details are as given in Fig. 3.1.

Table 3.2. Dark-deactivation of light-activated PEPC from leaf discs of C_3, C3-C4 intermediate and C_4 species. Values are % of dark adapted control (100%).

Photosynthetic type/ Species	Elapsed time in darkness after extraction (min)			
	0	30	60	90
C_3 Species				
Alternanthera sessilis	160	124	100	100
Arachis hypogea	149	122	100	100
Commelina benghalensis	106	103	100	100
Lycopersicon esculentum	145	131	101	100
Pisum sativum	129	107	101	100
Tagetes erecta	129	119	101	100
Tridax procumbens	110	100	100	100
Average	133	114	100	100
C3-C4 Intermediate Species				
Alternanthera ficoides	152	123	116	100
A.tenella	149	119	100	100
Mollugo nudicaulis	144	127	108	100
Parthenium hysterophorus	126	108	100	100
Average	143	119	106	100
C_4 Species				
Alternanthera pungens	243	192	148	121
Amaranthus hypochondriacus	221	184	146	121
A.viridis	222	157	117	100
Gomphrena globosa	289	235	141	106
Portulaca oleracea	283	197	155	111
Sorghum bicolor	340	227	187	121
Zea mays	330	243	162	115
Average	275	205	151	113

Light Intensity Versus PEPC Activation

In order to find out the relative response of PEPC activation to light intensity, the leaf discs were illuminated at different intensities of white light. In leaf discs of a C_3 species, *A. sessilis,* light activation of PEPC reached the maximum level at a light intensity of 800 µmol photon m^{-2} s^{-1} (Fig. 3.4A), compared to 1000 to 1200 µmol photons m^{-2} s^{-1} (Fig. 3.4C) that required for maximum activation of PEPC in leaf discs of C_4 species. PEPC from the C3-C4 intermediate species *A. ficoides* exhibited similar pattern to that of C3 species (Fig. 3.4B).

Sensitivity to L-malate and Glc-6-P

Upon light activation, the sensitivity of PEPC from the C4 species, *A. pungens* to L-malate (feedback inhibitor) and to Glc-6-P increased (Table 3.3). For example, 1 mM L-malate inhibited 85% of the enzyme activity in extracts prepared from darkened leaf discs, but the inhibition by L-malate was only about 50% in extracts prepared from preilluminated leaf discs. Due to illumination the K_I (L-malate) of C4 PEPC increased nearly four-fold compared to the dark form (Table 3.4). During the dark-deactivation, the sensitivity of the enzyme to L-malate gradually increased along with a decrease in catalytic activity of the enzyme. There was no significant change in the sensitivity of PEPC from C3 species *(A. sessilis)* or C3-C4 intermediate species (A. *tenella)* to L-malate or Glc-6-P upon illumination.

Effect of Medium pH on PEPC Activity

The activity of PEPC in relation to pH was assessed by incubating dark- and light-form of the enzyme in a medium of varying pH, between 7 and 8.5 (Fig. 3.5). The activity of dark-form of PEPC increased steadily as the pH was increased and reached

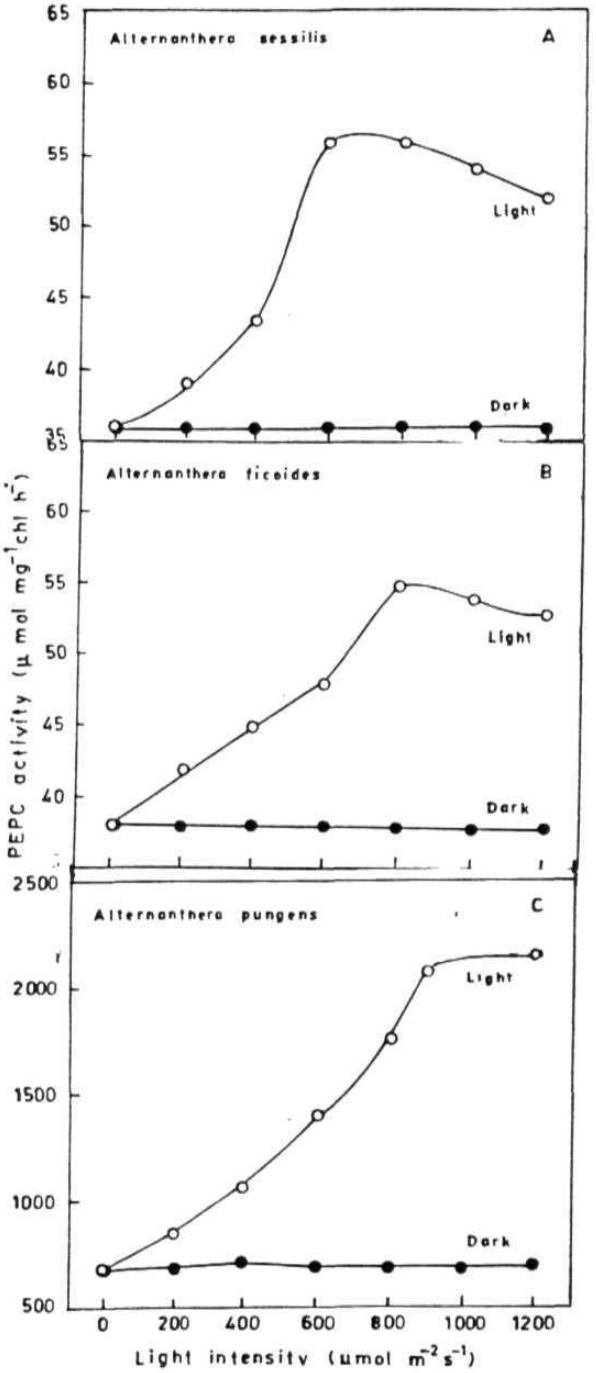

Figure *£4.* Effect of varying the light intensity on the light-activation of PEPC from *A. sessilis* (C_3; A), *A. ficoides* (C_3-C_4 intermediate; B) and *A. pungens* (C_4; C). In leaf discs of the C_3 species light-activation of PEPC reached the maximum level at a light intensity of 800 μmol m^{-2} s^{1} (A), compared to 1000 to 1200 μmol m^{-2} s^{-1} that required for maximum activation of PEPC in leaf discs of the C_4 species (C). PEPC activation by light saturated at around 800 μmol m^{-2} s^{-1} in the C_3-C_4 intermediate species (B).

Table 3.3 Effect of 1 mM L-malate on dark and light forms of PEPC activity from leaf discs of C3, C3-C4 intermediate and C4 species.

Photosynthetic type/ Species	PEPC activity (μmol mg^{-1} Chl h^{-1})			
	Dark		Light	
	Control	+Malate	Control	+Malate
C_3 Species				
Alternanthera sessilis	30 ±3 (100)[a]	10±1 (33)[a]	51 ±4 (100)[a]	19±2 (37)[a]
Arachis hypogea	28 ±3 (100)	11±1 (39)	45 ±4 (100)	19±2 (42)
Commelina benghalensis	30±3 (100)	9±1 (30)	42±4 (100)	14 ±1 (33)
Lycopersicon esculentum	30 ±3 (100)	8±1 (27)	48 ±4 (100)	14 ±1 (29)
Pisum sativum	22 ±2 (100)	5±1 (23)	29 ±2 (100)	8±1 (28)
Tagetes erecta	14±1 (100)	5±1 (36)	15±1 (100)	5 ±0.5 (33)
Average	26 (100)	8 (31)	38 (100)	13 (34)
C3-C4 Intermediate Species				
Alternanthera ficoides	41 ±4 (100)	9±1 (22)	69 ±4 (100)	20±2 (29)
A. tenella	30±3 (100)	8±1 (27)	44±4 (100)	13±1 (30)
Mollugo nudicaulis	33 ±1 (100)	8±1 (24)	14±1 (100)	14 ±1 (28)
Parthenium hysterophorus	18 ± 2 (100)	5±1 (28)	24 ±5 (100)	7±1 (29)
Average	31 (100)	8 (26)	38 (100)	14 (37)

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C_4 Species				
Alternanthera pungens	686 ± 54 (100)	66 ±5 (10)	2127 ± 141 (100)	1148 ±84 (54)
Amaramthus hypochondriacus	752 ± 61 (100)	114 ± 10 (15)	2106 ±168 (100)	1006 ±71 (48)
A. viridis	486 + 41 (100)	59 ±7 (15)	1574 ±121 (100)	789 ±71 (54)221
Gomphrena globosa	1011 ± 54 (100)	140 ±106 (14)	2990 ±51 (100)	1435 ±50 (48)
Portulaca oleracea	744 ±71 (100)	119±11 (16)	2083 ±141 (100)	1026 ±94 (49)
Sorghum bicolor	781 ±67 (100)	125 ±10 (16)	2410 ±224 (100)	1226 ± 95 (51)
Zea mays	306 ±21 (100)	46 ±5 (15)	1146 ±104 (100)	648 ±56 (57)
Average	681 (100)	96 (14)	2062 (100)	1042 (51)

Table 3.4 The effect of L-malate (a feedback inhibitor) and Glc-6-P (an allosteric activator) on the activity of PEPC extracted from darkened- or illuminated leaf discs

Species/Photosynthetic type	K_I (L -malate) mM		K_A(Glc-6-P) mM	
	Dark	Light	Dark	Light
Alternanthera sessilis (C3)	1.2	1.3 (1.08)[a]	2.0	2.0 (1.00)[a]
A. ficoides (C3.C4)	1.0	1.4 (1.40)	2.3	2.2 (0.96)
A. pungens (C4)	0.5	2.0 (4.00)	2.5	1.3 (0.52)

[a]Values in parentheses indicate light/dark ratios of K_I (L-malate) or K_A (Glc-6-P).

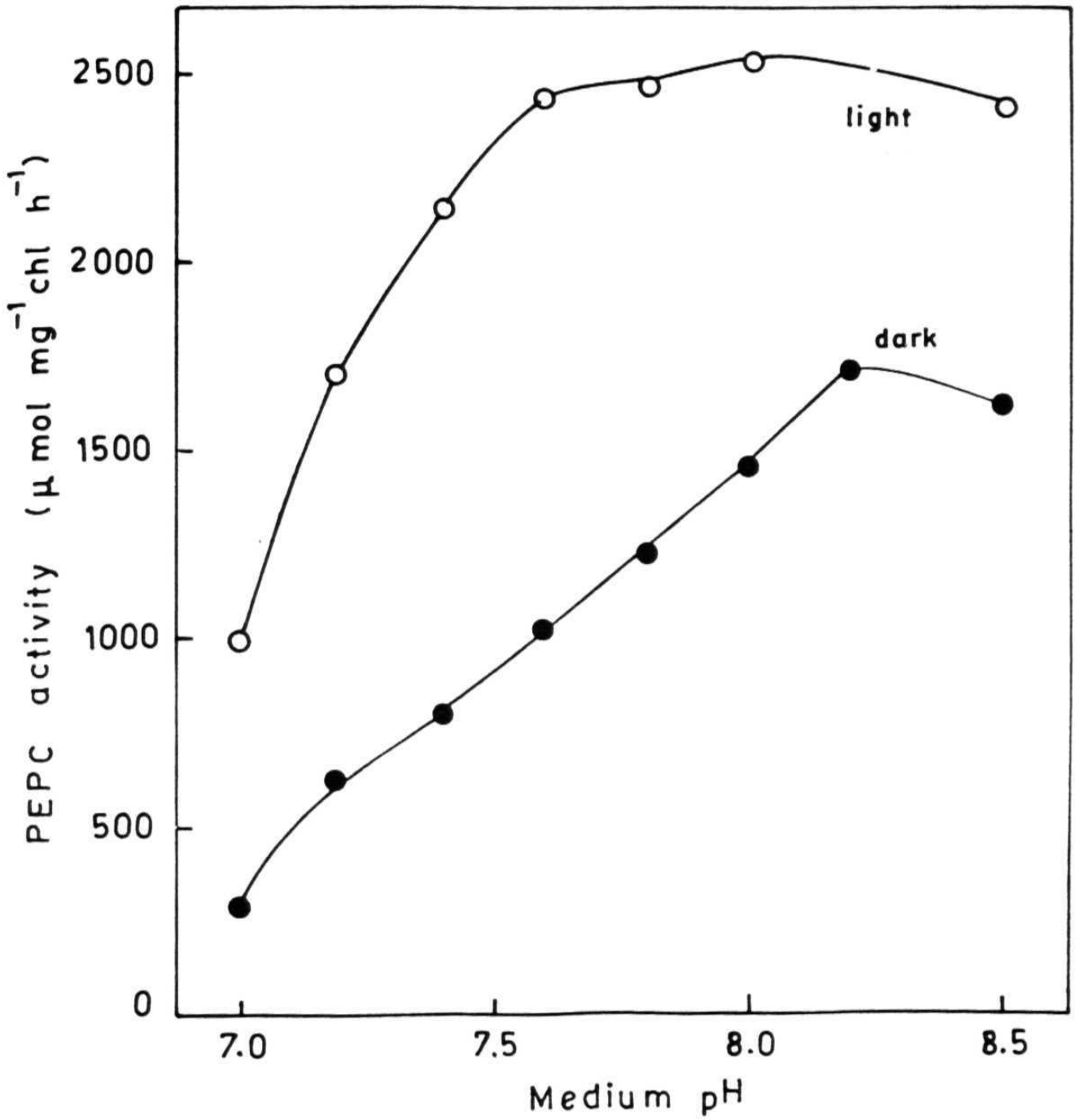


Figure 3.5. The effect of medium pH on PEPC activity extracted from darkened (closed symbols; O) or preilluminated (open symbols; O) leaf discs of *A. pungens*. The discs were illuminated at 1000 μmol m^{-2} s^{-1} for 20 min at 30 °C). The leaf discs were predarkened for 2-h period prior to illumination. The activity of the dark-form of PEPC grdually increased as the pH was increased and reached the optimal level around pH 8. Compared to the light-form PEPC, the enzyme extracted from darkened leaf discs was more sensitive to incubation medium pH. PEPC assay was performed at pH 7.3 with 1.5 mM PEP.

the optimum level at about pH 8 (Fig. 3.5). Compared to the light-form PEPC, the enzyme extracted from darkened leaf discs was more sensitive to medium pH. While the activity of PEPC raised by 5.7-fold in the pH range 7 to 8.2 , the activity of the light-form increased by only 2.7-fold. We speculate that the dark form of the enzyme is more sensitive to changes in cytosolic pH and thus, for phosphorylation. Since light induces cytosolic alkalinization, the light-form of the enzyme will be already phosphorylated and hence less sensitive to changes in pH.

Relationship Between Light-Induced Cytosolic Alkalinization and Light-Activation ofPEPC

Mechanical extraction of cell sap poses problems, since grinding of the leaf tissue damages cellular organelles, particularly vacuoles. Since vacuoles occupy nearly 90% of the plant cell, the rupture of vacuoles can alter the cytosol pH and makes it difficult to establish the exact pH of the cytosol. However, inclusion of 0.3 M sorbitol can reduce the breakage of vacuoles and minimize the dilution of cell sap with vacuolar contents. The cell sap prepared in water tended to be acidic, presumably due to vacuolar breakage. On the other hand, the use of 0.3 M sorbitol resulted in alkaline cell sap, presumably due to the enrichment of cytosol and reduction in the contribution from cell organelles including vacuoles.

Dlumination of leaf discs caused a measurable increase in pH of cell sap prepared from the leaves of *Alternanthera* ***pungens***, a C_4 species. The pH of cell sap increased with time during illumination and decreased after transfer to darkness. The extent of light-induced cell sap alkalinization was three-fold higher in leaves of C4 species evaluated than in the C3 species (Table 3.5). The degree of cell sap

Table 3.5. Light induced alkalinization of cell sap in C_3, C_4, and C3-C4 intermediate species, extracted in either distilled water or unbuffered 0.3. M sorbitol

Photosynthetic type/ Species	pH of cell sap					
	Extracted in water			Extracted in 0.3 M sorbitol		
	Dark	Light	ApH	Dark	Light	ApH
C_3 *Species*						
Alternanthera sessilis	6.78	6.84	0.06	7.06	7.13	0.07
Arachis hypogea	6.70	6.75	0.05	7.03	7.09	0.06
Commelina benghalensis	6.61	6.65	0.04	7.00	7.08	0.08
Lycopersicon esculentum	6.88	6.95	0.07	7.00	7.09	0.09
Pisum sativum	6.84	6.88	0.04	7.10	7.16	0.06
Tridax procumbens	6.84	6.87	0.03	7.14	7.20	0.06
Average	6.78	6.82	0.05	7.08	7.15	0.07
C_3-C_4 *Intermediate Species*						
Alternanthera ficoides 6.84	6.90		0.06	7.14	7.23	0.09
A.tenella	6.86	6.93	0.07	7.11	7.20	0.09
Mollugo nudicaulis	6.91	6.95	0.04	7.16	7.24	0.08
Parthenium hysterophorus	6.92	6.98	0.06	7.13	7.21	0.08
Average	6.88	6.94	0.06	7.14	7.22	0.09
C4 Species						
Alternanthera pungens	6.88	6.94	0.06	7.26	7.45	0.19
Amaranthus hypochondriacus	6.90	7.02	0.12	7.24	7.43	0.19
A.viridis	6.98	7.09	0.11	7.29	7.48	0.19
Gomphrena globosa	6.94	7.04	0.10	7.22	7.40	0.18
Sorghum bicolor	6.88	7.00	0.12	7.29	7.47	0.18
Zea mays	6.90	7.00	0.10	7.25	7.46	0.21
Average	6.94	7.02	0.11	7.26	7.44	0.19

alkalinization in C3-C4 intermediate species was more or less similar to that observed in C_3 species (Table 3.5).

The buffering capacity of cell sap, extracted from darkened and illuminated leaf discs, was estimated by titrating with 0.1 M NaOH, in order to evaluate whether there exists any difference between the cell saps prepared from darkened and illuminated leaf discs. There was no significant change in the buffering capacity as indicated by the magnitude of pH shifts due to the addition of NaOH.

The activity of PEPC and the change in pH were followed simultaneously in the same cell sap prepared in unbuffered 0.3 M sorbitol prepared from illuminated leaf discs of *Alternanthera pungens* (Fig. 3.5). The PEPC activity, as well as the pH of the cell sap, increased on a similar course with duration of illumination (Fig. 3.6) and similarly decreased during the dark-deactivation of PEPC, on subsequent darkness (Fig. 3.7).

Effect of Cycloheximide (CHX) on Light Activation of PEPC

It is possible that the increase in PEPC activity during illumination is due to the *de novo* synthesis of PEPC or any other protein such as PEPC-protein kinase. The effect of CHX, a eukaryotic cytosolic protein synthesis inhibitor, on light activation of PEPC has therefore been studied. When leaf discs of *Alternanthera pungens*, incubated in darkness for 6 h in 5 μM cycloheximide, were illuminated, the activity of PEPC increased, only to a limited extent, much less than that in the leaf samples illuminated in the absence of preincubation (Table 3.6). The light/dark PEPC activity ratios decreased from 2.5 to 1.9 even though no changes observed in the cell sap pH.

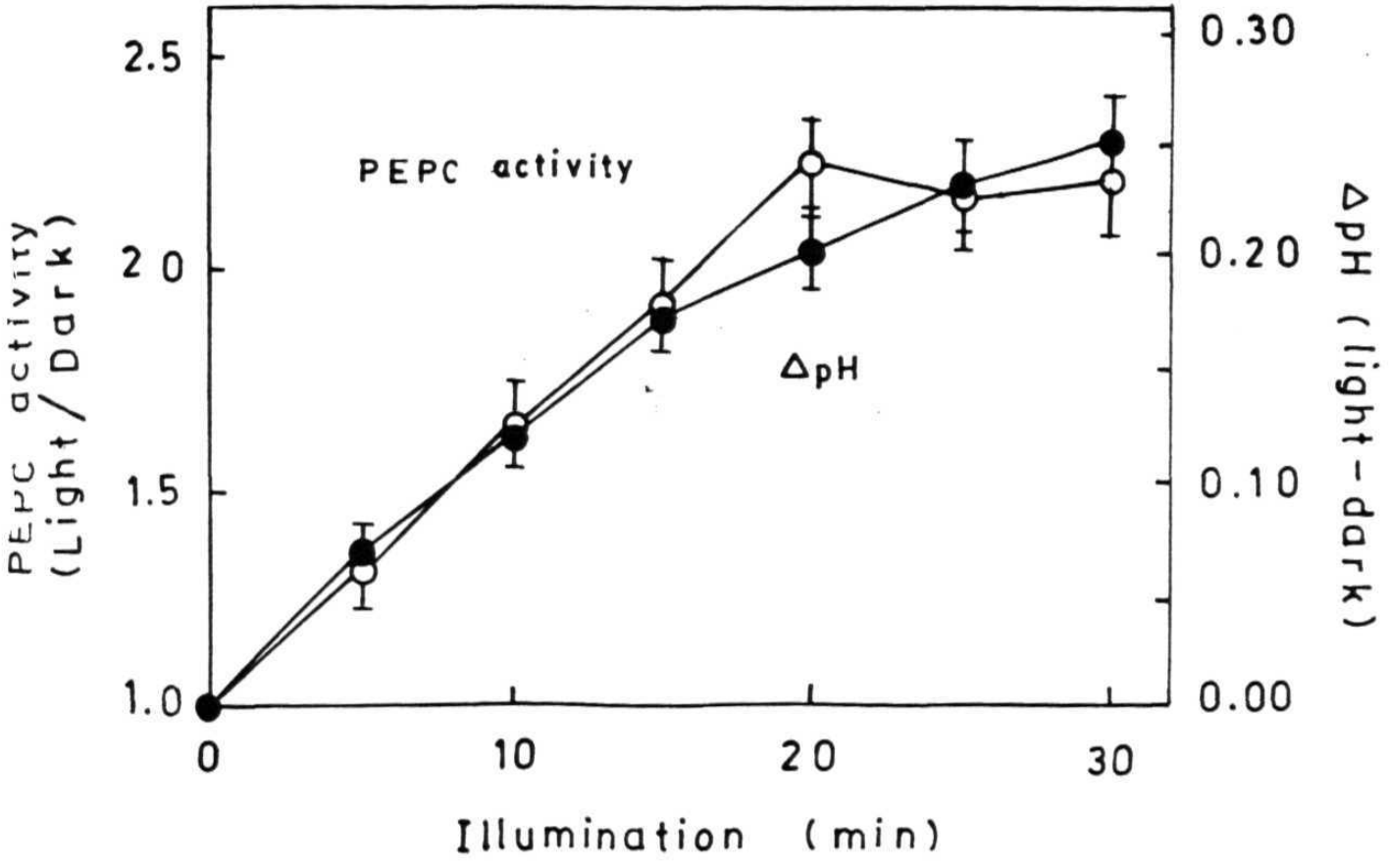


Figure 3.6. The effect of illumination on pH of cell sap (●) and activity of PEPC (○) in leaf discs of the C_4 species *Alternanthera pungens* measured simultaneously in the same cell sap, prepared in unbuffered 0.3 M sorbitol. Samples of cell sap were pepared at different time intervals of illumination (1000 μmol m^{2-} s^{-1}, 30 °C). The pH values of dark-adapted samples were subtracted to calculate ΔpH. PEPC activity (light) was represented as the ratio to that of the corresponding dark-sample.

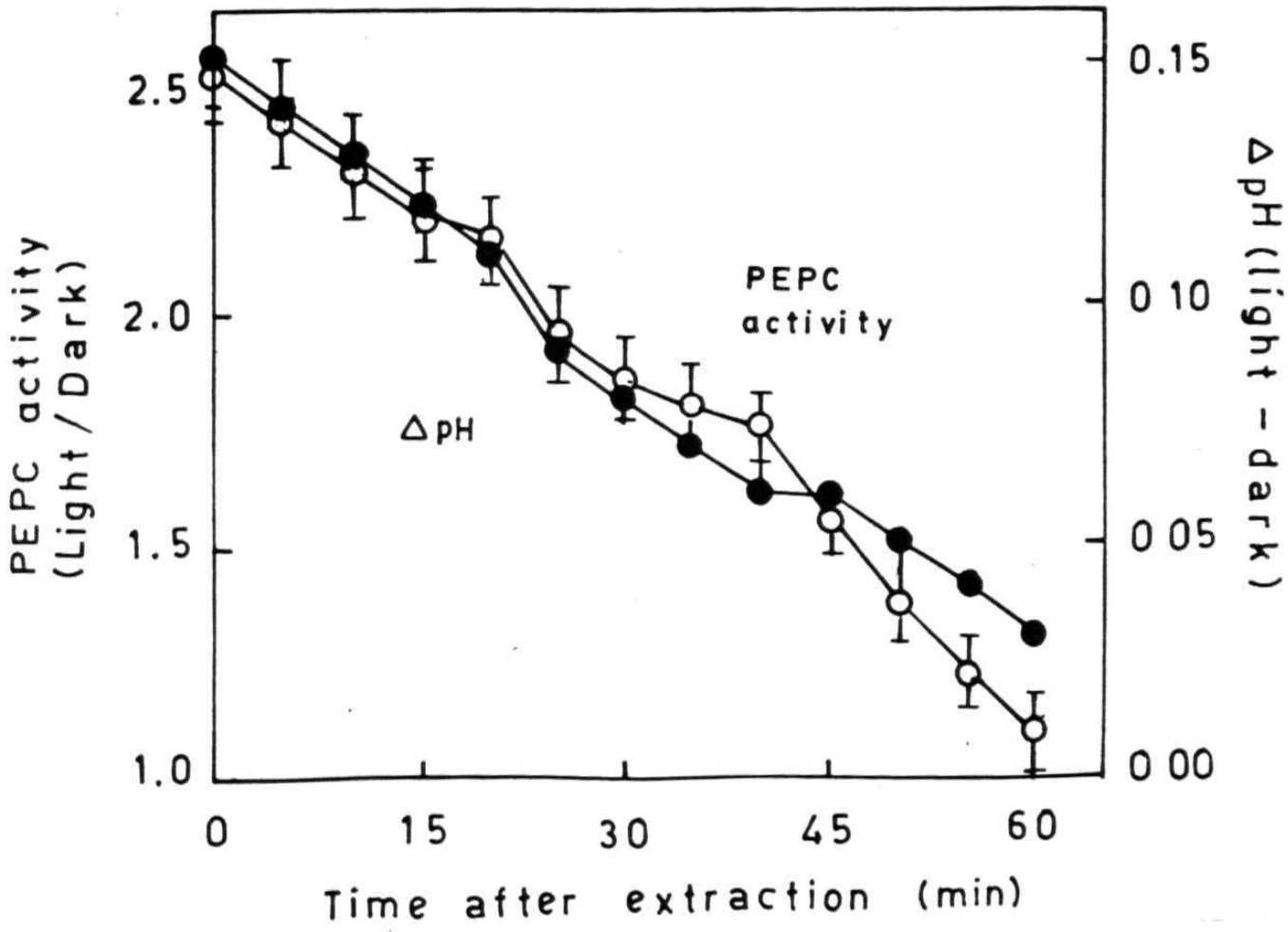


Figure 3.7. The time course of PEPC deactivation (O) and decrease in cell sap pH (●) of the extracts prepared in unbuffered 0.3 M sorbitol upon transfer to darkness. The ΔpH of cell sap (pH [light-dark]) and light/dark PEPC activity ratios were simultaneously measured in the same extract.

Table 3.6 Effect of *in vivo* feeding of 5 μM cycloheximide (CHX), a eukaryotic protein synthesis inhibitor, on light activation of PEPC from leaf discs of *Alternanthera pungens*

Treatment	Control	CHX treated
PEPC activity (μmol mg^{-1} Chl h^{-1})		
Dark	701 ±59	694 ±61
Light	1750 ± 162	1308 ± 124
Light/Dark activity ratio	2.5	1.9
Cell sap pH		
Dark	7.25 ± 0.03	7.23 ± 0.02
Light	7.44 ± 0.03	7.41 ± 0.02
ApH (light *minus* dark)	0.19	0.18

Discussion

The present chapter describes the pattern of light-activation of PEPC from C3, C_4 and C3-C4 intermediate species. The extent of light activation of PEPC varied considerably within each photosynthetic group (Table 3.1). These results demonstrate that the light activation of PEPC in the C4 plants is quite pronounced and is distinct from that in the C3 plants or the C3-C4 intermediate species, in terms of either the extent or the pace of light-activation/dark-deactivation (Tables 3.1, 3.2).

On the other hand the level of activation of PEPC, in the extracts prepared from leaf discs of C3-C4 intermediate and C_3 species, was low, ranging from 10 to 60% (an average of 35%) (Table 3.1). Chastain and Chollet (1989) reported slight activation of PEPC from C3-C4 intermediate species of *Flaveria* genus, while

reporting no detectable light activation in C_3 species. On the other hand, a marked light activation (2-3 fold) of the enzyme was observed among the C_4 species. The present study demonstrated that leaf-PEPC from C3-C4 intermediate and C3 species undergoes marginal activation by light.

Although the species of *Alternanthera ficoides*, *A. tenella*, *Mollugo nudicaulis* and *Parthenium hysterophorus* were identified as C3-C4 intermediate species based on their anatomical, physiological and biochemical characteristics (Edwards and Ku 1987), PEPC from these species showed a trend similar to those of C3 species (Tables 3.1, 3.2, 3.4).

Upon illumination, there was a three-fold enhancement in PEPC activity (when the enzyme was assayed at suboptimal conditions, pH 7.3 and 1.5 mM PEP) in extracts prepared from leaf discs of C4 species. There is a great deal of literature on the pattern of light activation of PEPC in leaves of C4 plants. Most of the studies used intact leaves and indicated a requirement of 40 to 60 min to reach maximum activation (Jiao and Chollet 1989, Van Quy *et* al. 1991, Van Quy and Champigny 1992). A few studies used leaf slices and reported that 15 min was long enough to activate PEPC from C4 plants (Doncaster and Leegood 1987). Variation in the duration of illumination needed for PEPC activation in C4 plants may be due to difference in the experimental system: intact leaves, leaf slices, leaf discs. Our experiments with leaf discs confirm that a period of 20-min illumination is necessary to activate PEPC in leaves of C_4 plants (Rajagopalan *et al.* 1993). Further, the sensitivity of PEPC to the effectors, L-malate (negative) and Glc-6-P (positive) was influenced only slightly by illumination in the C3 species. Similar results were recently reported by Gupta *et al.* (1994).

L-malate is a feedback inhibitor of PEPC. The sensitivity of PEPC to L-malate changes markedly on illumination particularly in C_4 species. However, the malate sensitivity of PEPC from leaf discs of C3 and C3-C4 intermediate species, did not change much on exposure to light. Consequently, the K_I (L-malate) slightly altered due to illumination of leaf discs from C_3 or C3-C4 intermediate species, whereas it increased by nearly four-fold in the C_4 species upon illumination. When assayed at pH 7.3 and 1.5 mM PEP and in the presence of 1 mM L-malate, the light/dark PEPC activity ratios ranged from 8 to 15 in the case of C4 species, compared to 1.5-1.9 in the C3 or 1.7 - 2.2 in the C3-C4 intermediate species. These results again demonstrate that light exerts a differential effect on PEPC between the C3 or C3-C4 intermediate and C4 species and illumination greatly reduces the feedback inhibition of PEPC by L-malate in the C_4 but only slightly in the C3-C4 intermediate species.

Glc-6-P is an allosteric activator of PEPC. The degree of Glc-6-P activation differed between the two groups: C_4 species appeared to have a greater activation of their PEPC by Glc-6-P than C_3 or C3-C4 intermediate species. There was about 80% activation of PEPC by Glc-6-P in C_4 species while only 60% activation in C3 or C3-C4 intermediate species. The K_A(Glc-6-P) also varied between these two groups: while the K_A value changed from 2.5 to 1.3 mM upon illumination, only marginal change was observed in the C3-C4 intermediate species and no change in C3 species. These results are consistent with those reported that Glc-6-P activates C4-PEPC to a larger extent than C3-PEPC (Nakamoto *et al.* 1983, Gupta *et al* 1994).

The efficient operation of the C_4 pathway is thought to require high malate concentration in the mesophyll cells to establish a large intercellular gradient to the bundle sheath cells (Hatch 1987, Raghavendra and Das 1993, Furbank and Taylor

1995, Ku *et al.* 1996). The light-dependent decrease of K_I (L-malate) for PEPC in the mesophyll cells of C_4 plants may allow this gradient to develop.

The slow pace of light-activation/dark-deactivation of PEPC in C4 plants is strikingly similar to the slow but steep rate of light-dependent cytosolic-alkalinization/dark-acidification in leaves of C_4 species, compared to the quick but small changes in cytosolic pH of C3 species (Yin *et al.* 1990, 1993, Raghavendra *et al.* 1993). The present results suggest that the pattern of light-activation can be taken as an additional criterion to distinguish PEPC from C3 and C4 plants (Rajagopalan *et al.* 1993). Comparative studies on C3, C3-C4 intermediate and C4 species of *Flaveria* genus indicated a distinct isoform of PEPC in the leaves of C4 species (Nakamoto *et al* 1983, Adams *et al.* 1986, Bauwe and Chollet 1986).

PEPC activity decreases at pH values closed neutrality, as compared to that at the optimal pH of 8. In contrast, the effects of L-malate, Glc-6-P, and phosphorylation is progressively amplified and becomes extremely sensitive to pH, at values of 7 to 7.3. We speculate that phosphorylation of PEPC appears to compensate for this pH-induced loss in enzyme activity by increasing activity at pH 7.3 to approach the optimal rate measured at pH 8. Therefore it appears to be one of the main factors which could help to regulate the light-dependent activation of C_4-leaf photosyntthetic PEPC .

Light-dependent cytosolic alkalinization *in vivo* has been reported in leaves of C_3 and C_4 plant species, using pH-dependent dyes (Raghavendra *et al.* 1993, Yin *et al.* 1993).). The uptake of 3-phosphoglyceric acid (3-PGA) into mesophyll chloroplasts during C4 photosynthesis may lead to an elevation of mesophyll cytosolic pH in C_4 leaves (Yin *et al.* 1993). That the cytosolic pH could be an important factor

regulating the activity of PEPC, is indicated by the observation that illumination raised the pH of cell sap. Since the dark-form of the enzyme is very sensitive to medium pH, *in vitro,* we propose that light-induced cytosolic alkalinization 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 C_4 leaves. Despite the technical limitation, our data suggest that illumination of leaves or leaf discs causes alkalinization of cytosol pH. Upon illumination, the degree of cytosolic alkalinization was much greater than vacuolar acidification in both C3 and C4 species as 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 changes in pH of the cell sap observed in this study is probably the result of cytosolic alkalinization.

It was also shown that pH affects the phosphorylation status of PEPC from mesophyll protoplasts of *Sorghum* (Pierre *et al.* 1992) and *Digitaria sanguinalis* (Giglioli-Guivarc'h *et al.* 1995, 1996, Duff *et al* 1996). Our results provide a strong evidence for the regulation of PEPC in leaf discs of *Alternanthera pungens* by pH.

The major mechanism of light regulation of C4 PEPC is the reversible phosphorylation of the enzyme during light/dark transitions (Rajagopalan *et al.* 1994, Chollet *et al.* 1996). A soluble protein-serine kinase catalyzes the phosphorylation of PEPC (Nimmo *et* al. 1987, Jiao and Chollet 1988, 1989, 1990, 1991, Echevarria *et al.* 1990, 1994), while a type 2A phosphatase brings out the dephosphorylation of the enzyme (Carter *et al.* 1990). The activity of PEPC-PK is stimulated by light

(Echevarria *et al.* 1990, Jiao *et al.* 1991, Jiao and Chollet 1992) and by cytosolic alkalinization of mesophyll *in situ* (Pierre *et al.* 1992, Giglioli-Guivarc'h *et al.* 1995, 1996, Duff *et al.* 1996).

PEPC is a classic example of marked modulation of enzyme activity by pH (Davies 1986, Kurkdjian and Guern 1989). Apart from its extreme sensitivity to even small variations in H^+ concentration, PEPC has a tendency to aggregate or dissociate depending on microenvironment and effectors (Meyer *et* al. 1990, Wu *et al.* 1990).

The results presented in the present chapter clearly provide evidence of a marked correlation between the light-induced increase in pH of cell sap and PEPC activation due to illumination. We suggest that 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 state of the enzyme, (b) change in the oligomeric state of the enzyme, and (c) an unknown change in the conformational state of the enzyme. All the three factors could be interrelated. For example, the change in oligomeric/ conformational state of the enzyme, due to cytosolic alkalinization, may increase the activity of PEPC or promote the extent of phosphorylation of the enzyme or both (Rajagopalan *et al.* 1993).

Chapter Four

Modulation by Weak Bases or Weak Acids of the pH of Cell Sap and PEP Carboxylase Activity in Leaf Discs of C_4 Plants

Introduction

Eukaryotic cells keep up their cytoplasmic pH at 7.0-7.4 by ion transport mechanisms and high buffer capacity of the cytosol (Raven 1985, 1995, Felle 1988, Kurkdjian and Guern 1989, Guern *et al.* 1991). Several factors influence the cytoplasmic pH, for e.g., acidic gases such (such as SO_2, NO2), anaerobiosis, light to dark transition, temperature and external pH (Guern *et* al. 1991, Heber *et al.* 1994). Cytoplasmic pH modifications also occur through intracellular production or consumption of protons or through the exchange of protons or proton equivalents across the boundary membranes (Raven 1985, Kurkdjian and Guern 1989).

PEPC is one of the enzymes known to be involved in the `biochemical pH-stat' and is located in the cytosol (Davies 1973, 1986). Intracellular pH is important for the activity of a number of enzymes with a sharp pH optimum, within the physiological pH range. The activity of PEPC also depends markedly on pH (Guern *et al.* 1983, Gonzalez *et al.* 1984, Rajagopalan *et al.* 1993). The enzyme is highly active at pH values above 7.8, while at acidic pH, the enzyme is much less active (Rajagopalan *et al.* 1993, Echevarria *et al.* 1994). Above pH 7.0, there is an increase in the affinity of PEPC for PEP and Mg^{2+} and a decrease in inhibition by L-malate (Gonzalez *et al.* 1984). Dlumination of leaves caused a marked alkalinization of the cytosolic compartment in mesophyll cells of C3 and C_4 plants (Yin *et al.* 1990, 1993, 1996, Raghavendra *et al.* 1993). Since PEPC is localized in the mesophyll cytosol, the light-induced cytosolic alkalinization could have profound effects on the kinetic/regulatory properties of the enzyme.

Acid-base loading has been used as a strategy for studying short-term pH regulation in plants (Felle 1988, Guern *et al.* 1991, Gout *et al.* 1992). Various lipophilic weak acids (e.g., acetic, propionic, butyric, benzoic, salicylic acid) induce a cytoplasmic acidification in a variety of plant systems. These weak acids permeate into cells in their undissociated form and they dissociate in the alkaline cytosol. The released protons acidify the cytosol (Hager and Moser 1985, Basso and Ulrich-Eberius 1987). Cytosolic pH decreased by about 0.1 to 1.0 unit after acid-loading, depending on the experimental conditions such as external pH, concentration/type of acid and plant species (Kurkdjian *et al.* 1989, Guern *et al.* 1991, Gout *et al.* 1992). Similarly, weak bases (such as NH_4^+ salts, methylamine or procaine) are capable of elevating the intracellular pH (Felle and Bertl 1986, Danthuluri *et al.* 1990, Guern *et al.* 1991, Friedman *et al.* 1992). The changes induced by weak acids or weak bases in cytosolic pH are usually reversible (Guern *et al.* 1986, Frachisse *et al.* 1988, Beffagna and Romani 1991).

The present work attempts to modulate the cytosolic pH in leaf discs of two NAD-ME type C_4 dicot species, *viz.*, *Alternanthera pungens* and *Amaranthus hypochondriacus*, using compounds capable of causing either alkalinization or acidification of the cytosol. Species of a few C_3 and C_3-C_4 intermediate photosynthetic types are also included for comparison. Our results demonstrate that PEPC, particularly in leaves of the C_4 plant species, responds markedly to changes in cytosol pH brought about these agents. PEPC activity is stimulated when the cytosol was alkalinized and decreased upon acidification of the cytosol due to base- or acid-loading of leaf discs, respectively. To our knowledge, this is the first report on acid-base modulation of PEPC in leaf tissue of C_4 species.

Results

Cytosolic pH was modulated *in vivo* by pretreatment of leaf discs with various concentrations of weak bases (in 5 mM Tricine-KOH, pH 8.5) or weak acids (in 5 mM sodium acetate buffer, pH 4.5). The activity of PEPC was assayed in the same extract (prepared in unbuffered 0.3 M sorbitol, unless specified otherwise) used for determining the pH of cell sap. Thus, it was possible to make a direct comparison of cytosolic pH with PEPC activity in the same leaf extract.

Incubation of Leaf Discs in Alkalinizing Agents

Incubation of leaf discs in different concentrations of alkalinizing agents revealed that 7.5 mM NH_4Cl (Fig. 4.1) and 10 mM procaine (Fig. 4.2) were optimal for cytosolic alkalinization. When the leaf discs were incubated in 7.5 mM NH_4Cl (in 5 mM Tricine-KOH, pH 8.5), the pH of the cell sap increased by about 0.2 unit within 120 min and then declined.

The activity of PEPC was enhanced markedly upon incubation of leaf discs with NH_4Cl (base-loading). The stimulation in PEPC activity ranged from 80 to 120% over the control (incubated in buffer alone), depending on the concentration of NH_4Cl (Fig. 4.1) or the duration of incubation (Fig. 4.3). Maximum stimulation of cell sap pH or PEPC activity occurred by 120 min of incubation (Fig. 4.3)..

Incubation of leaf discs in these test solutions had little or no effect on the Chl content. This rules out the possibility that the observed changes in activity of PEPC were due to variation in Chl content.

Acid-Loading of Leaf Discs

The incubation of leaf discs in weak lipophilic acids (propionic, butyric or salicylic acid) decreased pH of the cell sap by a maximum of about 0.3 unit. A concentration

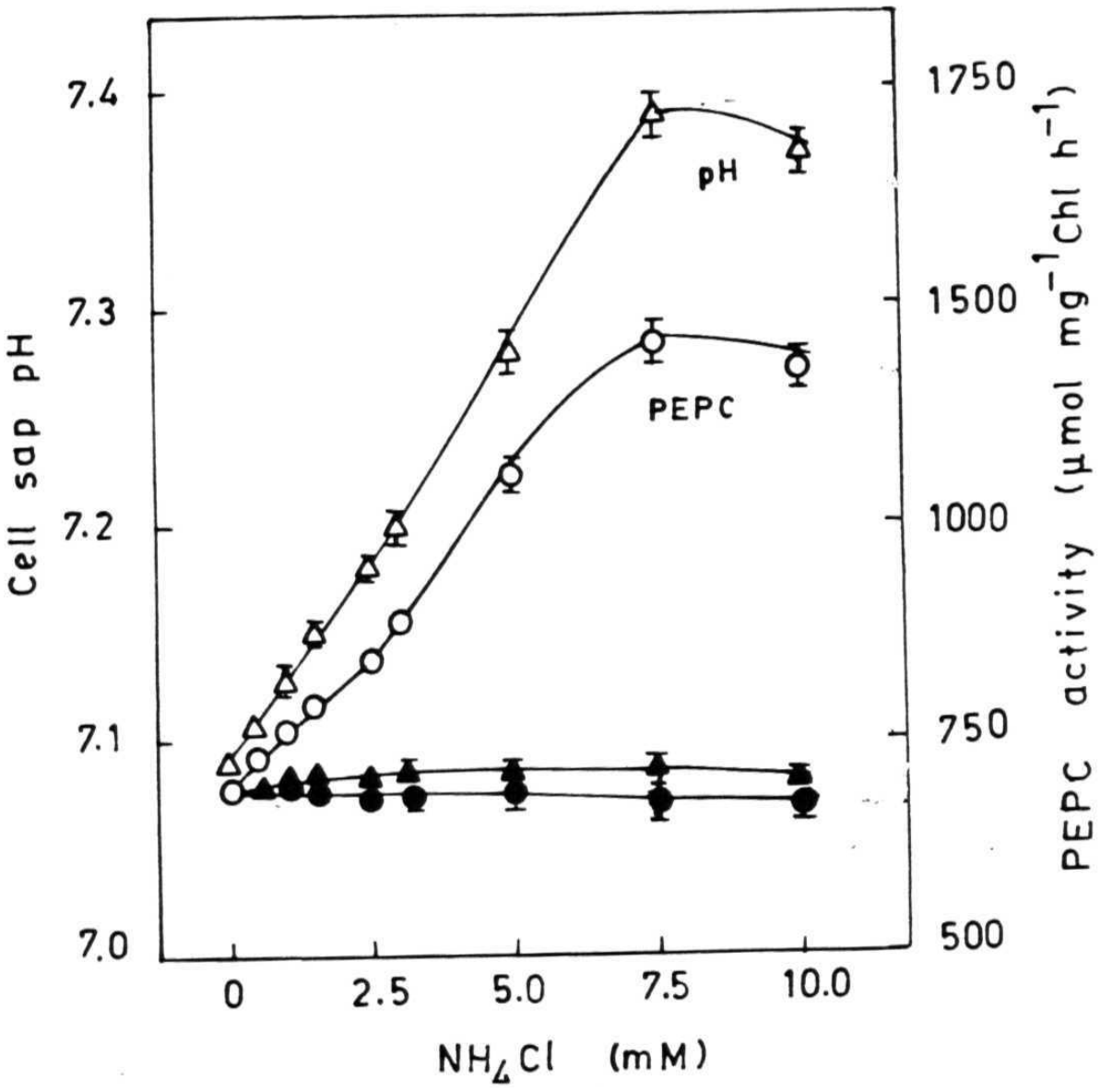


Figure 4.1. Effect of NH_4Cl treatment (base-loading) on the pH (O) and PEPC (A) activity in cell sap prepared from leaves of the C4 species A. ***pungens***. The leaf discs, predarkened for 2-h, were floated on various concentrations of NH_4Cl (prepared in 5 mM Tricine-KOH, pH 8.5). The extracts were prepared in unbuffered 0.3 M sorbitol after an incubation period of 90 min in NH_4Cl and used for determining the cell sap pH and PEPC activity. The control leaf discs were floated on buffer alone (closed symbols).

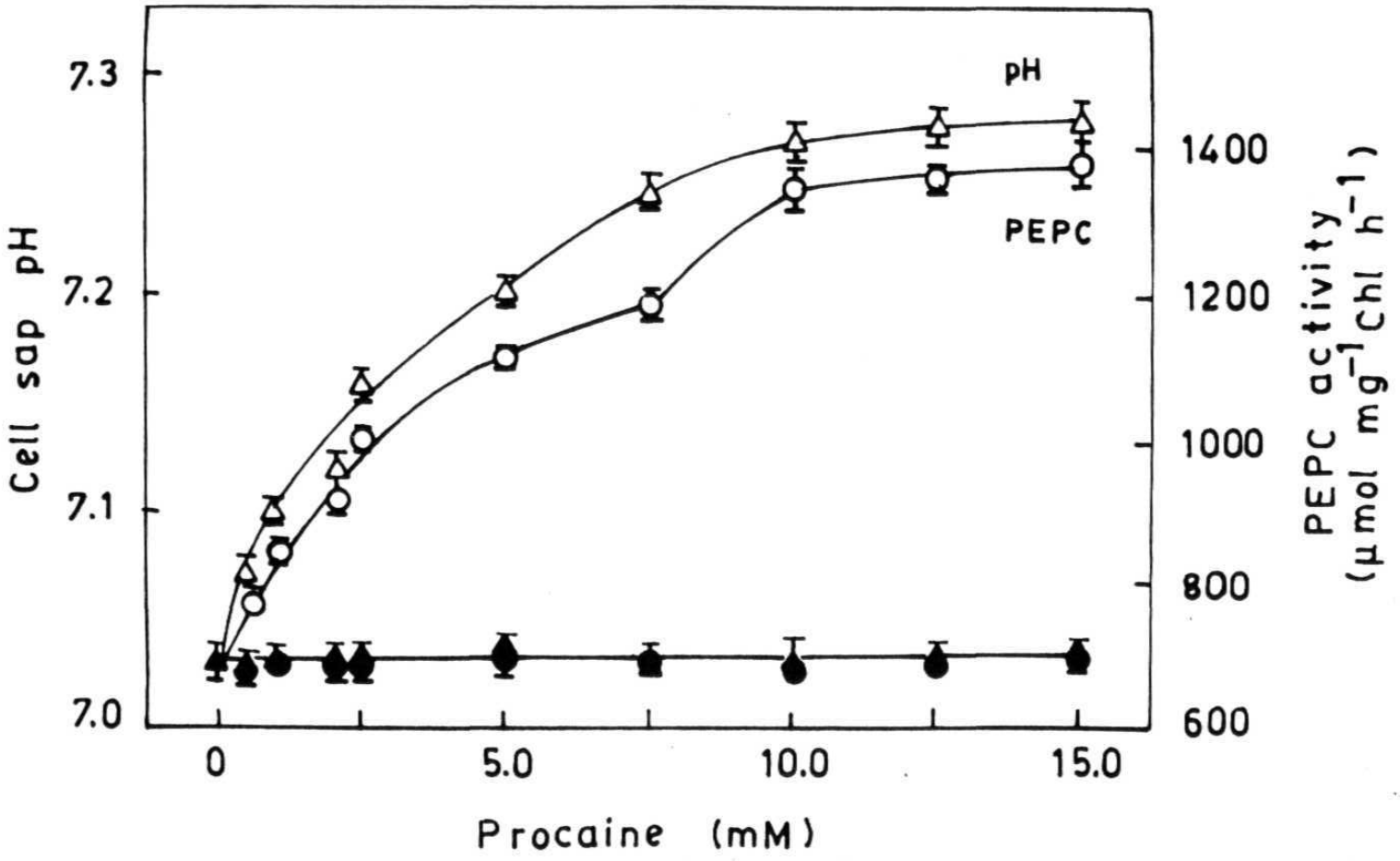


Figure 4.2. Effect of feeding procaine (base-loading) on the pH (O) and PEPC (Δ) activity in cell sap prepared from leaves of the C_4 species *A. pungens*. The leaf discs, predarkened for 2-h, were floated on various concentrations of procaine (prepared in 5 mM Tricine-KOH, pH 8.5). The extracts were made in unbuffered 0.3 M sorbitol after an incubation period of 90 min in procaine and used for determining the cell sap pH and PEPC activity. The control leaf discs were floated on buffer alone.

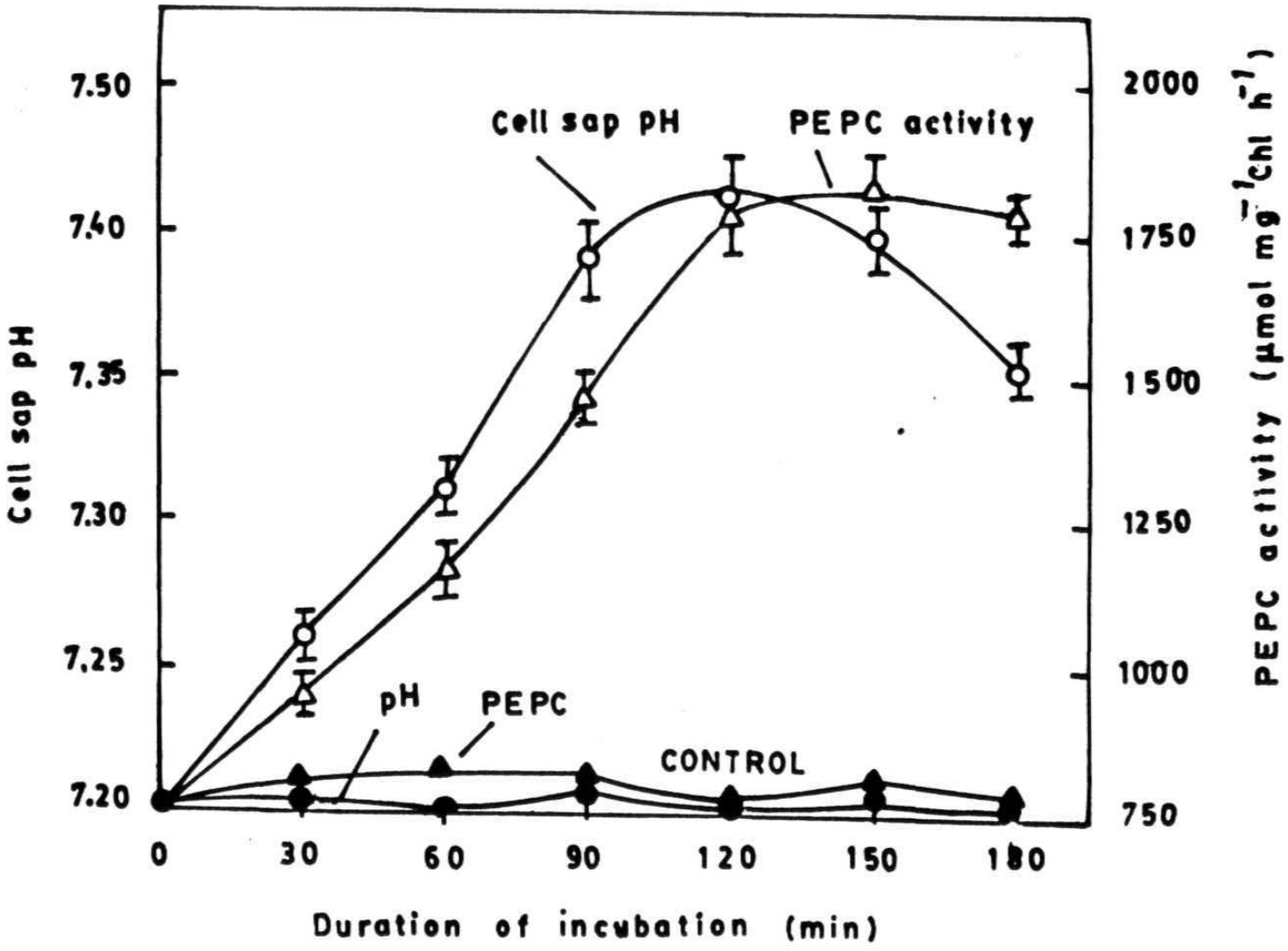


Figure 4.3. Time course of changes in cell sap pH (O) and PEPC (Δ) activity of crude extracts prepared from leaf discs of *A. pungens* incubated in 7.5 mM NH_4Cl (in 5 mM Tricine-KOH buffer, pH 8.5). Cell sap pH and PEPC activity were determined at different time intervals, as indicated, using the same extract prepared in unbuffered 0.3 M sorbitol.

of 5 mM propionic acid (Fig. 4.4) and an incubation period of 120 min (Fig. 4.5) were optimal. Acid-loading of leaf discs markedly decreased PEPC activity, with the pattern being quite similar to the acidification of cytosol. The activity of the enzyme decreased by about 50% (compared to the control sample incubated in buffer alone) when the leaf discs were incubated for 90 min in 5 mM propionic acid (in 5 mM acetate buffer, pH 4.5).

Effect of External pH

The modulation of cell sap pH in leaf discs by weak bases or weak acids depended on the pH of the external (incubation) medium. The cell sap pH became markedly alkaline upon incubation with NH_4Cl only when the external pH was above 8.0 (Fig. 4.6). Incubation of leaf discs in 5 mM sodium acetate or 5 mM Tricine-KOH buffer, in the absence of cytosolic acidifying/alkalinizing agents, had no effect on cell sap pH. The cell sap pH decreased markedly upon incubation with propionic acid only when the external pH was below 6.0. The pH of the cell sap remained steady, despite the treatment with propionic acid or NH_4Cl, when the external pH was within the range of 6.0 to 7.5 (Fig. 4.6).

Properties of PEPC Extracted from Base- or Acid-Loaded Leaf Discs

Changes in various properties of PEPC extracted from base-loaded leaf discs were studied, including the extent of light-activation and the response of the enzyme to two effectors, L-malate (a feedback inhibitor) and Glc-6-P (an allosteric activator).

Despite the marked stimulation in activity, light activation of PEPC in base-loaded leaf discs remained high. The malate sensitivity (inhibition by 1 mM malate at

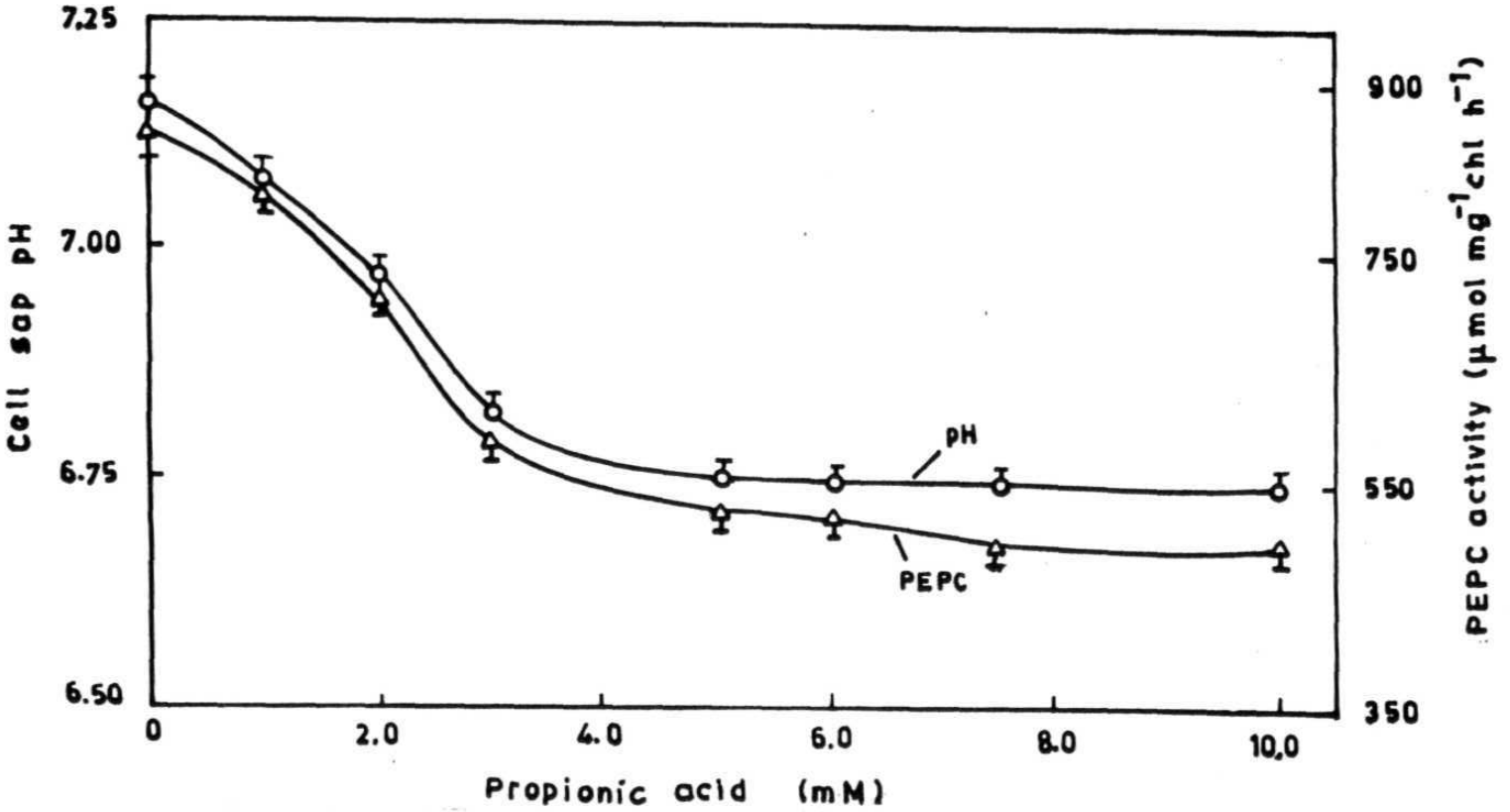


Figure 4.4. Effect of propionic acid (acid-loading) on the pH (O) and PEPC (Δ) activity in cell sap prepared from leaves of the C_4 species *A. pungens*. The leaf discs, predarkened for 2-h, were floated on various concentrations of propionic acid (prepared in 5 mM sodium-acetate buffer, pH 4.5). The extracts were made in unbuffered 0.3 M sorbitol after an incubation period of 90 min in propionic acid and used for determining the cell sap pH and PEPC activity. The control leaf discs were floated on buffer alone.

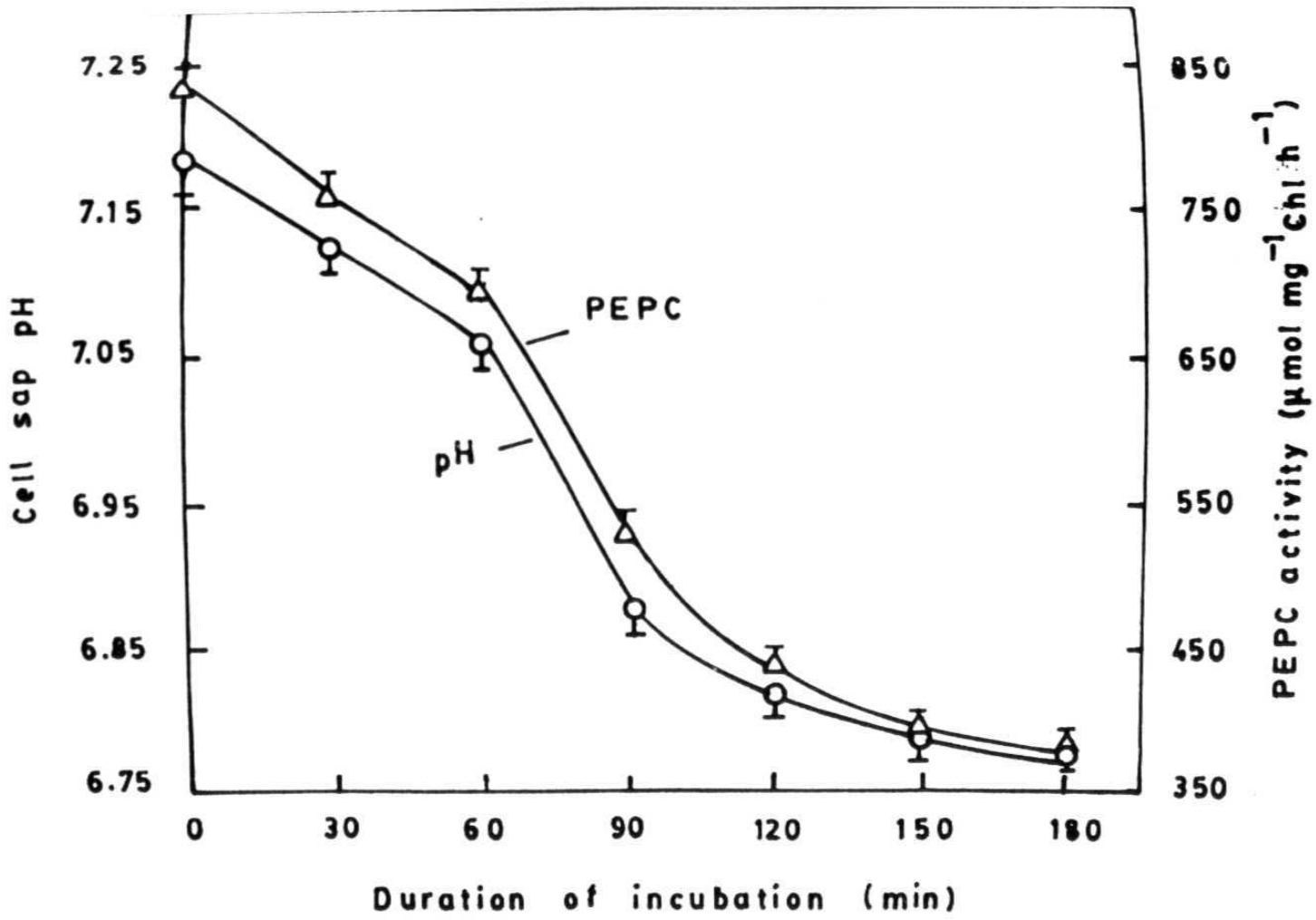


Figure 4.5. Time course of changes in cell sap pH (O) and PEPC activity (Δ) of crude extracts prepared from leaf discs of *A. pungens* incubated in 5 mM propionic acid (in 5 mM sodium-acetate buffer, pH 4.5). Cell sap pH and PEPC activity were determined at different time intervals, as indicated, using the same extract prepared in unbuffered 0.3 M sorbitol.

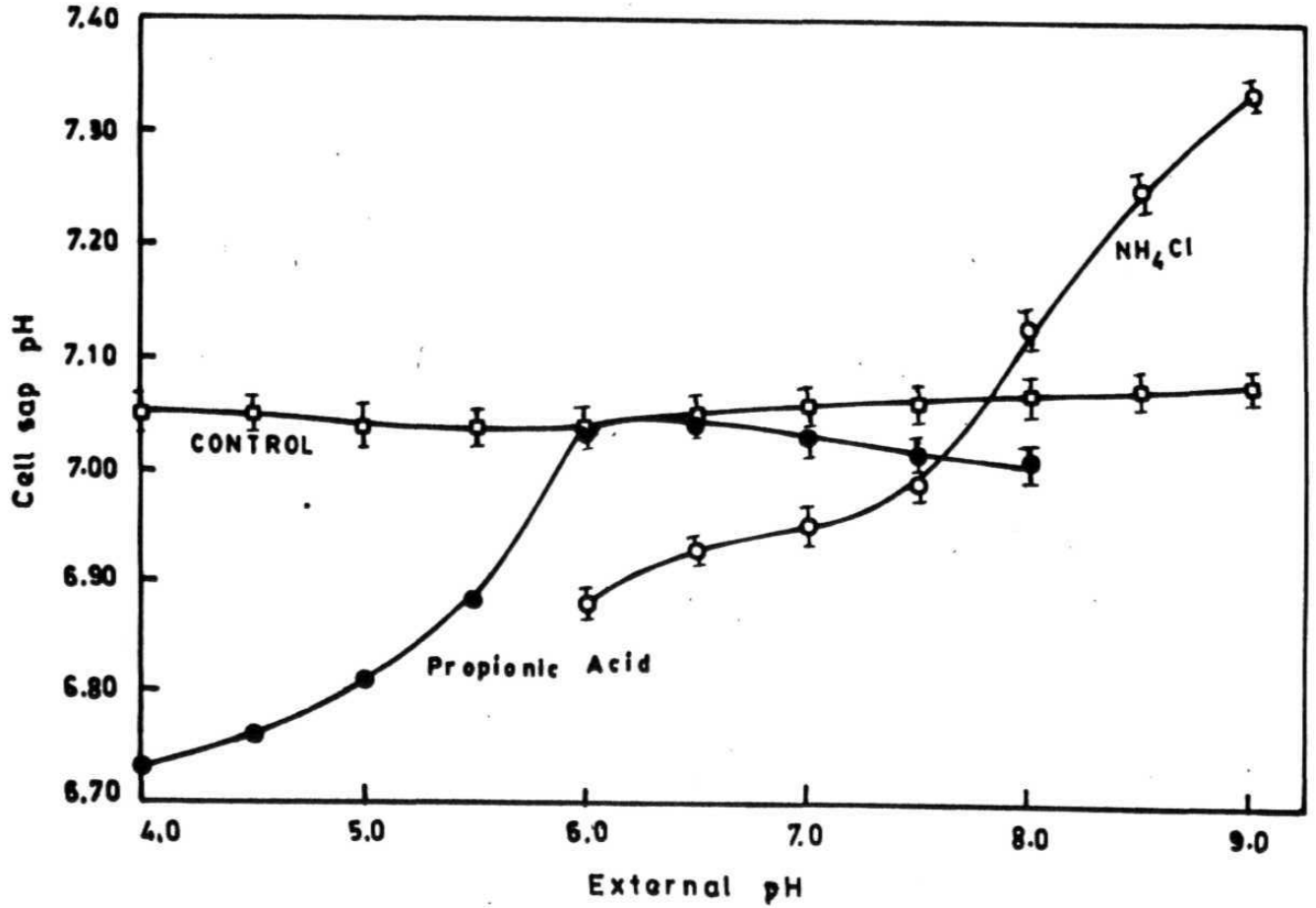


Figure 4.6. Effect of external pH (acid-/base-modulation) on cell sap pH. Leaf discs of *Alternanthera pungens*, predarkened for 2-h, were floated on either 7.5 mM NH_4Cl (O) or 5 mM propionic acid (O) prepared in the following buffers: sodium acetate - pH 4.5-5.5; Mes-KOH - pH 5.5-6.5; phosphate - pH 6.5-7.5; Tricine-KOH - pH 7.5-8.5; Ches-KOH - pH 8.5-9.0) for 90 min duration. Leaf discs floated on buffer alone (without NH_4Cl or propionic acid) constituted the control set ( ). After the completion of incubation period, the leaf discs were washed thoroughly with distilled water, blotted dry and used for the preparation of cell sap measurement of pH.

suboptimal pH and PEP concentration) of PEPC decreased slightly compared to the control enzyme (incubated in 5 mM Tricine-KOH, pH 8.5) (Table 4.1).

When leaf discs were preincubated for 6 h in the presence of 5 μM CHX, the extractable activity of dark form of PEPC from control or acid-, base-loaded leaf discs did not change much, while the light activation of the enzyme was impaired (but the stimulation by base-loading remained unchanged). There was no marked changes in either K_M(PEP) or extent of activation by Glc-6-P due to base-loading.

The pace of light activation of PEPC from NH_4Cl-loaded leaf discs was slightly faster than that of the enzyme in control leaf discs (incubated in buffer alone), whereas incubation in propionic acid restricted severely the pace of PEPC light-activation (Fig. 4.7). The malate sensitivity of PEPC was examined in order to assess changes in the phosphorylation status of PEPC, upon illumination, of the enzyme in leaf discs exposed due to base-/acid-loading. The sensitivity to L-malate remained unchanged (about 90% inhibition) by 1 mM L-malate due to acid-loading of leaf discs upon illumination. However, inhibition by L-malate of PEPC decreased markedly (by 56% and 60% from control) in both control (incubated in buffer alone) and base-loaded leaf discs (Figs. 4.8).

Probing the levels of PEPC protein in leaf extracts using Western blots, revealed that there was no change in the levels of PEPC protein after incubation of leaf discs with NH_4Cl or propionic acid (Fig. 4.9).

In vivo *reversibility of acid-/base-modulated changes*

Acid- or base-induced changes in cell sap pH and PEPC activity were reversible. When the base-loaded leaf discs of A. ***pungens*** were transferred to a medium

Table 4.1. Characteristics of PEPC extracted from leaf discs of *Alternanthera pungens* after pretreatment with 7.5 mM NH_4Cl (in 5 mM Tricine-KOH, pH 8.5) or propionic acid (in 5 mM sodium acetate buffer, pH 4.5).

Parameter	Pretreatment					
	Control (Buffer)		NH_4Cl		Propionic acid	
	Dark	Light	Dark	Light	Dark	Light
PEPC activity[b]	656 ±61	1594 ±136	1209 ±104	3201 ±168	387 ±31	738 ±64
L/D ratio		2.4		2.6		1.9
K_M (PEP) mM	1.26	1.16	1.21	1.01!	1.54	1.21
% Inhibition by 1 mM L-malate	88	54	74	46	91	87
K_I (malate) mM	0.48 ± 0.05	2.10 ± 0.24	0.60 ± 0.05	2.70 ± 0.23	0.49 ±0.05	0.49 ±0.04
% Activation by 5 mM Glc-6-P	61	85	60	88	82	78
K_A (Glc-6-P) mM	2.09 ±0.25	2.09 ±0.14	1.81 ±0.16	1.51 ±0.14	2.08 ±0.11	1 .89 ±0.13
PEPC activity after pretreatment with 5 µM CHX	609 ±46	908 ±68	1183 ±84	2021 ±99	n.d.[b] n.d.	n.d. n.d.
Inhibition of activity due to CHX	7	43	2	37	n.d.	n.d

[a] µmol mg^{-1} Chl h^{-1}.
[b] n.d. - not determined.

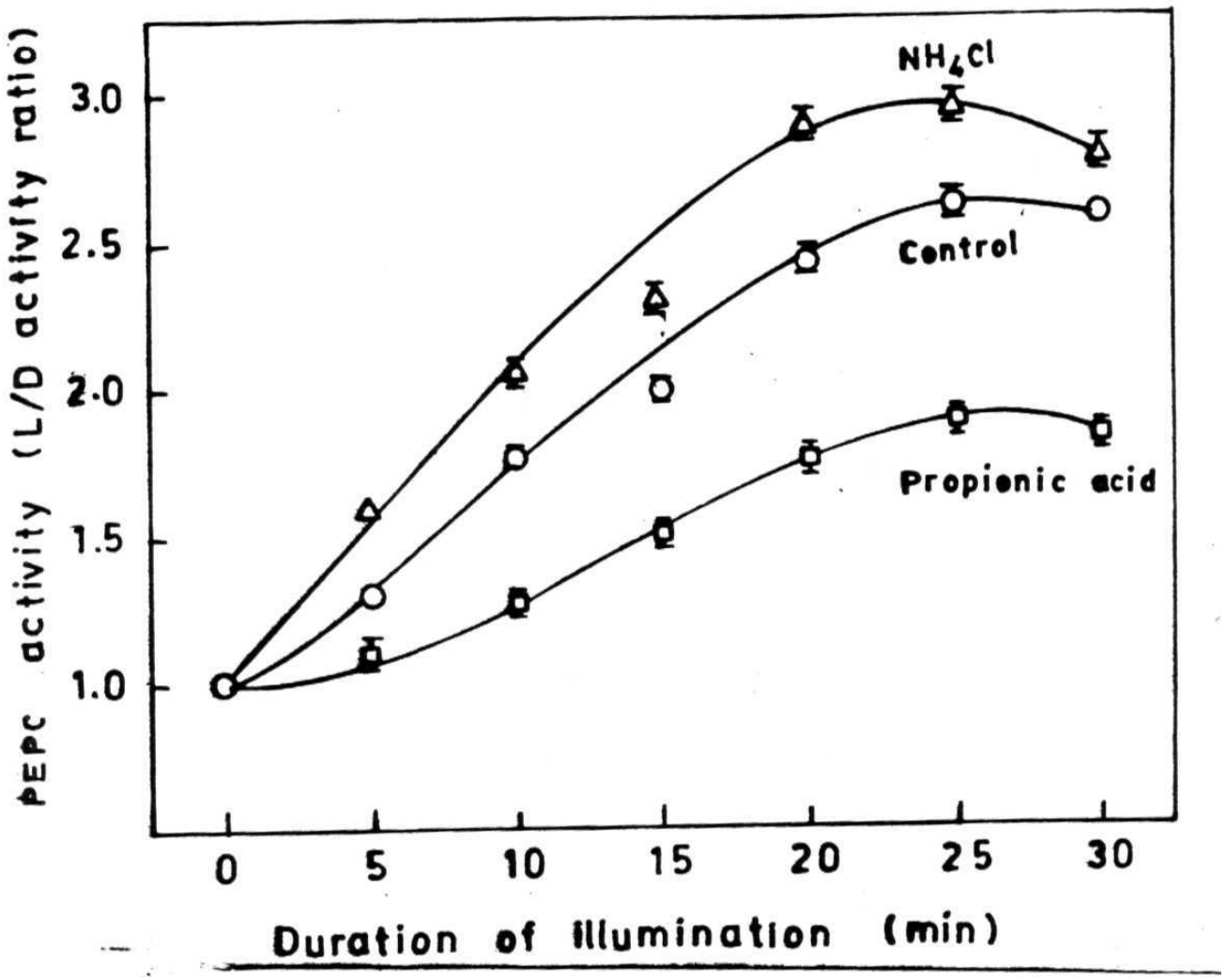


Figure 4.7. The pace of PEPC light-activation, as indicated by the light/dark activity ratio of the enzyme prepared from acid-loaded (□) or base-loaded ( ) leaf discs of *A. pungens*. Leaf discs incubated in buffer alone (5 mM Tricine-KOH, pH 8.5 for NH_4Cl and 5 mM sodium acetate, pH 4.5 for propionic acid, O). Illumination was at a light intensity of 1000 μmol m^{-2} s^{-1}.

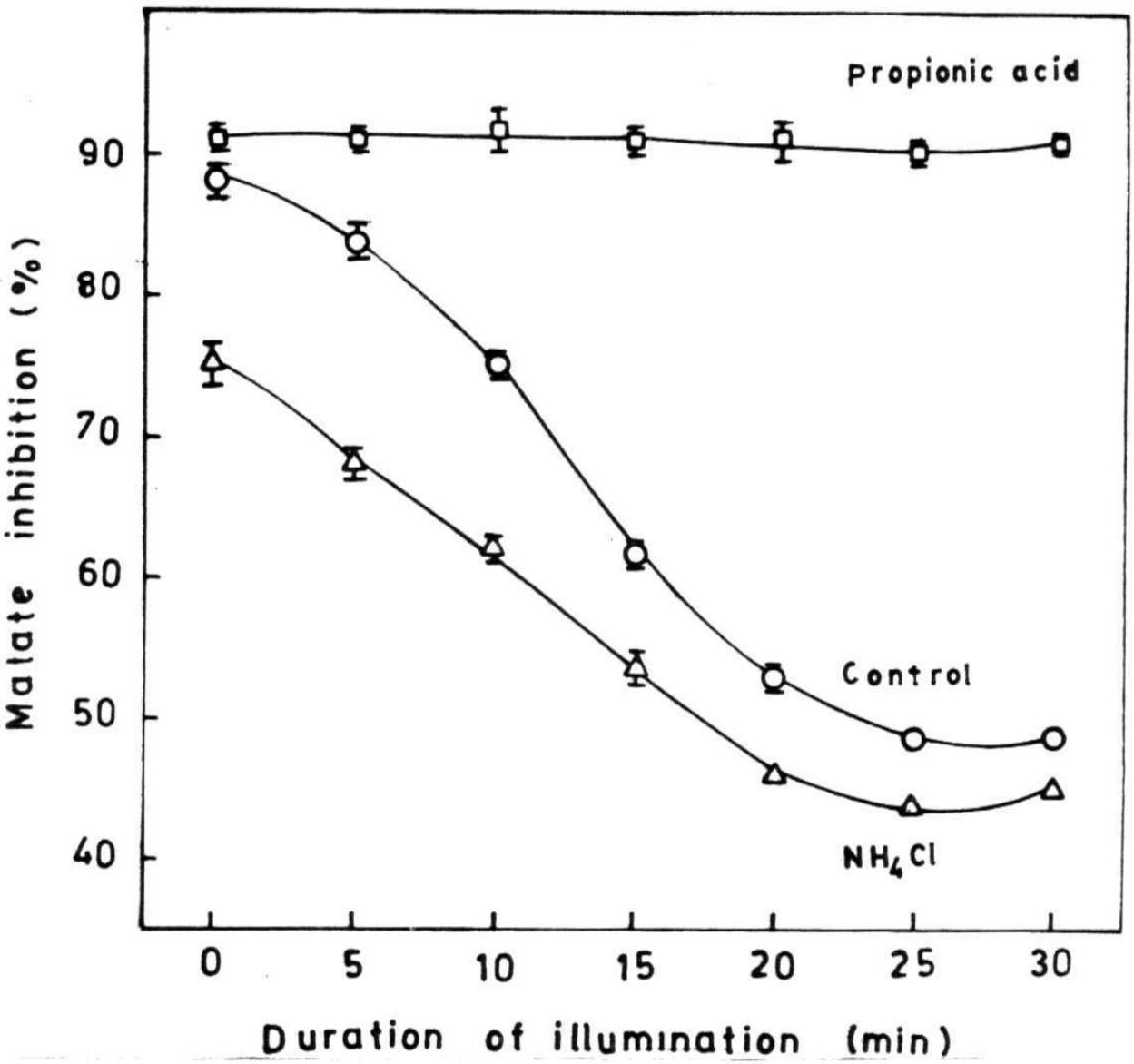
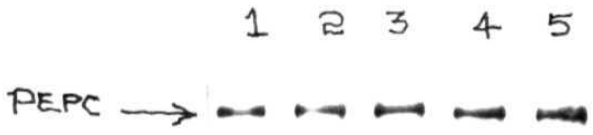


Figure 4.8. The L-malate sensitivity of PEPC extracted from acid- or base-loaded leaf discs of *A. pungens* after illumination. The inhibition of PEPC by 1 mM L-malate was assayed at suboptimal pH (7.3) and PEP (1.5 mM), in order to assess the phosphorylation status of PEPC, upon illumination, of the enzyme prepared from leaf discs subjected to acid- or base-loading. The sensitivity to L-malate remained unchanged (about 90% inhibition) due to acid-loading (p). However, inhibition by L-malate decreased markedly (O) control and base-loaded (A) leaf discs.

Figure 4.9. Probing the levels of PEPC protein in leaf extracts (subjected to various treatments), as indicated by Western blot analysis. The leaf discs were incubated for 90 mm in various test solution in darkness at 30 °C. After the completion of the incubation period, the discs were washed thoroughly, blotted dry, and used for preparing the extracts. Probing was done by using anti-*Amaranthus hypochondriacus*-PEPC serum. Equal amounts of protein (5 $\mu g\ lane^{-1}$) were loaded.

The number of lanes indicate leaf discs incubated in :

(1) water,
(2) 5 mM Tricine-KOH buffer, pH 8.5,
(3) 7.5 mM NH_4Cl in 5 mM Tricine-KOH, pH 8.5,
(4) 5 mM sodium-acetate buffer, pH 4.5, and
(5) 5 mM propionic acid in 5 mM sodium acetate buffer, pH 4.5.


1 2 3 4 5
PEPC

containing a weak acid (5 mM propionic acid in 5 mM sodium acetate buffer, pH 4.5), the cell sap pH and PEPC activity returned to the levels in control sample (Fig. 4.10A). Similarly, the low activity of PEPC in extracts from acid-loaded leaf discs recovered and increased upon transfer of the discs to an alkalinizing medium (7.5 mM NH_4Cl in 5 mM Tricine-KOH, pH 8.5; Fig. 4.1 OB).

Effect of other compounds capable of modifying cytosolic pH

Besides NH_4Cl, two other alkalinizing agents, procaine, 2-deoxyglucose were also effective in elevating cell sap pH and PEPC activity (Table 4.2). Similarly, at least

Table 4.2. Effect of alkalinizing (weak bases) or acidifying (weak acids) agents on cell sap pH and PEPC activity extracted from leaf discs of *Alternanthera pungens*, an NAD-ME type C4 species.

Treatment/ concentration	Cell sap pH	PEPC activity	% Inhibition by L-malate	L/D rati ratio
		$\mu mol\ mg^{-1}\ Chl\ h^{-1}$		
Control	7.07	774 ± 49 (100)[b]	82	2.6
Weak bases				
NH_4Cl (7.5)	7.33	1625 ± 104 (210)	72	2.8
Procaine (10)	7.32	1549 ± 113 (200)	75	2.8
2-deoxyGlc (5)	7.30	1396 ± 113 (180)	76	2.7
Average	7.32	(197)	74	2.8
Weak acids				
Propionic (5)	6.69	441 ± 38 (57)	87	1.9
Butyric (5)	6.64	387 ± 31 (50)	91	1.9
Salicylic (5)	6.64	347 ± 39 (45)	90	1.8
Average	6.66	(51)	89	1.9

[a] % Inhibition by 1 mM L-malate..

[b] % over control (incubated in respective buffer alone).

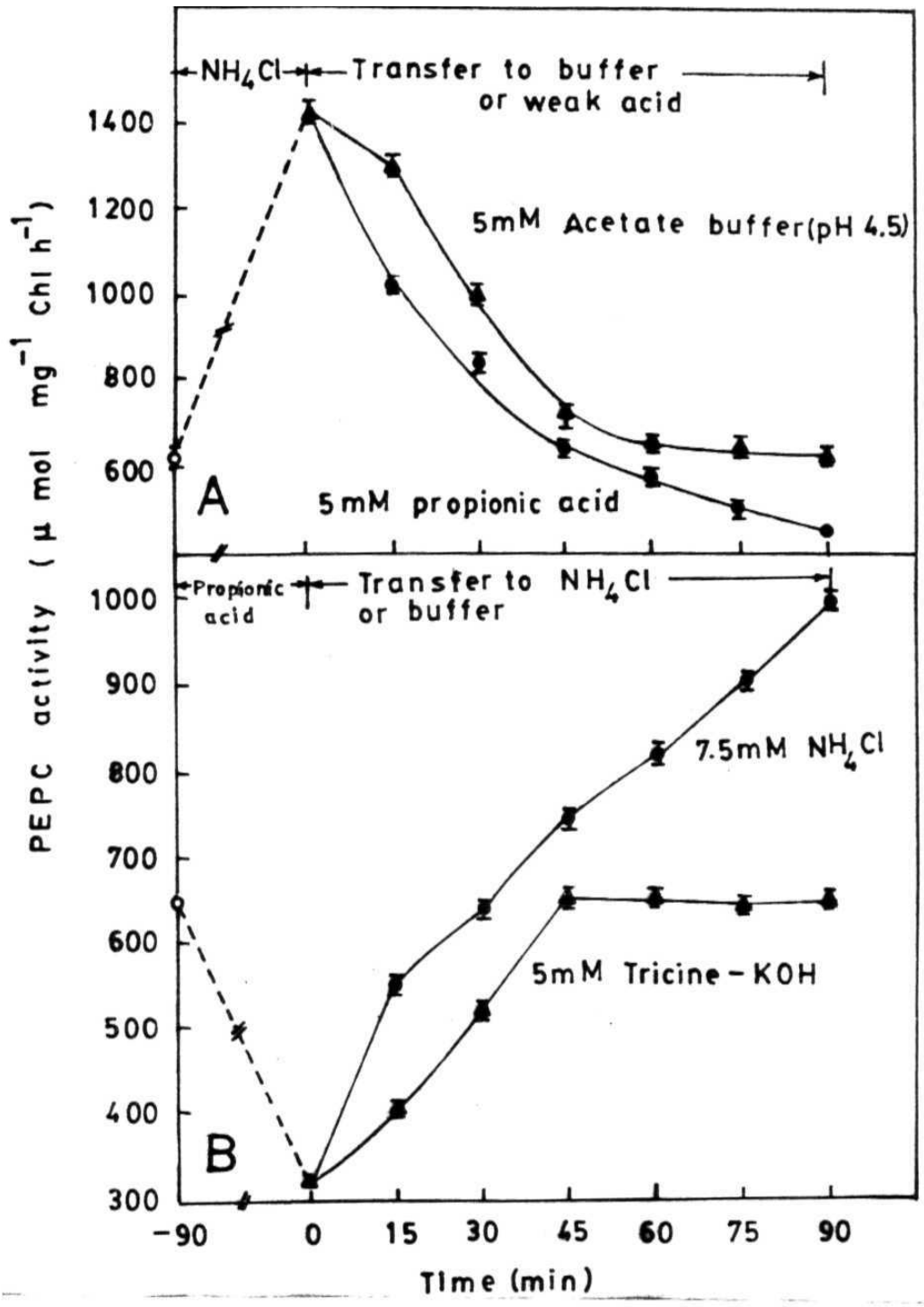


Figure 4.10. The reversibility *in vivo* of changes in PEPC activity in leaf discs of the C_4 species (*A. pungens)* after either base-loading (A) or acid-loading (B). After 90-min incubation in 7.5 mM NH_4Cl , the discs were transferred to either 5 mM sodium acetate buffer (pH 4.5) alone or 5 mM propionic acid in 5 mM sodium acetate buffer (pH 4.5). The extracts were prepared at different time intervals (as indicated) and used for the determination of PEPC activity. Similarly, in B after 90 min incubation in 5 mM propionic acid (pH 4.5), the discs were transferred either to 5 mM Tricine-KOH, pH 8.5, or 7.5 mM NH_4Cl. At different time intervals extracts were made from the discs and used to determine PEPC activity.

three weak acids, propionic, butyric and salicylic acid, decreased PEPC activity and cell sap pH in leaf discs of A. *pungens* (Table 4.2).

Comparative Studies with Leaf Discs of C_3, C_4 and C_3-C_4 Intermediate Species

The effect of cytosolic alkalinization on cell sap pH, PEPC activity and its sensitivity to L-malate were studied in leaf discs of a few C_3 species, C4 plants and C3-C4 intermediates (Table 4.3). On incubation with NH_4Cl, the average increase in pH and PEPC activity from leaf discs of C_4 plants were 0.29 unit and >200% (over control), respectively. On the other hand, the pH and PEPC activity in leaf discs of C3 and C3-C4 intermediate plants rises (on base loading) only by 0.1 pH unit and <25%, respectively. Thus, the leaf discs of C4 species were more responsive to cytosolic alkalinization than those from C3 or C3-C4 intermediate species (Table 4.3).

Discussion

Our results demonstrate the marked modulation of PEPC activity in leaf discs of C4 species in response to alteration in cytosolic pH by acid-/base-loading. Incubation of leaf discs in 7.5 mM NH_4Cl or 10 mM procaine (in 5 mM Tricine-KOH, pH 8.5) not only elevated the cell sap pH but also the activity of PEPC (Figs. 4.1 to 4.3 and Table 4.1). The enhancement of PEPC activity can therefore be attributed to the elevation of cytosolic pH brought about by the exogenous weak bases. A significant increase in cytosolic pH after exposure to NH_4^+ was reported in maize root tips (Kurkdjian *et al.* 1978), *Acer pseudoplatanus* root cells (Roberts *et al.* 1982) and very recently in mesophyll protoplasts and cells from an NADP-type C4-monocot *Digitaria sanguinalis* (Duff *et al.* 1996, Giglioli-Guivar'ch *et al.* 1996). Felle and Bertie (1986) showed that the cytoplasmic pH in *Riccia fluitans* cells increased from 7.5 to 8.1 on preincubation with 0.2 mM procaine, while the membrane potential dropped down.

Table 4.3. Effect of NH_4Cl (7.5 mM in 5 mM Tricine-KOH, pH 8.5) pretreatment on cell sap pH and PEPC activity from leaves of C_3, C4, and C_3-C_4 intermediate plant species.

Photosynthetic type/ Species	Cell sap pH		PEPC activity		% Inhibition by 1 mM L-malate	
	Control	NH_4Cl	Control	NH_4Cl	Control	NH_4Cl
			$\mu mol\ mg^{-1}\ Chl\ h^{-1}$			
C_3 Plants						
Alternanthera sessilis	6.92	7.00 **(0.08)[a]**	37 **(100)[b]**	38 **(103)[b]**	52	51 **(1)[c]**
Arachis hypogea	6.83	6.93 (0.10)	31 (100)	37 (120)	47	45 (2)
***Pisum** sativum*	6.91	7.00 (0.09)	30 (100)	32 (105)	52	50 (2)
Lycopersicon esculentum	6.84	6.95 (0.11)	31 (100)	37 (119)	55	49 (6)
Tagetes erecta	6.91	7.00 (0.09)	28 (100)	32 (114)	50	47 (3)
*Tridax **procumbens***	6.92	7.02 (0.11)	21 (100)	24 (114)	57	54 (3)
Average		**(0.10)[a]**	**(100)[b]**	**(113)[b]**	52	**(3)[c]**
C3-C4 Intermediates						
Alternanthera ficoides	7.02	7.14 (0.12)	54 (100)	72 (133)	57	50 (7)
A. tenella	7.04	7.16 (0.12)	48 (100)	62 (129)	58	50 (8)
*Mollugo **nudicaulis***	7.00	7.10 (0.10)	39 (100)	48 (123)	59	54 (5)
Parthenium hysterophorus	7.00	7.09 (0.09)	21 (100)	24 (112)	57	50 (7)
Average		**(0.11)[a]**	**(100)[b]**	**(124)[b]**	58	**(7)[c]**

(Continued in the next page)

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C_4 Plants						
Alternanthera pungens	7.09	7.35 (0.26)	621 (100)	1467 (236)	86	72 (14)
Amaranthus *hypochondriacus*	7.10	7.39 (0.29)	450 (100)	1100 (264)	83	70 (13)
A. viridis	7.18	7.47 (0.29)	813 (100)	1635 (201)	86	69 (17)
Gomphrena *globosa*	7.06	7.32 (0.26)	568 (100)	1159 (204)	83	72 (11)
Sorghum bicolor	7.05	7.36 (0.31)	909 (100)	1624 (179)	84	73 01)
Zea mays	7.07	7.39 (0.32)	428 (100)	728 (170)	83	70 (13)
Average		**(0.29)[a]**	**(100)[b]**	**(209)[b]**	84	**(13)[c]**

[a] Δ pH, due to NH_4Cl.; [b] % activity over control ; [c] Δ Change due NH_4Cl.

When the leaf discs were preincubated in darkness for 90 min in 5 mM sodium acetate buffer alone the extractable PEPC activity did not change much. However, when 5 mM propionic acid (in the above mentioned buffer) was used as the incubation medium, PEPC activity decreased by about 50%, compared to those of control (Figs. 4.4 and 4.5). There are reports on induction of cytosolic acidification by weak acids (Reid *et al.* 1985, Basso and Ulrich-Eberius 1987).

Cytoplasmic pH is maintained close to neutrality by powerful ion exchange mechanisms at the plasmalemma (Raven 1985, 1995, Guern *et al* 1991). In our experiments pH of the cell sap remained relatively constant when the external pH was

in the range of 6 to 7.5 (Fig. 4.6). Cytosolic acidification or alkalinization occurred only when the external pH was either above (>8) or below (<6) these limits.

Activation (by ammonia or procaine) or inactivation (by propionic acid) of PEPC appears to be a result of alkalinization or acidification, respectively. When ammonia (a weak base; pK_a 9.25) or a weak acid enters the cell, it dissociates, thereby producing OH" or liberating H^+, respectively. The cell sap became markedly alkaline upon incubation with NH_4Cl only when the external pH was above 8. This could be due to the high pK_a value of ammonia (Muller *et al.* 1980, Kleiner 1993). Acid-/base-induced changes in cytosolic pH are reversible. A strong decrease in cytosolic pH induced by propionic acid loading is followed by a partial recovery, even when the acid is still present in the external medium in *Acer pseudoplatanus* cells (Guern *et al.* 1986). This clearly demonstrates the operation of strong mechanisms able to compensate for the proton-load caused by the entry of propionic acid. Similar results were also obtained for *Riccia fluitans* cells using weak acids (Franchisse *et al.* 1988).

The use of buffered medium during extraction made it possible to examine the kinetic characteristics of PEPC from the leaf discs pretreated with NH_4Cl. The properties of the enzyme prepared from base-loaded leaf discs were marginally different from the control (buffer treated) leaf discs. Due to cytosolic alkalinization, the activity of PEPC increased by almost two-fold and its sensitivity to L-malate (dark) decreased by ≤14% compared to the control. The K_I(malate) increased by 25% as a result of alkalinization. In illuminated leaves, L-malate sensitivity of PEPC decreased by 34% and the K_I(malate) increased by about four-fold. Thus, the changes observed in the kinetic and regulatory properties of PEPC due to alkalinization were not exactly comparable to those induced by light (Table 4.1). In addition, the rate and

extent of light-activation even increased (marginally) due to the alkalinization (Fig. 4.7).

Studies using isolated protoplasts from *Sorghum* (Pierre *et* al. 1992) and *Digitaria sanguinalis* (Giglioli-Guivar'ch *et al.* 1996) have shown that cytosolic pH is an important factor in regulating the light-induced changes in activity and kinetic/regulatory properties of PEPC. However, cytosolic pH may modulate either PEPC or PEPC-PK or both (Rajagopalan *et al.* 1993, 1994). PEPC-PK from maize as well as *Mesembryanthemum crystallinum* have been found to require alkaline pH for maximal activity (Wang and Chollet 1993a, Li and Chollet 1994).

The activity of C4-PEPC at pH 7.3 is stimulated nearly two- to three-fold upon illumination (Table 4.1; Rajagopalan *et al.* 1993). Such light/dark modulation of PEPC in C4 plants is mediated by reversible protein phosphorylation (Rajagopalan *et al.* 1994, Chollet *et al.* 1996), which results in decreased feedback inhibition by L-malate. The sensitivity to L-malate is therefore considered as an indicator of the apparent phosphorylation state of the enzyme (Jiao and Chollet 1991, Nimmo 1993). Although the activity of PEPC was stimulated by about two-fold as a result of base-loading, the decrease in malate sensitivity was much less than that caused by illumination alone (Table 4.1). We therefore suggest that the effect of cytosolic alkalinization was more on PEPC activity *per se* than on the PEPC-PK activity.

Illumination enhances the activity of PEPC-PK in leaves of C_4 species by a CHX-sensitive mechanism (Jiao and Chollet 1991). Treatment of leaf discs for 6 h with CHX decreased the extent of light-activation of PEPC but did not alter the increase in PEPC activity due to alkalinization (Table 4.1). Thus, the possibility of the synthesis of PEPC or PEPC-PK during alkalinization can be ruled out.

We also propose that these effects of acid-/base-modulation of PEPC are not due to the interaction of limited proteolysis or phosphorylation/dephosphorylation of the enzyme. Inclusion of PMSF or benzamidine (protease inhibitors) or NaF (inhibitor of phosphatase, though not specific) in the extraction medium had no effect on the increase in PEPC activity due to base-loading.

Western blotting using anti-PEPC antiserum confirmed that there was no detectable change in PEPC protein during acid-/base-modulation of leaf discs (Fig. 4.9). We therefore suggest that the rise in cytosolic pH mainly enhances the activity of PEPC. It is also possible that the changes in cytosolic pH would affect free Ca^{24} levels, subsequent light-activation of C4-PEPC-PK, as has been suggested recently (Giglioli-Guivar'ch *et al.* 1996, Duff *et al.* 1996).

An interesting observation is the marked increase in cytosolic pH and PEPC activity in leaf discs of several C4 plants, upon incubation in NH_4Cl, with only marginal effects in leaf tissue from C3 or C3-C4 intermediate species on incubation in NH_4Cl (Table 4.3). While we do not have any explanation for this phenomenon, one of the reasons could be a possible difference in buffer capacity of different leaves and cellular organelles (Discussed in *Chapter Seven* of this thesis).

The kinetic and regulatory properties of PEPC from the acid- or base-loaded leaf discs (Table 4.1) indicate a relatively stable alteration in the enzyme. Marked modulation of PEPC activity *in vitro* by pH (or metabolites or protein concentration) has been recorded and related to changes in association/dissociation state of maize PEPC (Kruger and Kluge 1987, Wedding *et al.* 1992, Willeford and Wedding 1992). The importance of such changes in oligomeric status in regulation of PEPC is however questioned (Chollet *et al.* 1996). However, we speculate that cytosolic

alkalinization may induce conformational changes in PEPC activity making the enzyme more phosphorylatable by PEPC-PK.

The effect of pH modulation on PEPC activity reported in this study may be relevant under other conditions besides exposure to light/darkness. For example, the pH of cytosol is known to decrease under anoxia or gaseous (NO_2 or SO2) pollution and increase after nitrate feeding (Pfanz and Heber 1989, Wagner *et al.* 1990, Heber *et al.* 1994).

The present study emphasizes the significant role of cytosolic pH in modulating the activity of C4-PEPC. The changes in cytosolic pH and PEPC activity induced by the weak bases or weak acids are not only significant but reversible, both *in vivo* (Fig.. 4.10) and *in vitro.* Besides the physiological importance of this basic observation, acid-/base-modulation of PEPC *in vivo* could be an important tool to study the C_4 enzyme.

Chapter Five

Indirect Assay of Phosphorylation and Dephosphorylation of PEP Carboxylase *In Vitro*

Introduction

The reversible phosphorylation of enzymes plays an important and ubiquitous role in the regulation of cellular metabolism. The phosphorylation state of an enzyme-protein is a dynamic process, controlled by protein kinases (which phosphorylate the substrate protein) and protein phosphatases (which dephosphorylate the phosphorylated protein). This mode of covalent modulation of enzymes has been investigated extensively in animal systems, but for a few plant enzymes. For example, at least three cytosolic enzymes, *viz.*, PEPC, nitrate reductase (NR) and sucrose-phosphate synthase (SPS), have been subjected to posttranslational regulation by phosphorylation-dephosphorylation cascade (Huber *et al.* 1994, Rajagopalan *et al.* 1994, Verslues *et al.* 1996).

The light-form (phosphorylated) of C4-photosynthetic-PEPC is three- to four-fold more active and less sensitive to feedback inhibition by its products of carboxylation (oxalacetate, L-malate or aspartate), than the corresponding dark-form (dephosphorylated) when assayed at suboptimal, but physiological, concentrations of PEP and pH. One of the reasons for such changes in the activity and regulatory properties of the enzyme is the phosphorylation (in light) and dephosphorylation (in darkness) of an N-terminus Ser residue (Jiao and Chollet 1991).

Experiments conducted *in vivo* as well as *in vitro* have established that C_4-photosynthetic-PEPC undergoes reversible phosphorylation/desphosphorylation, mediated by PEPC-PK/PEPC-PP (Rajagopalan *et al.* 1994, Chollet *et al.* 1996). This posttranslation mechanism plays a cardinal role in regulating the PEPC activity during

light/dark transitions and the enzyme's sensitivity to feedback inhibitors (L-malate, aspartate) and allosteric activators (Glc-6-P, triose-P) (Jiao and Chollet 1991, Rajagopalan *et al.* 1994).

Further studies on the light- and dark-form of PEPC including *in vitro* phosphorylation or dephosphorylation using incubation with Mg^{2+}-ATP or alkaline phosphatase (AP), respectively, would be helpful to understand the optimal conditions and regulation of phosphorylation/dephosphorylation of the enzyme. These analyses are likely to provide critical insight into the relative contribution of covalent modification and regulatory metabolites to the overall light-activation of the enzyme *in vivo.*

In this chapter, the *in vitro* phosphorylation and dephosphorylation of PEPC was assessed by using agents capable of phosphorylating (ATP) or dephosphorylating (AP) the enzyme. In some of the experiments α-D-Man is used, which results in sequestration of intracellular Pi (Moore 1981).

Base-loading (with NH_4Cl or other weak bases) of leaf discs enhances the activity of PEPC by >100% *(Chapter Four* of this thesis). However, it is not clear if such base-loading stimulates the activity of PEPC or increases the phosphorylation status of PEPC/PEPC-PK or both. An attempt has therefore been to study the effect of base-loading (7.5 mM NH_4Cl, pH 8.5) on the phosphorylation status of the enzyme.

Results

On preincubation of leaf extracts (particularly from the illuminated leaf discs) with Mg^{2+}-ATP enhanced the activity of PEPC, whereas the activity of the control enzyme (incubated in phosphorylating mixture without Mg^{2+}-ATP) remained almost constant. Since L-malate sensitivity indicates the phosphorylation status of PEPC, the inhibition

by 1 mM L-malate of PEPC was also monitored during all these experiments of *in vitro* phosphorylation by Mg^{2+}-ATP, dephosphorylation by AP or restriction of phosphorylation by α-D-Man..

One millimolar ATP and an incubation period of 30 min was optimal for ATP-dependent stimulation (obviously due to *in vitro* phosphorylation) of PEPC (Fig. 5.1 A). The activity of PEPC in extracts from illuminated leaf discs increased by nearly 2.5-fold over control set (without ATP).

The L-malate sensitivity decreased from 82% to 67% and from 51% to 35% as a result of *in vitro* phosphorylation in extracts from darkened and illuminated leaf discs of *Alternanthera pungens,* respectively (Fig. 5.1B).

***In Vitro* Dephosphorylation of PEPC**

The use of AP is a common approach to desphosphorylate an enzyme-protein and assess the enzyme activity in relation to phosphorylation (Nimmo *et* al. 1986). Our preliminary experiments showed that 5 U mL^{-1} AP and a period of 60 min incubation as the optimum (Figs. 5.2A). The activity or L-malate sensitivity of dark-form PEPC was not significantly altered by treatment with AP.

When crude extracts of PEPC prepared from darkened leaf discs incubated in AP mixture, no significant (<5% inhibition) change in the activity and malate sensitivity (inhibition increased by 2%) observed (Fig. 5.2B). AP had significant effect on PEPC extracted from illuminated leaf discs. The activity decreased by 53% and sensitivity to L-malate enhanced from 53% to 85% (Fig. 5.2B).

Effect of α-D-Manrose Feeding

a-D-Man, an analogue of Glc, was employed to assess whether light-activation of PEPC involves protein phosphorylation and examined the effects of Man on light/dark

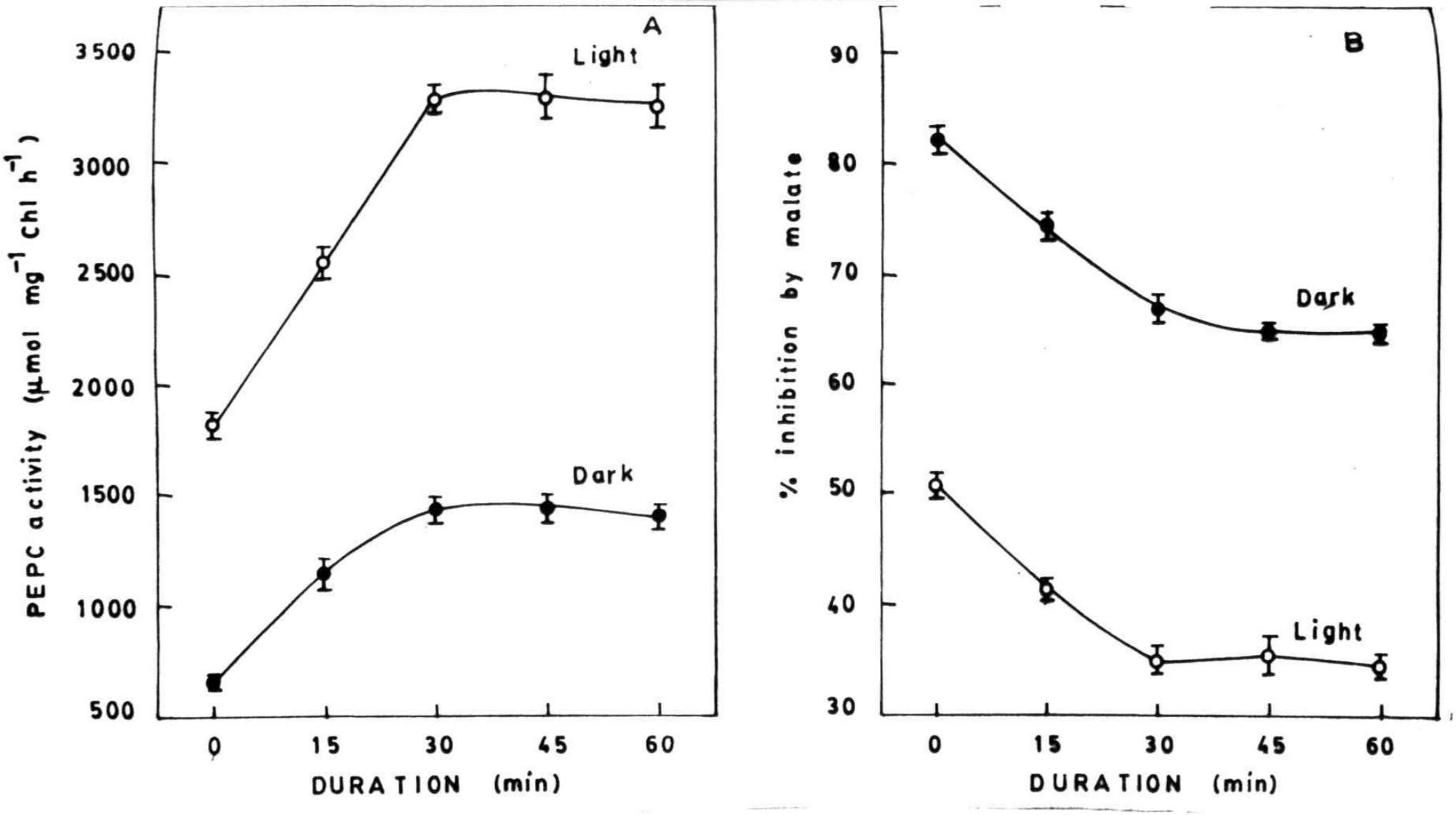

Figure 5.1. *In vitro* phosphorylation of PEPC extracted from leaf discs of *A. pungens*. The activation of PEPC by preincubation with ATP and $MgCl_2$ of leaf extracts prepared from either leaf discs either illuminated (O) or kept in dakrkness (O). at the indicated times, 10 µL aliquots were removed from incubation mixture and assayed for PEPC avtivity (A) and sensitivity to 1mM L-malate (B). The decrease in L-malate sensitivity indicates the phosphorylation of PEPC.

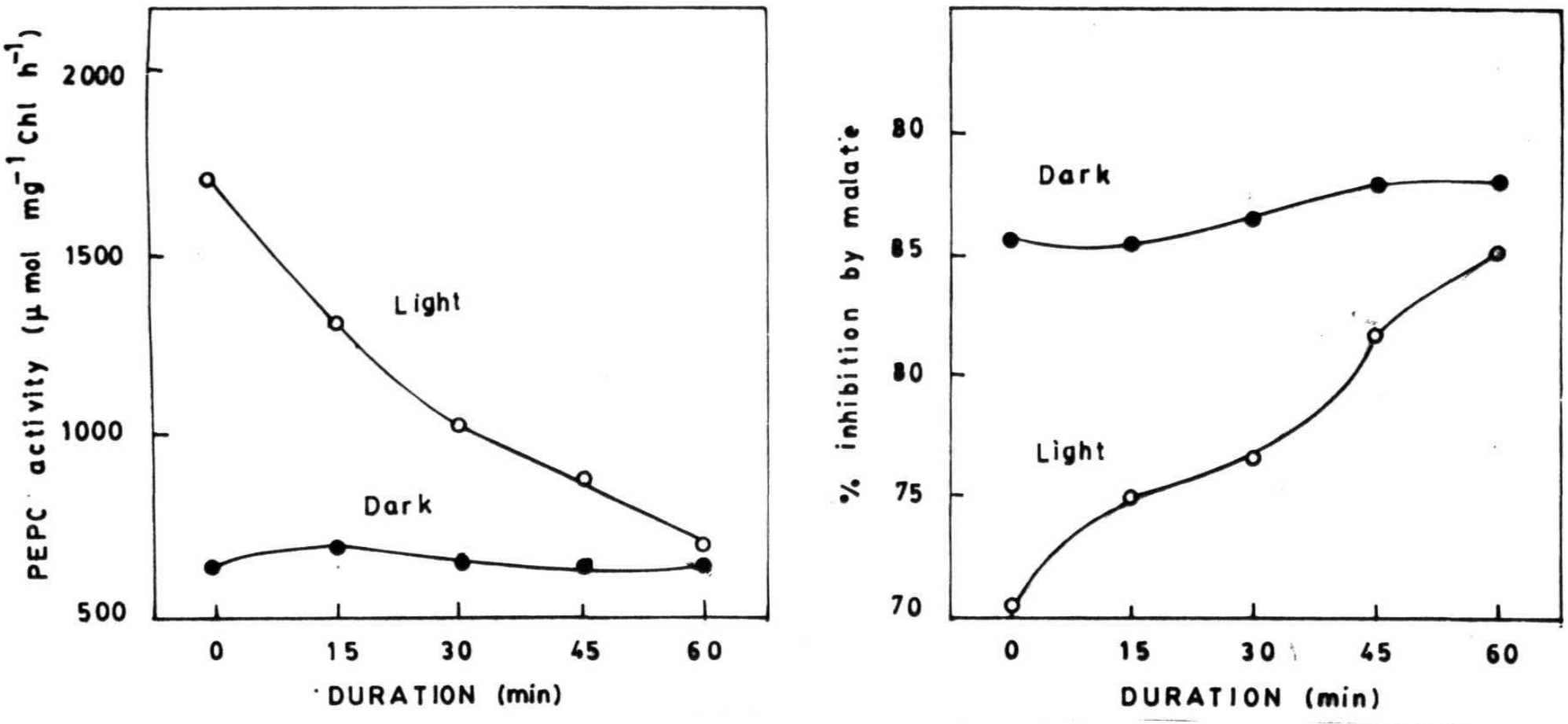

Figure 5.2. Dephosphorylation of PEPC by incubation of leaf extracts with exogenous alkaline phosphatase. AP-dependent dephosphorylation mixture contained (final concentrations): 20 mM Hepes-KOH, 5 U mL^{-1} AP and 5 mM $MgCl_2$, and the dephosphorylation was carried out 30 °C. PEPC activity (A) and L-malate inhibition (B) were measured, at intidicated time intervals, by taking 10-μL aliquots of the mixture.

transition and the changes in PEPC activity *in vivo.* Man was supplied to the leaf discs in order to decrease the cytosolic Pi by sequestering Pi as Man-6-P, thereby inhibiting the synthesis of ATP and the kinase activity without inhibiting the phosphatase reaction.

There was no marked change (≤10%) in the activity of PEPC extracted from darkened leaves (Fig. 5.3A). However, the activity decreased by 70% (as a result of Man feeding) upon illumination (Fig. 5.3A). In addition, L-malate sensitivity of PEPC decreased and reached to that of control (Fig. 5.3B).

Effect of Base-Loading on Phosphorylating/Dephosphorylating Agents

Effect of* In Vitro *Phosphorylation of PEPC with ATP

PEPC activity from crude extracts prepared from base-loaded leaf discs (in darkness) of *Alternanthera pungens* altered as a result of *in vitro* phosphorylation by incubation with Mg^{2+}-ATP. The activity of PEPC extracted from NH_4Cl-incubated leaf discs (in darkness) increased by more than two-fold, while L-malate sensitivity decreased from 75% to 54% (Figs. 5.4A,B).

Effect of* in vitro *dephosphorylation by AP of PEPC

Incubation with 5 U mL^{-1} decreased PEPC activity by 47% in extracts prepared from leaf discs kept in darkness. On the other hand, AP-treatment decreased PEPC activity in extracts prepared from base-loaded leaf discs by 65% (Table 5.1). Similarly, the L-malate sensitivity increased from 70 to 80% (in base-loaded leaf discs kept in darkness), while the L-malate sensitivity enhanced from 30 to 85% in base-loaded and illuminated leaf discs after AP-treatment. The light/dark activity ratios increased marginally changed from 3.0 to 3.6 due to base-loading (Figs. 5.5 A,B and Table 5.1).

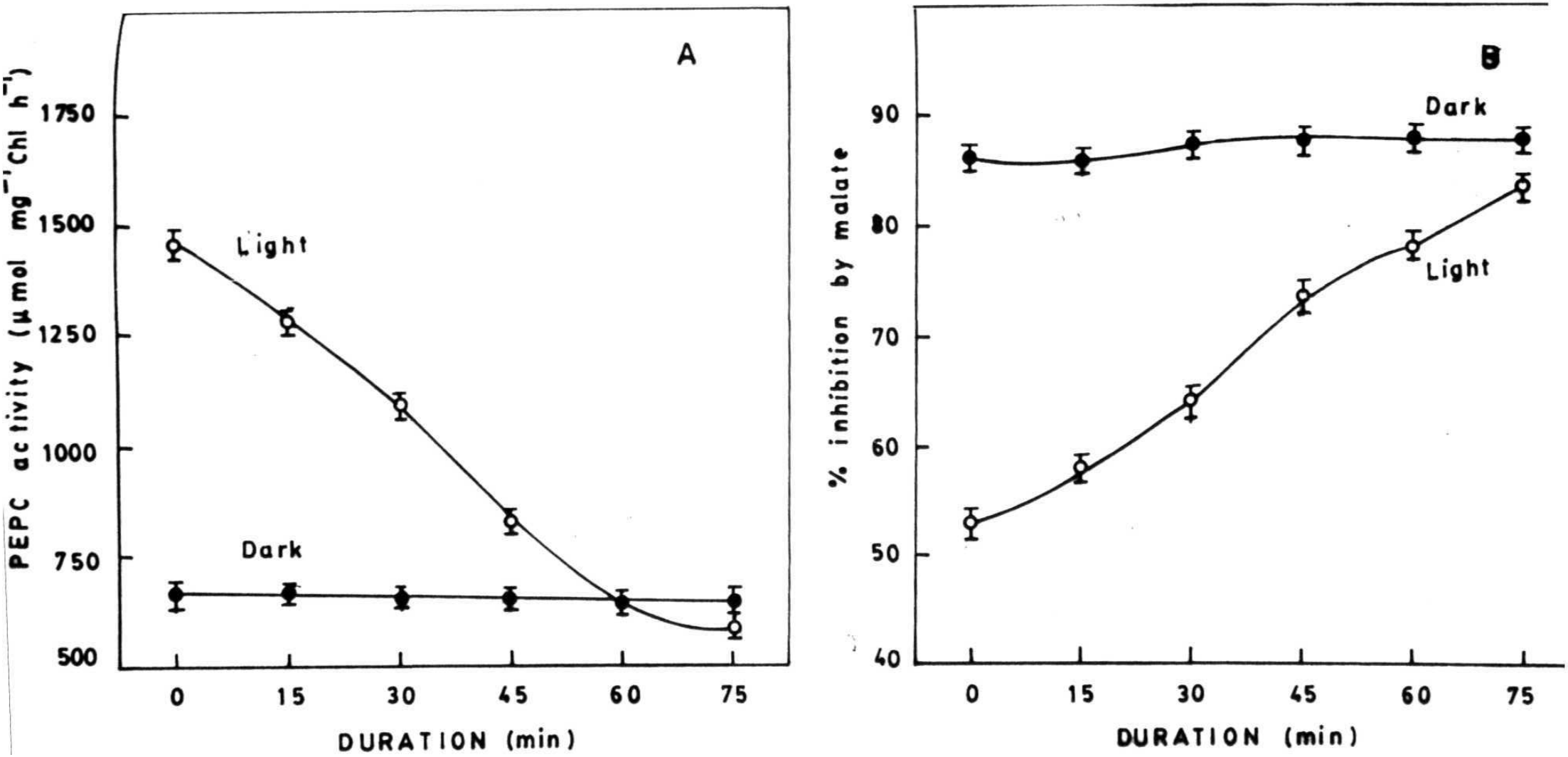

Figure 5.3. Effect of *in vivo* feeding of 5 mM α-D-mannose on ATP-activation of PEPC.. 5 mM Man (prepared in 5 mM Hepes-KOH, pH 7) was fed to the leaf discs predarkened for 2-h in darkness. After the completion of incubation, the discs were either illuminated (at 1000 µmol m^{-2} s^{-1} for 30 min at 30°C) or kept in darkness for the same duration. After illumination, leaf extracts were rapidly prepared and used for PEPC activity (A) and for to test 1 mM L-malate inhibition (B).

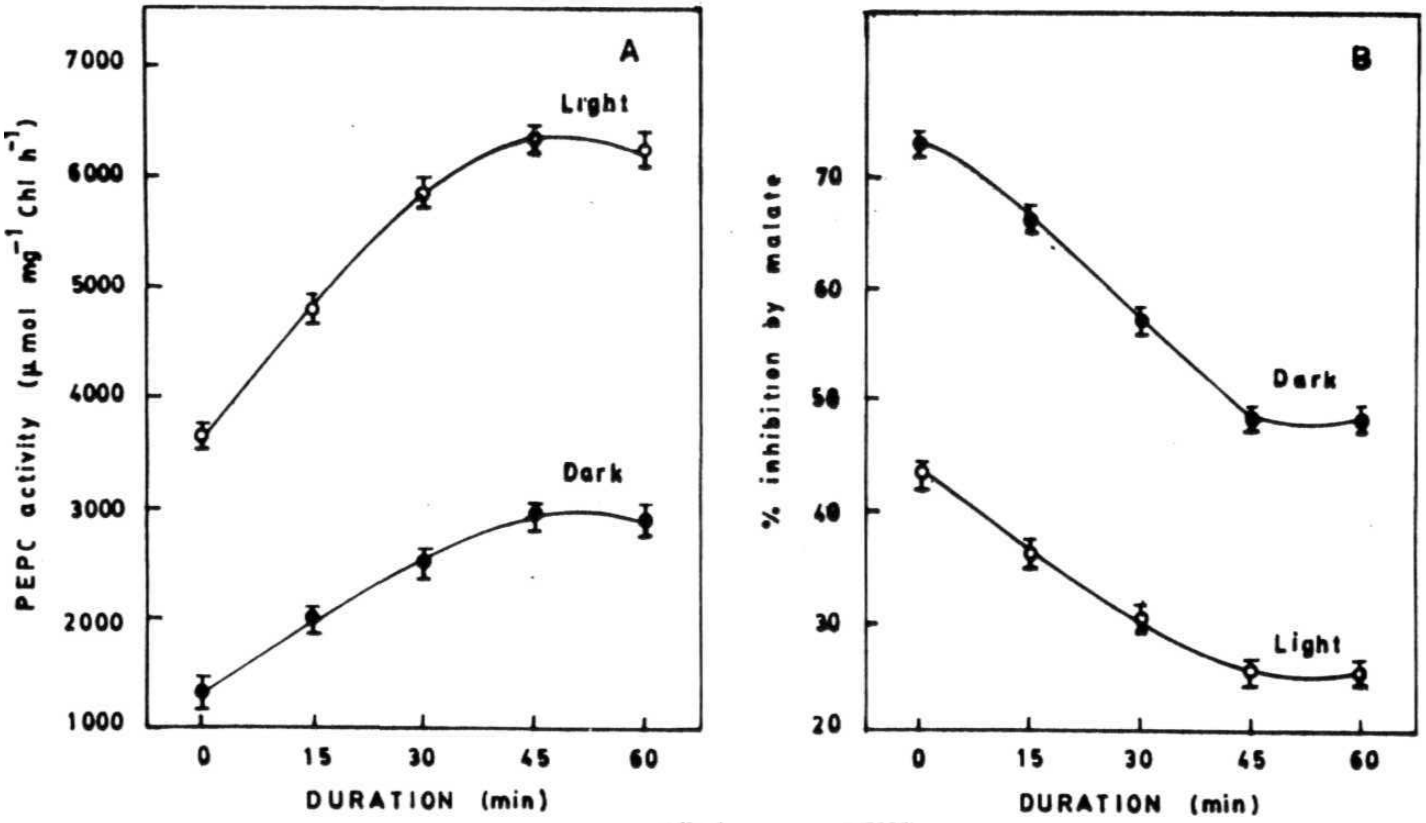

Figure 5.4. *In vitro* phosphorylation of PEPC extracted from leaf discs of *A. pungens* fed exogenously with NH_4Cl (base-loading). The activation of PEPC by preincubation with ATP and $MgCl_2$ of leaf extracts prepared from either leaf discs either illuminated (O) or kept in dakrkness (O). At the indicated times, 10 μL aliquots were removed from incubation mixture and assayed for PEPC avtivity (A) and sensitivity to 1mM L-malate (B). The decrease in L-malate sensitivity indicates the phosphorylation of PEPC. The pattern may be compared to that in Fig. 5.1.

Table 5.1. Effect of *in vitro* phosphorylating and dephosphorylating agents on PEPC in extracts from darkened or illuminated leaf discs of *Alternanthera pungens*, with or without base-loading with 7.5 mM NH_4Cl.

Pretreatment/ Parameter	Control		7.5 mM NH_4Cl-treated	
	Dark	Light	Dark	Light
Control				
PEPC activity[a]	661 ±29	1831 ± 53	1583 ±78	4106 ± 68
% inhibition by malate[b]	82	51	68	37
ATP(1 mM)				
PEPC activity	1420 ± 64	3300 ± 126	3750 ± 185	7400 ± 221
% inhibition by malate	67	35	40	25
AP (5 U mL^{-1})				
PEPC activity	350 ±21	702 ± 89	752 ±89	1450 ±85
% inhibition by malate	83	80	81	78
Mannose (5 mM)				
PEPC activity	556 ± 23	550 ±29	700 ± 21	2500 ± 86
% inhibition by malate	84	70	85	51

[a] PEPC activity in µmol mg Chl h .
[b] 1 mM L-malate.

When potassium phosphate (20 mM) was included in the assay medium while incubation with AP, the effect of AP significantly reduced, suggesting that the specific removal of phosphate from light-form of PEPC resulted in the AP-induced changes in its activity and L-malate sensitivity.

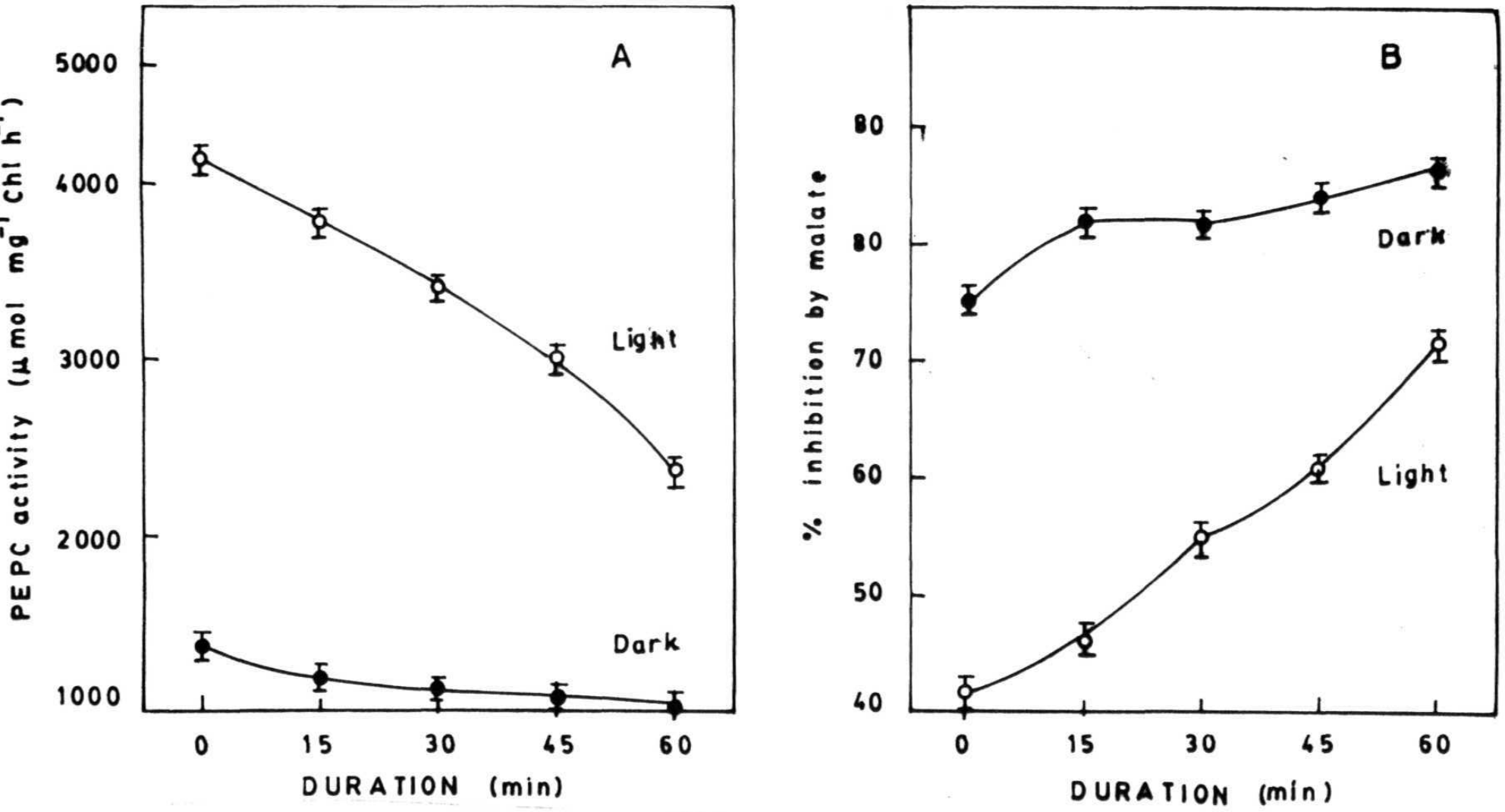

Figure 5.5. Dephosphorylation of PEPC extracted from base-loaded leaves and then incubation of leaf extracts with exogenous alkaline phosphatase. AP-dependent dephosphorylation mixture contained (final concentrations): 20 mM Hepes-KOH, 5 U mL^{-1} AP and 5 mM $MgCl_2$, and the dephosphorylation was carried out 30 °C. PEPC activity (A) and L-malate inhibition (B) were measured, at intidicated time intervals, by taking 10-μL aliquots of the mixture. The pattern may be compared to that in Fig. 5.2.

Effect of Man feeding

There was about a two-fold decrease in PEPC activity as a result of feeding of Man to leaf discs. Further, the degree of light-activation decreased markedly compared to the control (Table 5.1).

Discussion

Regulatory phosphorylation can lead to either activation or inactivation of the target enzyme. However, the extent of change in activity varies greatly among different enzymes. For some enzymes, like the PPDK, the difference in catalytic activity between the phosphorylated forms is very large, to the extent that one form is active and the other considered kinetically inactive. On the other hand, changes in phosphorylation status of other enzymes result in only several-fold changes in activity, so that one of the two forms is less active compared to the other. In addition, protein phosphorylation can also alter the regulatory properties of the target enzyme (Huber *et al* 1994, Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

The indirect assessment of the phosphorylation status of PEPC using Mg^{2+}-ATP is simple and easy to carry out and to interpret and very useful in assessing the *in vitro* phosphorylation status of the enzyme. Further, the *in vitro* phosphorylation studies indicated that PEPC is phosphorylated *in vitro*, obviously by an endogenous protein-kinase present in crude leaf extracts.

Incubation of light-or dark-form of PEPC, extracted from leaf discs of *A. pungens*, with Mg^{2+}-ATP resulted in a marked increase in the activity of particularly PEPC from illuminated leaf discs. The extent of activation by **ATP** was quite small in the case of dark-form PEPC (Table 5.1). The sensitivity to L-malate markedly changed as a result of *in vitro* phosphorylation.

The extent of PEPC activation, in the presence of Mg^{2+}-ATP, measured in the absence of 1 mM L-malate, increased dramatically, during the 30 min of preincubation with Mg^{2+}-ATP (Fig. 5.1 A). Earlier reports with maize PEPC (Jiao and Chollet 1989) and nodule PEPC (Shuller and Werner 1993) have shown that a period of 30 min as the optimum, while Arrio-Dupont *et al.* (1992) reported a duration of 200 min for the ATP-dependent *in vitro* phosphorylation of *Sorghum* leaf PEPC.

The activity of PEPC extracted from leaf discs, incubated in NH_4Cl, increased as a result of *in vitro* phosphorylation. PEPC activity increased by more than two-fold and malate sensitivity decreased from 75% to 54% (Figs. 5.4A,B). This indicates that PEPC extracted from base-loaded leaf discs is more phosphorylatable compared to the control enzyme. This further indicates that cytosolic alkalinization plays an important role in the phosphorylation of PEPC by making the enzyme more responsive to phosphorylation (Rajagopalan *et al.* 1993).

The *in vitro* phosphorylation of maize leaf C4-PEPC was dependent on temperature. Heat treatment (100 °C, 5 min) of the enzyme preparation led to a complete loss of phosphorylation effect. A low degree of activation occurred when *in vitro* phosphorylation was performed at <5 °C (Jiao and Chollet 1989).

Typically, the ratios of PEPC activity following preincubation with the endogenous protein kinase and Mg^{2+}-ATP to those obtained in the absence of ATP (control) were about two when PEPC was assayed under standard conditions (pH 7.3, 1.5 mM PEP). The presence of 1 mM L-malate, during the assay, increased the ratio to four. From the *in vitro* phosphorylation studies it is evident that the changes in L-malate sensitivity of PEPC are kinetically coupled to the concomitant increase in catalytic activity. For example, after 45 min incubation in phopshorylation mixture,

the increase of about 200 to 250% in dark-form of PEPC activity, was accompanied by the decrease in L-malate inhibition from about 82% at zero time to 67% (68 to 40% in the case of PEPC extracted from base-loaded leaf discs) (Figs. 5.4A,B). Thus, like the light-induced effects on C_4 PEPC activity *in vivo,* treatment of the dark-form enzyme *in vitro* with Mg^{2+}-ATP resulted in marked changes in its catalytic activity and feedback inhibition by L-malate.

However, the changes in the properties of PEPC as a result of Mg^{2+}-ATP-dependent *in vitro* phosphorylation are comparable to those induced by light *(in vivo* phosphorylation) in that there is an increase in PEPC activity and a decrease in sensitivity to L-malate (Table 5.1). However, the change in sensitivity to L-malate was only marginal, unlike light-induced change. This indicates that in addition to *in vivo* phosphorylation, some of the key photosynthetic metabolites (for example Glc-6-P, triose-P) may also help in the reduction of L-malate sensitivity of C4-PEPC.

Earlier studies (Jiao and Chollet 1989) indicated that Mg^{2+} is essential for the activity of the PEPC-PK. Activation was not observed in the absence of exogenous Mg^{2+}. Similar concentration of Mn^{2+} was able to partially substitute for Mg^{2+} in the *in vitro* phosphorylation mixture, resulting in 50% reduction in the extent of PEPC activation when assayed in the presence of L-malate.

Exogenous AP has shown to affect the property of a variety of phosphorylated enzymes *in vitro* (Walker and Huber 1987, Taniguchi and Pyerin 1987) including phosphorylated PEPC purified from a CAM plant (Nimmo *et al.* 1986), maize (Jiao and Chollet 1988) and *Sorghum* (Arrio-Dupont *et al.* 1992). In the present study, the *in vitro* dephosphorylation, induced by 5 U mL^{-1} AP on PEPC, and thus change in activity and malate sensitivity (Figs. 5.2 and 5.5), was similar to those reported by Jiao

and Chollet (1988) and Arrio-Dupont *et al.* (1992). The decrease in activity due to AP was rapid in the initial phase, and the activity decreased to 50% within the first 30 min (Fig. 5.2A), similar to the reports of Jiao and Chollet (1988) but differs from the reports of Arrio-Dupont *et al* (1992), who reported the requirement of 1 h for the *in vitro* dephosphorylation of *Sorghum* leaf-PEPC.

Analogues of Glc like α-D-Man, β-D-galactose, L-sorbose or 3-*o*-methylGlc, have also been used extensively in eukaryotes to study various aspects of carbohydrate and energy metabolism (Moore 1981). These Glc-analogues perturb normal metabolic sequences by substituting for Glc. These metabolic perturbations are in most cases initiated by the phosphorylation of the sugar analogue by hexokinase to the 6-P derivatives. If the species in question lacks the ability to rapidly metabolize the phosphorylated analogue, its continued accumulation may deplete intracellular Pi leading to a variety of intracellular disturbances (Herold and Lewis 1977, Moore 1981).

Earlier studies on a-D-Man metabolism in maize have shown that in scutellum and root tissues, Man feeding resulted in the accumulation of Man-6-P and depletion of Pi adenylates, Fru-6-P and Glc-6-P (Herold and Lewis 1977, Kime *et al.* 1982). This study demonstrated that, when millimolar concentrations of the Glc analogue a-D-Man were provided to corn leaves through the transpiration stream, photosynthesis was rapidly inhibited. In general, Man toxicity has been found to be correlated with an inability to metabolise extensively Man and accumulation of Man-6-P.

We hypothesize that the presence of Man in the mesophyll cells could create a powerful ATP sink which could potentially deplete cytosolic Pi. Another ramification

of the presence of an ATP sink in the mesophyll cytosol might be an initial acceleration of ATP turnover. One could expect a futile cycle inhibiting PPDK, in the chloroplast, and pyruvate kinase and hexokinase in the cytoplasm. We speculate that Man metabolism can directly impact the synthesis of PEP, its pool size, and its translocation from chloroplast.

Van Quy and Champigny (1992) reported that feeding of mannose to low-NO_3^- leaves of wheat (a C_3 species) restricted the light-activation of PEPC, while causing deactivation of the previously light-activated PEPC of high-NO_3^- leaves and making the activity-levels similar to that of dark-adapted low-NO_3^- leaves.

In the case of C4-PEPC, both the light- and dark-forms of the enzyme have shown to contain phosphoserine and possess catalytic activity. However, further phosphorylation of the dark-form PEPC, in both *in vivo* and *in vitro,* results in an enhancement of the enzyme's catalytic activity and a decrease in its sensitivity to feedback inhibition by malate (Jiao and Chollet 1988, 1991).

The properties of PEPC extracted from base-loaded leaf discs were different from that of the enzyme extracted from illuminated leaf discs (Table 5.1). The malate-inhibition of the enzyme, upon ATP-induced (and presumably) *in vitro* phosphorylation, decreased to 40% in base-loaded leaf discs, compared to the 67% of the control leaf discs (Figs. 5.1B and 5.4B). However, in illuminated leaf discs, the L-malate sensitivity decreased to 51% and 37% in case of PEPC extracted from control- and base-loaded leaf discs, respectively (Table 5.1). As a result of *in vitro* phosphorylation, the malate sensitivity decreased significantly for the enzyme extracted from base-loaded (7.5 mM NH_4Cl) or control leaf discs (25% and 35% inhibition, respectively; Table 5.1). These results suggest that the changes observed in

the kinetic and regulatory properties of PEPC due to alkalinization were not exactly comparable to those induced by light. However, the rate and extent of ATP-dependent *in vitro* phosphorylation increased marginally but consistently due to alkalinization. Similarly, the dephosphorylation of PEPC extracted from base-loaded leaf discs mediated by AP was distinct from that of the control leaf discs. These results demonstrate that the changes in properties of PEPC brought by base-loading of leaf discs are distinct from that of illumination (Table 5.1). In addition, the enhanced phosphorylation of PEPC from base-loaded leaf discs clearly indicate the role of light-induced cytosolic alkalinization playing an important role, besides other factors, in regulating the phosphorylation status of PEPC.

Chapter Six

Effects of Inorganic Salts fed *In Vivo* on PEP Carboxylase Activity

Introduction

Dissolved inorganic salts alter the physical properties of the (biological) solvent water and thus affects the hydration status, stability and activity of enzymes (Wyn Jones and Pollard 1983). Several plant enzymes are activated by low concentration of neutral salts. The stimulation in enzyme activity (for example, pyruvate kinase, 6-phosphofructokinase) often occurs at 50 to 150 mM concentration of univalent salts, particularly potassium and sodium (Wyn Jones and Pollard 1983). The enzymes are affected by the inorganic salts both *in vivo* and *in vitro.* For example, potassium, the major cytoplasmic cation in plant cells (and frequently the main cation in the cell sap) performs a number of interrelated and integrated roles: enzyme activation, stabilization of the native conformations of enzymes and membranes, maintenance of cell turgor, osmotic regulation and regulation of cytoplasmic pH (Epstein 1972, Zimmermann 1978).

PEPC, the enzyme responsible for the primary carboxylation in C4-photosynthetic metabolism and CAM, is predominantly localized in the cytosol of mesophyll cells (Rajagopalan *et al.* 1994). Since the inorganic ion composition of the cytosol effects a number of processes including the activity of cytosolic enzymes (as discussed earlier), it is expected that *in situ* feeding of inorganic salts can affect the properties of PEPC, such as light-activation, sensitivity to L-malate (a feedback inhibitor) and response to Glc-6-P (an allosteric effector).

In vivo feeding of nitrate starved leaves with nitrate salts enhanced the light-activation of PEPC from the C_3 species, e.g., wheat (Van Quy *et al.* 1991, Van Quy and Champigny 1992, Duff and Chollet 1995, Li *et al.* 1996) and the NADP-ME type C_4 monocot maize (Gupta *et al.* 1994). However, Gupta *et al.* (1994) reported that

feeding of nitrate to nitrate-starved plants had no effect on light-activation in wheat Thus, reports on the stimulation by the feeding of nitrate on the stimulation of PEPC in leaves of C_3 and C_4 plants, are contradictory. Sugiharto *et al* (1990, 1992b) observed a selective accumulation of PEPC protein (and its mRNA) in nitrogen starved leaves of maize, when supplied with nitrate (or glutamine).

Therefore, it is quite interesting to evaluate the short-term *in vivo* effects of nitrate on the activity and light-activation of PEPC from plant species, which have grown under normal environmental (field) conditions. The objective of the present study is to know whether nitrate or other inorganic ions, affect PEPC activity in leaves of only C3- or C4-species, or both. The activation status of PEPC was examined in leaves of C3 and C4 species of *Alternanthera* after *in vivo* treatment with seven different inorganic salts. During these studies, we observed nitrate salts increased the activity of PEPC from *Alternanthera pungens,* a C_4 species, much more markedly than that of the enzyme from *Alternanthera sessilis,* a C3 species.

Results

Effect of* In Vivo *Feeding of Inorganic Salts on PEPC Activity

When leaves of the C4 species *(A. pungens)* were fed in darkness with potassium or sodium chloride, the extractable PEPC activity was enhanced by more than three-fold, by 50 mM KC1, while NaCl had almost no effect (Table 6.1). Unless otherwise mentioned, leaves were fed through petiole with various concentrations of salts for 120 min (or as otherwise mentioned). After the feeding, the leaf extracts were examined for the activity and effector (L-malate or Glc-6-P) sensitivity of PEPC. The effect on PEPC was maximal after feeding of the inorganic salts *in vivo* for a period of

Table 6.1. Effect of exogenously fed inorganic salts (50 mM) on extractable PEPC activity from the leaves of *Alternanthera pungens*, a C4-dicot. Leaves incubated in distilled water were used as the control.

Inorganic salt/ Treatment	PEPC activity $\mu mol\ mg^{-1}\ Chl\ h^{-1}$	% of control
Control	625 ±28	100
KC1	734 ± 27	115
NaCl	674 ±29	108
KNO_3	1475 ± 39	236
$NaNO_3$	1636 ± 53	262
NH4NO3	2387 ± 95	382
K2SO4	426 ± 28	68
Na_2SO_4	619 ± 38	99

90 to 120 min, in case of *A. pungens,* a C_4 species (Figs. 6.1 to 6.3) or *A. sessilis,* a C3 species (Figs. 6.4, 6.5). Concentrations above 50 mM of either KC1 or NaCl were inhibitory. For instance, feeding of leaves with 200 mM KC1 reduced the *in vivo* PEPC activity by 53%. Exogenous feeding of KNO_3, $NaNO_3$ or NH4NO3 enhanced the extractable PEPC activity in the leaves of A. *pungens.* Feeding of 50 mM KNO3 or $NaNO_3$ enhanced PEPC activity by nearly three-fold (Table 6.1). Among the three nitrate salts, NH_4NO_3 caused maximal stimulation (almost four-fold) PEPC activity. On the other hand, feeding with K2SO4 (in darkness) lowered the *in vivo* **PEPC** activity by 30%, comparable to that of the control leaves, while sodium sulfate had no effect (Table 6.1).

Exogenous feeding of leaves of the C3 species *{Alternanthera sessilis)* in darkness with 50 mM KC1 enhanced the *in vivo* PEPC activity by about 60%, whereas

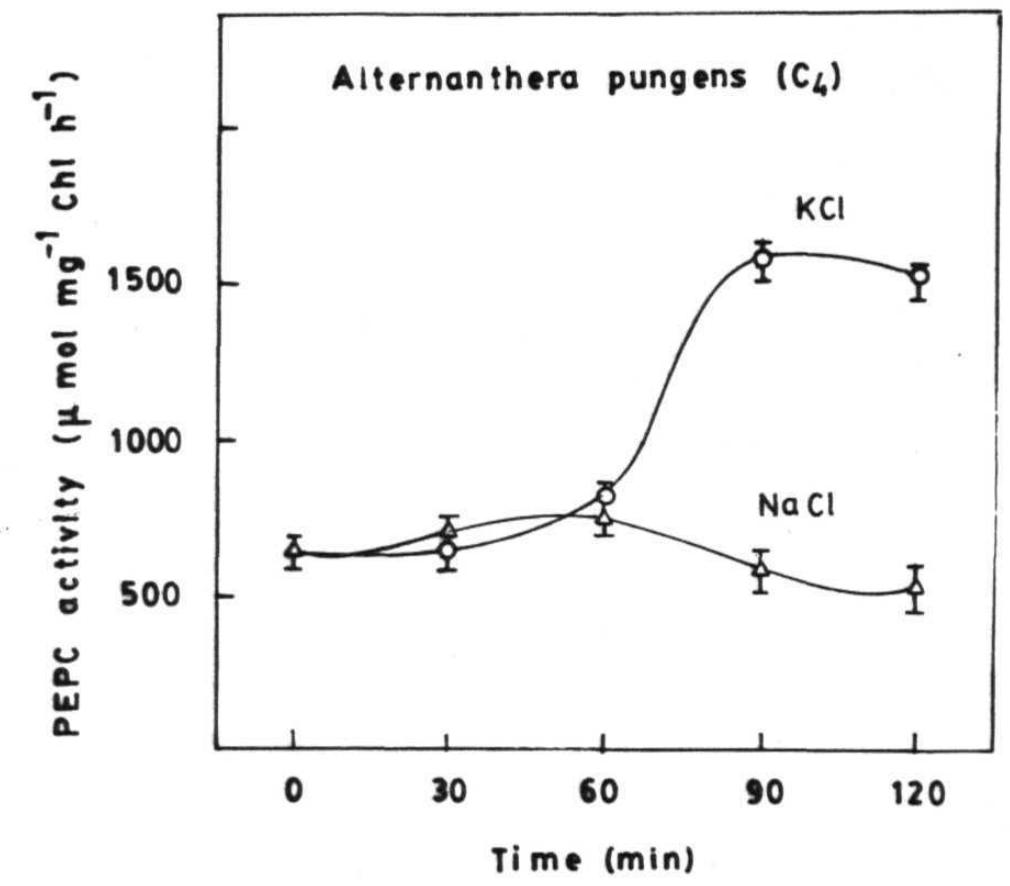

Figure 6.1. The pattern of changes in PEPC activity in extracts prepared from leaves of ***Alternanthera*** *pungens* (C4 species) fed with *in vivo* 50 **mM** chloride salts of potassium and sodium. The extractable activity of PEPC was measured, at various time intervals (as indicated), at pH 7.3 and 1.5 mM PEP. **KCl** stimulated PEPC activity by nearly 3-fold, while NaCl had almost no slight effect on *in vivo* PEPC activity.

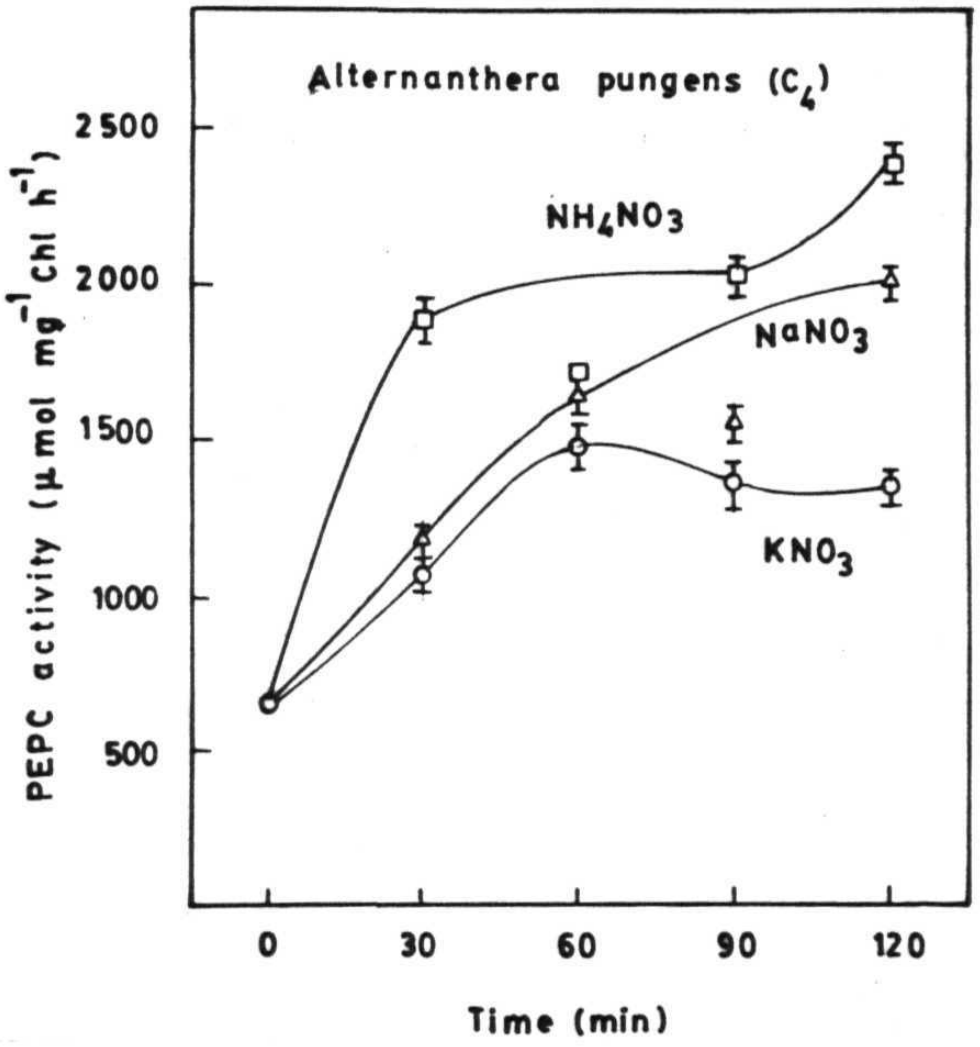

Figure 6.2. Changes in PEPC activity in extracts prepared from the excised leaves of *Alternanthera pungens* (C4 species) fed *in vivo* with 50 **mM** nitrate salts of potassium, sodium, or ammonium. PEPC activity in the extracts were assaying at pH 7.3 and 1.5 mM PEP. Nitrate salts caused a very high stimulation of PEPC and the order of nitrate salts enhancement of PEPC activity was: NH_4NO_3 > $NaNO_3$ > KNO3.

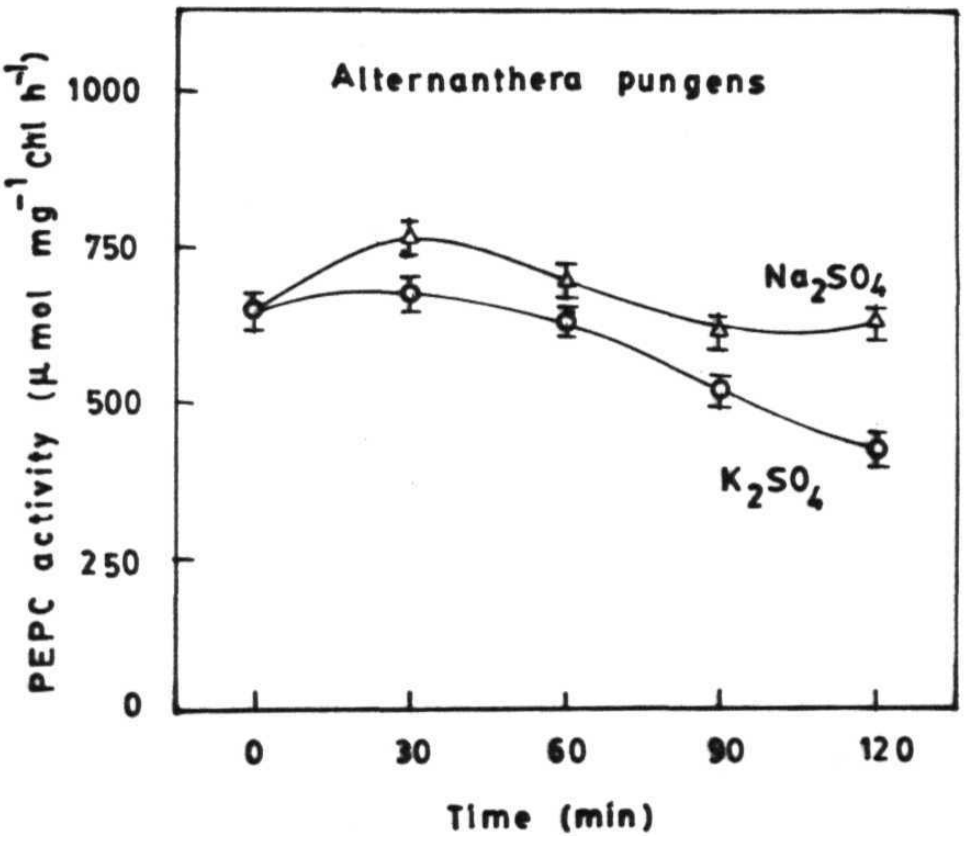

Figure 6.3. The pattern of changes in **PEPC** activity in extracts prepared from leaves of ***Alternanthera*** *pungens* (C4 species) fed with *in vivo* 50 **mM** sulfate salts of potassium and sodium. The extractable activity of PEPC was measured, at various time intervals (as indicated), at pH 7.3 and **1.5 mM** PEP. Both the salts have no effect on PEPC activity.

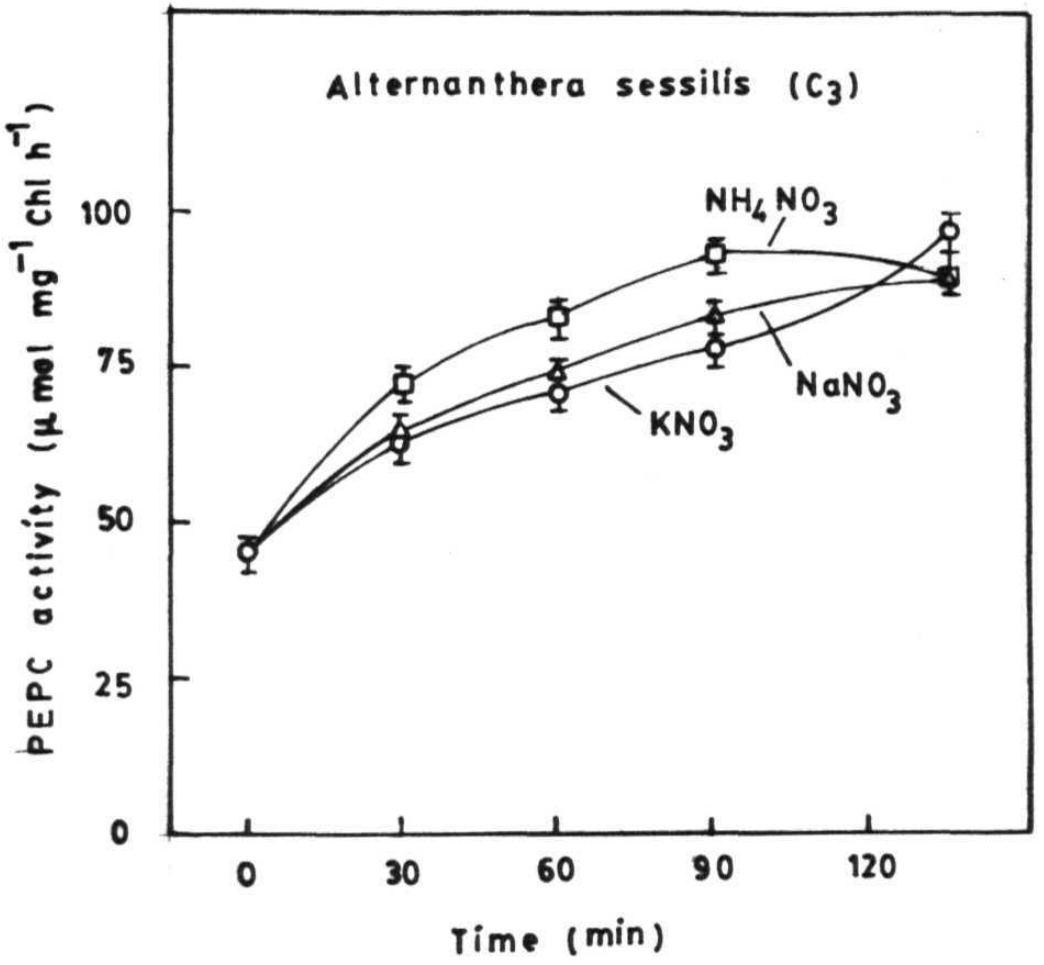

Figure 6.4. Changes in PEPC activity in extracts prepared from the excised leaves of *Alternanthera sessilis* (C3 species) fed *in vivo* with 50 mM nitrate salts of potassium, sodium, or ammonium. PEPC activity in the extracts were assaying at pH 7.3 and 1.5 mM PEP. Nitrate salts caused a very high stimulation of PEPC and the order of nitrate salts enhancement of PEPC activity was: NH_4NO_3 > $NaNO_3$ > KNO3. (see Fig.6.2).

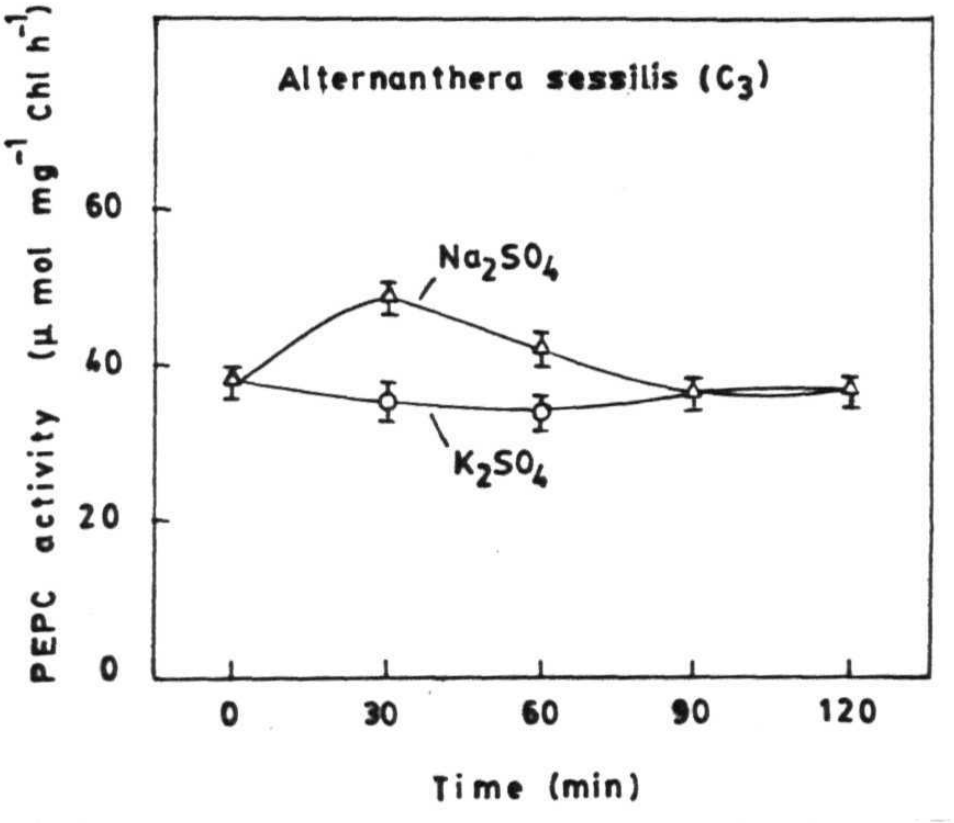

Figure 6.5. The pattern of changes in PEPC activity in extracts prepared from leaves of *Alternanthera sessilis* (C_3 species) fed with *in vivo* 50 mM sulfate salts of potassium and sodium. The extractable activity of PEPC was measured, at various time intervals (as indicated). Both the salts had any significant activation on PEPC from the C_3 leaves..

50 mM NaCl had no effect on the *in vivo* PEPC activity (Table 6.2). Concentrations of above 100 mM decreased the extractable PEPC activity.

Nitrate salts were the most effective, increasing the PEPC activity by nearly 2.5-fold. On the other hand, sulfate salts of sodium or potassium had no effect on the activity of PEPC in leaves of A. *sessilis* (Table 6.2).

*Effect on **Light-Activation of** PEPC*

In order to evaluate whether the *in vivo* feeding of inorganic salts had any effect on the activity of PEPC on illumination, we have studied the effects of light (1000 μmol m^2 s^{-1} at 30 °C for 30 min) on the enzyme. The light activation of PEPC is indicated by the light/dark (L/D) activity ratio of the enzyme extracted from leaves which are either illuminated (L) or dark-adapted (D).

Table 6.2. Effect of exogenously fed inorganic salts (50 mM) on extractable PEPC activity from the leaves of *Alternanthera sessilis,* a C3-dicot. Leaves incubated in distilled water were used as the control.

Inorganic salt/ Treatment	PEPC activity μmol mg^{-1} Chl h^{-1}	% of control
Control	39±4	100
KC1	62±7	159
NaCl	39±4	100
KNO_3	97 ±5	249
$NaNO_3$	92 ±7	236
NH4NO3	92 ±5	236
K_2SO_4	37±2	95
Na_2SO_4	38±3	97

Exogenous feeding of KCl or NaCl enhanced the extent of light activation of PEPC in the leaves of *A. **pungens***, a C4 species (Table 6.3). The most effective in stimulating

the light activation of PEPC are nitrate salts of sodium and potassium, which results in high L/D activity ratio nearing almost 6. Although K_2SO_4 inhibited the *in vivo* activity of dark PEPC, it has stimulated the *in vivo* PEPC activity upon illumination and the L/D activity ratio increased to 3.9 (Table 6.3).

KCl and NaCl had no effect on the L/D ratio of PEPC in leaves of *A. sessilis,* a C_3 species (Table 6.3). However, nitrate salts (of potassium and sodium) enhanced the L/D PEPC activity ratios to nearly four-fold, compared to the control value of 1.5 (Table 6.3). On the other hand, K_2SO_4 decreased marginally the L/D activity ratio to 1.1 (Table 6.3).

L-Malate Sensitivity of PEPC

The sensitivity of PEPC to L-malate (a feedback inhibitor) is an indicator of phosphorylation status of the enzyme (Jiao and Chollet 1988, 1991, Rajagopalan *et al.* 1994).

Feeding *in vivo* of leaves of *A. pungens,* with KCl, NaCl or K_2SO_4 had not much effect on the L-malate sensitivity of PEPC either in darkness or upon illumination (Table 6.4). However, feeding of nitrate salts of potassium or sodium (in darkness) decreased the inhibition 1 mM L-malate of PEPC from 86% (untreated dark-control) to 76% (Table 7.4). Similarly, feeding of leaves with exogenous nitrate salts and illumination, had reduced the inhibition by L-malate to 30% compared to 52% (untreated light-control). K_2SO_4 had no effect on the L-malate sensitivity of both the dark- and the light-forms of PEPC (Table 6.4).

Table 6.3. Effect of *in vivo* feeding of inorganic salts (50 mM) on light-activation of PEPCactivity as indicated by the ratio of enzyme activity extracted from either illuminated (L) or dark-adapted (D) leaves of *Alternanthera pungens* (C_4) and *A. sessilis* (C3). Leaves incubated in distilled water were used as the control.

Inorganic salt/ Treatment	PEPC activity (L/D ratio)	
	Alternanthera sessilis	*Alternanthera pungens*
Control	1.5	2.8
KC1	1.7	3.2
NaCl	1.6	3.6
KNO3	3.8	5.6
$NaNO_3$	3.9	5.8
K2SO4	1.1	3.9

Table 6.4. Effect of *in vivo* feeding of inorganic salts (50 mM) on the sensitivity to L-malate of of PEPC from *Alternanthera pungens* (C4). Leaves incubated in distilled water were used as the control.

Inorganic salt/ Treatment	% inhibition b>r 1 mM L-malate	
	Dark	Light
Control	86	52
KC1	85	49
NaCl	90	51
KNO3	76	32
$NaNO_3$	75	30
K2SO4	85	45

Nitrate salts were the most effective and increased K_I (malate) of PEPC in the leaves of A. pungens from 0.25 mM (control) to 0.41 mM (Table 6.5). However, feeding (in darkness) of other salts had no effect on the K_I(malate) of PEPC (Table 6.5). Illumination increased the K_I(malate) of PEPC in leaves of *A. pungens.* Feeding of

Feeding *of A. sessilis* (C_3) leaves with KC1, NaCl or K_2SO_4 did not change the L-malate sensitivity of PEPC extracted from either darkened or illuminated leaves discs (Table 6.6). However, feeding with nitrate salts decreased significantly the inhibition by L-malate of PEPC extracted both from dark as well as illuminated leaves. In addition, due to after feeding of the leaves with KNO_3 or $NaNO_3$, the K_I (L-malate) of PEPC prepared from the darkened and illuminated leaves of *A. sessilis* had increased to 1.38 and 1.83 mM (compared to the control values of 0.92 and 1.18 mM), respectively (Table 6.7).

Activation of PEPC by Glc-6-P

Unlike the marked changes in L-malate sensitivity of PEPC, there were only marginal effects on activation constant for Glc-6-P (K_A(Glc-6-P)). KNO_3 or $NaNO_3$ to the leaves in darkness and upon illumination decreased the KA(G1C-6-P) value to 2.2 mM (control 2.56 mM) and 1.3 mM (control 1.91 mM), respectively (Table 6.8). Other salts did not change $K_{A(Glc\text{-}6\text{-}P)}$ of PEPC (Table 6.8).

Table 6.5. Effect of exogenously fed inorganic salts on K_I(L-malate) of PEPC extracted from leaves of *Alternanthera pungens* (C_4 species). Leaves incubated in distilled water were used as the control.

Inorganic salt/ Treatment	K_I(malate) mM	
	Dark	Light
Control	0.25	1.19
KC1	0.27	1.19
NaCl	0.27	1.13
KNO3	0.41	2.93
$NaNO_3$	0.40	2.91
K2SO4	0.25	1.10

Table 6.6. Effect of *in vivo* feeding of inorganic salts on the sensitivity to L-malate of PEPC from *Alternanthera sessilis* (C3 species). Leaves incubated in distilled water were used as the control.

Inorganic salt/ Treatment	% inhibition by L-malate	
	Dark	Light
Control	51	45
KC1	50	45
NaCl	55	47
KNO3	45	39
$NaNO_3$	42	40
K2SO4	51	50

Table 6.7. Effect of exogenously fed inorganic salts on K_I(L-malate) of PEPC extracted from leaves of *Alternanthera sessilis* (C3 leaves). Leaves incubated in distilled water were used as the control.

Inorganic salt/ Treatment	K_I (malate) mM	
	Dark	Light
Control	0.92	1.18
KC1	0.91	1.18
NaCl	0.91	1.16
KNO_3	1.39	1.83
$NaNO_3$	1.37	1.83
K2SO4	0.92	1.19

Table 6.8. Effect of exogenous feeding of inorganic salts on K_A (Glc-6-P) of PEPC from *Alternantherapungens* (C_4). Leaves incubated in distilled water were used as the control.

Inorganic salt/ Treatment	K_A (Glc-6-P) mM	
	Dark	Light
Control	2.56	1.91
KC1	2.49	1.87
NaCl	2.57	1.94
KNO_3	2.21	1.34
$NaNO_3$	2.23	1.31
K2SO4	2.55	1.99

Effect of CHX on light-Activation of PEPC after feeding with Inorganic Salts

CHX was used as a eucaryotic protein synthesis inhibitor in order to examine whether there is any synthesis of PEPC- or PEPC-PK protein (Rajagopalan *et al.* 1994). Five μM CHX (fed for 6 h), had no major effect on PEPC activity from dark-control samples, while it had inhibited the activity of light form of PEPC by 46% and light/dark PEPC activity ratio decreased from 2.58 (control) to 1.53 (CHX-treated).

Feeding of 5 μM CHX caused a marked (nearly 30%) reduction in the activity of PEPC in the KNO_3- and $NaNO_3$-fed (dark) leaves, which were either illuminated or dark-adapted, after NO_3-feeding (Table 6.9). However, the L/D ratio of CHX treated control, KNO_3 or $NaNO_3$ leaves was quite high at 5.5 , while control gave a value of 1.53, PEPC from the nitrate fed-leaves gave about 5.5 (Table 6.9). Thus, the synthesis of the protein-kinase during feeding of nitrate salts can be one of the reasons for the enhanced activity, besides the direct interaction of nitrate salts with the enzymes directly.

Table 6.9. Effect of 5 μM CHX feeding (6 h) on PEPC activity from leaves of *Alternanthera pungens* (C_4 species) fed with 50 mM each of KNO_3 and $NaNO_3$.

Inorganic salt/ Treatment	PEPC activity		LTD ratio
	Dark	Light	
	μmol mg^{-1} Chl h^{-1}		
Control	675(100)	1742(100)	2.58
+ CHX	621(92)	948 (54)	1.53
KNO_3	1475(100)	8240(100)	5.60
+ CHX	1075 (73)	5768) 70)	5.37
$NaNO_3$	1636(100)	9469(100)	5.80
+ CHX	1136 (69)	6249(66)	5.50

Discussion

Van Quy *et al.* (1991) reported that the supply of nitrate to nitrate-starved (or low nitrate grown) leaves had enhanced significantly the extent of light-activation of PEPC in leaves of wheat, a C3 species. Duff and Chollet (1995) also reported enhanced PEPC-PK activity as a result of nitrate feeding to nitrate-starved wheat leaves. However, Gupta *et al.* (1994) reported that feeding of nitrate had no effect on already low the light-activation pattern in leaves of the C3 species (wheat), but had significantly increased the extent of photoactivation of PEPC from maize, a C4 species.

Nitrate may enhance the PEPC-PK activity which would phosphorylate PEPC, leading to a greater light-activation in leaves of C3 species upon illumination (Van Quy *et al* 1991, Van Quy and Champigny 1992, Manh *et al* 1993, Duff and Chollet 1995). Recently, Li *et al* (1996) reported that after 3 h of illumination PEPC activity from tobacco leaves increased by about 47% and its inhibition by L-malate

decreased from 72% to about 50%, and suggested that the primary reason for the light-activation of PEPC in this species is reversible phosphorylation.

All the above experiments were performed after creating an artificial nitrate deficiency in the plants (Van Quy *et al.* 1991, Manh *et al.* 1993, Gupta *et al.* 1994).

Our results indicate that nitrate salts (of K^+, Na^+ and NH_4^+) are quite effective in enhancing *in vivo* PEPC activity (Tables 6.1 to 6.3) from both the C_3 and the C_4 species of *Alternanthera*. The order of activation observed by nitrate salts is: NH_4NO_3 > $NaNO_3$ > KNO_3. This seems logical since NH_4NO_3 is a combined nitrogen source containing NH_4^+ and NO3" ions, both of which are known to activate the enzyme. The greater effect of $NaNO_3$ compared to KNO_3 is in intriguing, but may be interpreted due to the beneficial effect of sodium as a micronutrient for C_4 species (Brownell 1979).

Our results also suggest that the effects observed on feeding of leaves with various salts is mainly due to the anionic rather than the cationic effects. Chloride and sulfate anions at higher concentrations were in fact inhibitory.

The L-malate sensitivity of PEPC, which has been used to assess the phosphorylation status of the enzyme, indicates that there is possibly an enhanced phosphorylation of the enzyme on short-term NO_3 feeding (Tables 6.4 to 6.7). Salts of SO_4^{2} have not much effect on the L-malate inhibition of PEPC both from darkened and illuminated leaves.

Duff and Chollet (1995) suggested the existence of the regulatory phosphorylation of C3 PEPC (from wheat) by a protein-serine/threonine kinase in nitrate-deficient leaves. The results of the present study, though indirect, also supports the hypothesis that PEPC from the C_3 species, *Alternanthera sessilis,* can also undergo marginal phosphorylation on nitrate feeding.

Huber *et al* (1994) reported that feeding of excised leaves with inorganic phosphate or sulfate resulted in a slight reduction in the activity of nitrate reductase. However, the activation status of the enzyme (which corresponds to the dephosphorylation of phospho-NR), was not significantly affected by these ions. In contrast, both Pi and SO_4^{2} reduced the activation status of SPS in light. They have suggested that the interconverting enzymes that act on SPS are sensitive to certain inorganic ions while having no effect in the case of NR. We speculate that a similar hypothesis can be valid also with PEPC.

Sugiharto *et al* (1990, 1992b) observed a selective accumulation of PEPC protein in N-starved leaves of maize when supplied with nitrate or glutamine. The rise in level of PEPC-mRNA accumulation is more pronounced in maize plants supplemented with NH_4^+ or glutamine than those with nitrate (Sugiharto and Sugiyama 1992).

Pretreatment of excised leaves in the dark for 6 h caused only marginal reduction in the subsequent light + nitrate (Table 6.9). The decrease in activity is about 35% compared to the 46% reduction in the control. Thus, the present study indicates that though protein synthesis (of PEPC-PK) may be needed, although the enhanced light activation of PEPC in the C_3 or the C_4 species is not entirely dependent on protein synthesis machinery. We speculate that a possible activation of existing PEPC-PK is sufficient to enhance markedly the activity of PEPC.

Previous studies have suggested that C_3-leaf PEPC is regulated by reversible phosphorylation *in vivo* (Van Quy *et al* 1991, Van Quy and Champigny 1992, Manh *et al* 1993, Duff and Chollet 1995). Except the studies of Duff and Chollet (1995), other studies relied primarily on measurement of PEPC activity at near-optimal

(approximately V_{max}) conditions, to provide indirect evidence of *in vivo* or *in vitro* changes in apparent phosphorylation status of PEPC.

Our observations on the marked stimulatory effects of nitrate on the activity of C3-leaf-PEPC (both in light and in darkness) are similar to those of earlier reports (Van Quy *et al* 1991, Manh *et al* 1993). The decrease in the L-malate sensitivity of PEPC as a result of nitrate feeding (Table 6.4) suggests an apparent increase in the phosphorylation status of the enzyme.

Nitrate assimilation produces hydroxyl ions, in plant tisssues. Organic acids are required for counter-acting this phenomenon, so as to keep up a pH-homeostasis (Crawford 1995). It has long been known that the nitrate nutrition of plants is accompanied by a synthesis of organic acids, particularly malic acid. This is because the alkalinization of cells due to nitrate assimilation activates PEPC. The activity of PEPC and malic enzyme, being pH-sensitive, respond to the variations in proton and hydroxyl ion concentration and help to maintain the cytosplasmic pH at a value very close to neutrality (Davies 1973).

In spite of similarity in light activation of C_3 and C4 leaf PEPC, upon nitrate feeding, the details of their respective light transduction pathways are likely to differ. For example, light activation of C3 PEPC-PK is inhibited strikingly by inhibitors of glutamine synthatase, such as methionine sulfoximine or phosphinothricine, whereas these compounds have no detectable effect on the light-activation of maize (C4) PEPC-PK (Li *et al* 1996). These results indicate that a disruption of leaf N metabolism does not have the same impact on the regulatory phosphorylation of PEPC in illuminated leaves of C_3 and C4 species. Gln specifically antagonizes the inhibitory effect of MSX on light-activation of PEPC-PK in tobacco leaves,

suggesting that this product of catalysis by GS may be a critical component in the C_3 light-signal transduction mechanism.

Gln spcifically antagonizes the inhibitory effect of methionine sulfoximine on light activation of PEPC-PK in tobacco leaves, suggesting that this product of catalysis by GS may be a critical component in the C_3 light-signal transduction mechanism (Li *et al.* 1996). Gln would be one of the products, when nitrate or ammonium ions are assimilated in plant tissues. Gln has been implicated as a positive modulator in control of gene expression of leaf PEPC (Sugiharto *et al.* 1992). However, it is not clear how Gln regulates the expression of genes of either PEPC or of protein-serine/threonine kinase.

Chapter Seven

Buffer Capacity of Extracts from Tissues of C3, C_4 and C3-C4 Intermediate Species

Introduction

Plant cells can withstand large variations in the pH of their external environment (Bown 1985, Guern *et al.* 1991). In contrast, cellular processes are sensitive to pH changes and intracellular pH is therefore markedly regulated to keep it in constant. There may, however, be localized intracellular pH variations, for example, at membrane surface (Kurkdjian and Guern 1989). The majority of intracellular processes occur at a pH maintained near neutrality, and this neutral pH is the one at which various processes of cellular metabolism occur at their maximum rate (Raven 1985, Felle 1988). Such control of a virtually constant pH in biological systems is achieved by the action of efficient buffering systems whose chemical nature is such that they can resist pH changes due to the metabolic production of acids and bases (Madshus 1988).

The nature and relative significance of the mechanisms that contribute to intracellular buffering have been subjects of considerable debate (Guern *et al.* 1991 and references therein). The major buffering systems found in cellular compartments involve phosphate, phosphate esters, bicarbonate, amino acids, peptides and proteins (Kurkdjian and Guern 1989, Guern 1991). An understanding of intracellular buffering and of the parameters affecting its magnitude is crucial for gaining quantitative insight into the intracellular pH regulation and other transport processes involving H^+ or equivalent ions.

Titration of cellular homogenates is one of the simplest methods to estimate intracellular buffer capacity. Determination of buffer capacity by titration of cell homogenates of animal tissues (Roos and Boron 1981), and plant extracts/organelles (Pfanz and Heber 1986, 1989, Guern *et al.* 1991) have been reported. The values

obtained by titrating cellular homogenates reflect the physiochemical buffering but may also indicate degree of metabolic and cellular buffering. Further, the extracts may have also some contamination from mechanical breakdown of the tissue compartments.

The enzyme responsible for carboxylation of PEP, *viz.*, PEPC, is located in mesophyll cells of plants, and is highly sensitive to changes in pH (Gonzalez *et al.* 1984, Eschevarria *et al.* 1994). The activity of PEPC is markedly stimulated upon illumination in leaves of C_4 plants but not in those of C_3 species (Rajagopalan *et al.* 1993, Gupta *et al.* 1994). Another associate feature is that the light-induced cytosolic alkalinization is more in mesophyll cells of the C_4 leaves than those of the C_3 type (Yin *et al.* 1990, Raghavendra *et al.* 1993, Rajagopalan *et al.* 1993). One of the possible reasons for such light-induced increase in cytosolic pH and PEPC activity in mesophyll cells of C_4 plants (but not in C_3 species) could be the difference in the buffer capacity of the leaf tissues from these plants. An examination of existing literature did not reveal any information on the differences in the buffering strength of leaf tissues of plant species belonging to different photosynthetic types.

In the present study, an attempt is made to assess the buffer capacity of cell sap prepared from leaves of plant species belonging to different photosynthetic types, *viz.*, C3, C4, and C3-C4 intermediate species. In addition, the buffer capacity of different organs such as leaf, stem and root were also evaluated. Further, the buffer capacities of extracts prepared from leaves, stem and root tissues (of a few plant species belonging to different photosynthetic types), which were kept in darkness or illuminated, were determined.

Results

The variations in buffer capacities of extracts prepared in water were quite small. Use of water disrupts most of the organelles, making the extract a mixture of different compartments. The use of unbuffered 0.3 M sorbitol, can help (at least to some extent) to protect the organelle integrity (discussed in *Chapter Three* of this Thesis; Rajagopalan *et al.* 1993). Hence, we assessed the buffer capacity of tissue extracts prepared in also unbuffered 0.3 M sorbitol. A similar approach has earlier made while monitoring changes in PEPC activity and cell-sap pH in extracts of leaf discs upon illumination *{Chapter Three* of this Thesis).

Majority of the cellular metabolic processes occur at an intracellular pH values of 7 to 8. Most of the enzymes have their maximum catalytic activity at an optimal pH, close to this range. Hence, we have assessed the buffer capacity of extracts at the physiological range pH of 7 to 8, besides the determination of total buffer capacity in the range of pH 3 to 10.

Buffer Capacity of Extracts Prepared in Water

Leaves

Buffer capacity (at physiological pH range) of cell sap of leaf discs prepared in distilled water ranged between 720 and 880 $\mu Eq\ H^+$ pH $unit^{-1}\ g^{-1}$ FW (fresh weight) with a mean value of 810 $\mu Eq\ H^+$ pH $unit^{-1}\ g^{-1}$ FW for the C_3 species and 800 $\mu Eq\ H^+$ pH $unit^{-1}\ g^{-1}$ FW for C3-C4 intermediate species (Table 7.1). The buffer capacity of cell saps prepared from leaf discs of C_4 species (ranged between 700 and 790 $\mu Eq\ H^+$ pH $unit^{-1}\ g^{-1}$ FW), with an average value of 860 $\mu Eq\ H^+$ pH $unit^{-1}\ g^{-1}$ FW (Fig. 7.1, Table 7.1) were slightly less than those of C3 species. Similarly, the total

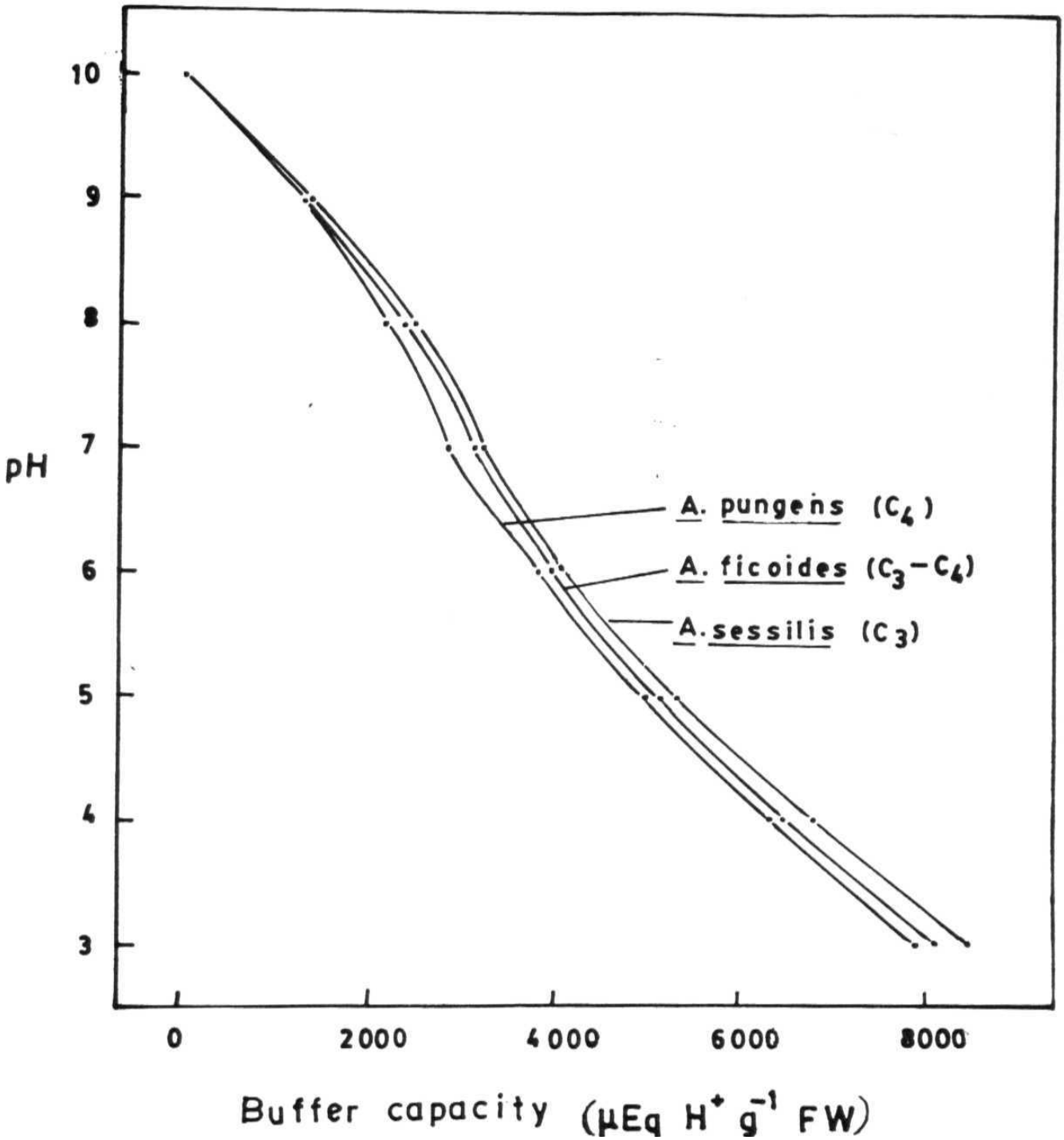

Figure 7.1. Buffer capacity of the cell sap extracted from predarkened (2-h) leaf discs of C3 (*Alternanthera sessilis),* C3-C4 intermediate *(A. ficoides)* and C_4 *(A. pungens)* species. After the predark-treatment, the leaf discs were blotted dry and then then extracted with, using water. The extracts were cleared by centrifuging and the supernatant (after centrifugation) was used for measruing the pH as indicated in Materials and Methods..

buffer capacity (pH range of 10 to 3) of the extracts from leaves of C_4 plant species was marginally less than that of C_3 and C_3-C_4 intermediate species (Fig. 7.1).

However, when the extracts were prepared from illuminated leaf tissue, the difference among the three different photosynthetic types was pronounced (Fig. 7.2, Table 7.2). While the buffer capacity was quite high in C_3 species, it was only 76% of the C_3 in C_4 species, measured at physiological pH range. The buffer capacity of leaf extracts (prepared in water) from the C3-C4 intermediate species showed intermediate values to those of C_3 and C_4 species (Fig. 7.2, Table 7.2). However, the total buffer capacity of extracts prepared from illuminated leaf tissues had only very small differences among the three different photosynthetic types (Fig. 7.2, Table 7.2).

Stem

There was no significant difference in the buffering pattern of stem extracts among the three photosynthetic types (Fig. 7.3). However, physiological buffer capacity (pH 7 to 8) of stem extracts (Fig. 7.3) was <50% of the leaf extracts (Fig. 7.1). In addition, the total buffer capacity of stem extracts was also about half of those of leaf tissue (Figs. 7.1, 7.3).

There was a small decrease in the buffer capacity (both physiological and total buffer capacity) as a result of illumination of stem tissue from C_3 species (Figs. 7.3, 7.4). Due to illumnation, the physiological buffer capacity of stem tissue from the C3-C4 intermediate species decreased by 12% and total buffer capacity by 11% (Figs. 7.3, 7.4). However, there was no significant difference in the buffer capacity of stem extract upon illumination from C_4 species.

Table 7.1. Buffer capacity of the cell sap extracted from darkened leaf discs of various plant species belonging to C_3, C_4, and C3-C4 intermediate photosynthetic types. The extracts were prepared in distilled water.

Photosynthetic type/ Species	Buffer capacity	
	Physiological (pH 7-8)	Total (pH 3-10)
	µEq H^+ pH $unit^{-1}$ g^{-1} FW	µEq H^+ g^{-1} FW
C3 Species		
Alternanthera sessilis	840	9200
A rachis hypogea	880	10530
Commelina communis	880	9760
Lycopersicon esculentum	780	9380
Pisum sativum	720	8770
Tagetes erecta	830	8110
Tridax procumbens	720	8710
Average	810 (100)	9280 (100)
C3-C4 Intermediate Species		
Alternanthera ficoides	800	8590
Alternanthera tenella	790	8840
Mollugo nudicaulis	830	8700
Parthenium hysterophorus	780	8830
Average	800 (99)[a]	8740 (91)[a]
C_4 Species		
Alternanthera pungens	730	8690
Amaranthus hypochondriac us	700	9080
Amaranthus viridis	770	8470
Gomphrena globosa	790	9190
Portulaca oleracea	760	8220
Sorghum bicolor	740	8740
Zea mays	770	8430
Average	760 (94)[a]	8690 (94)[a]

• Values in parentheses are % of C_3 species.

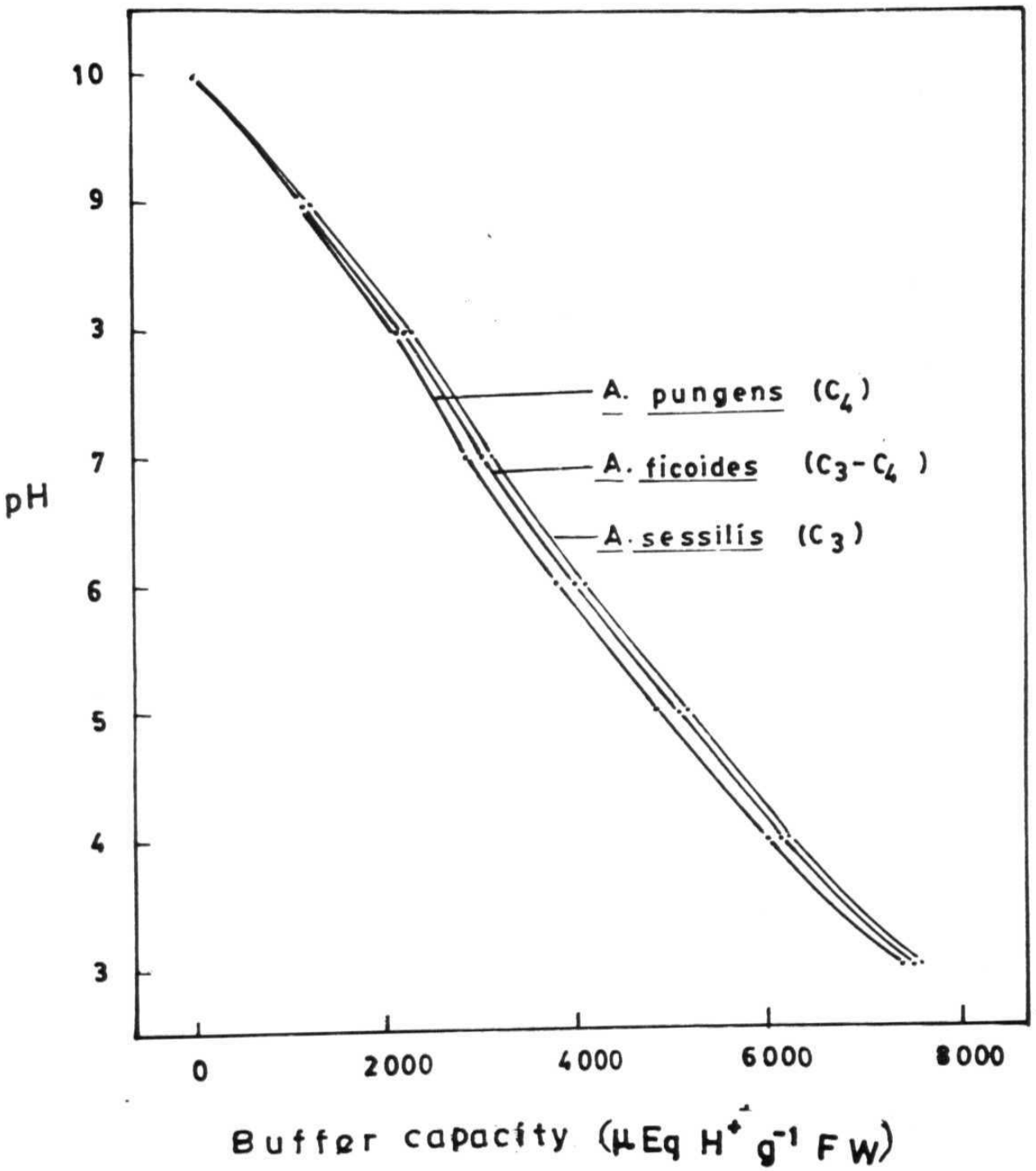

Figure 7.2. Buffer capacity of the cell sap extracted from iluminated leaf discs of C_3 (*Alternanthera sessilis),* C3-C4 intermediate (A. *ficoides)* and C_4 (A. *pungens*) species, using water. After the predark-treatment, the leaf discs were illuminated at 1000 $\mu molm^{-2}$ s^{-1} for 30 min at 30 °C and then extracts were prepared using distilled water and centrifuged at 15 000 *g* for 1 min to clear the debris. The supernatant (after centrifugation) was used for measuring the pH using a digital pH meter.

Table 7.2. Buffer capacity of the cell sap extracted from illuminated leaf discs of various plant species belonging to C_3, C_4, and C3-C4 intermediate photosynthetic types. The extracts were prepared in distilled water.

Photosynthetic type/ Species	Buffer capacity	
	Physiological (pH 7-8)	Total (pH 3-10)
	µEq H^+ pH $unit^{-1}$ g^{-1} FW	µEq H^+ g^{-1} FW
C_3 Species		
Alternantherasessilis	870	8520
Arachis hypogea	930	8860
Commelina communis	890	9300
Lycopersicon esculentum	930	9630
Pisum sativum	830	8840
Tagetes erecta	890	9130
Tridax procumbens	800	8900
Average	880 (100)	9020 (100)
C_3-C_4 Intermediate Species		
Alternanthera ficoides	720	8310
Alternanthera tenella	730	7780
Mollugo nudicaulis	800	8330
Parthenium hysterophorus	780	8790
Average	730 **(83)**[a]	8310 **(92)**[a]
C4 Species		
Alternanthera pungens	720	8170
Amaranthus hypochondriacus	730	8340
Amaranthus viridis	670	8400
Gomphrena globosa	620	8120
Portulaca oleracea	640	8410
Sorghum bicolor	630	8210
Zea mays	660	8230
Average	670 **(76)**[a]	8280 **(92)**[a]

•Values in parentheses are % of C3 species.

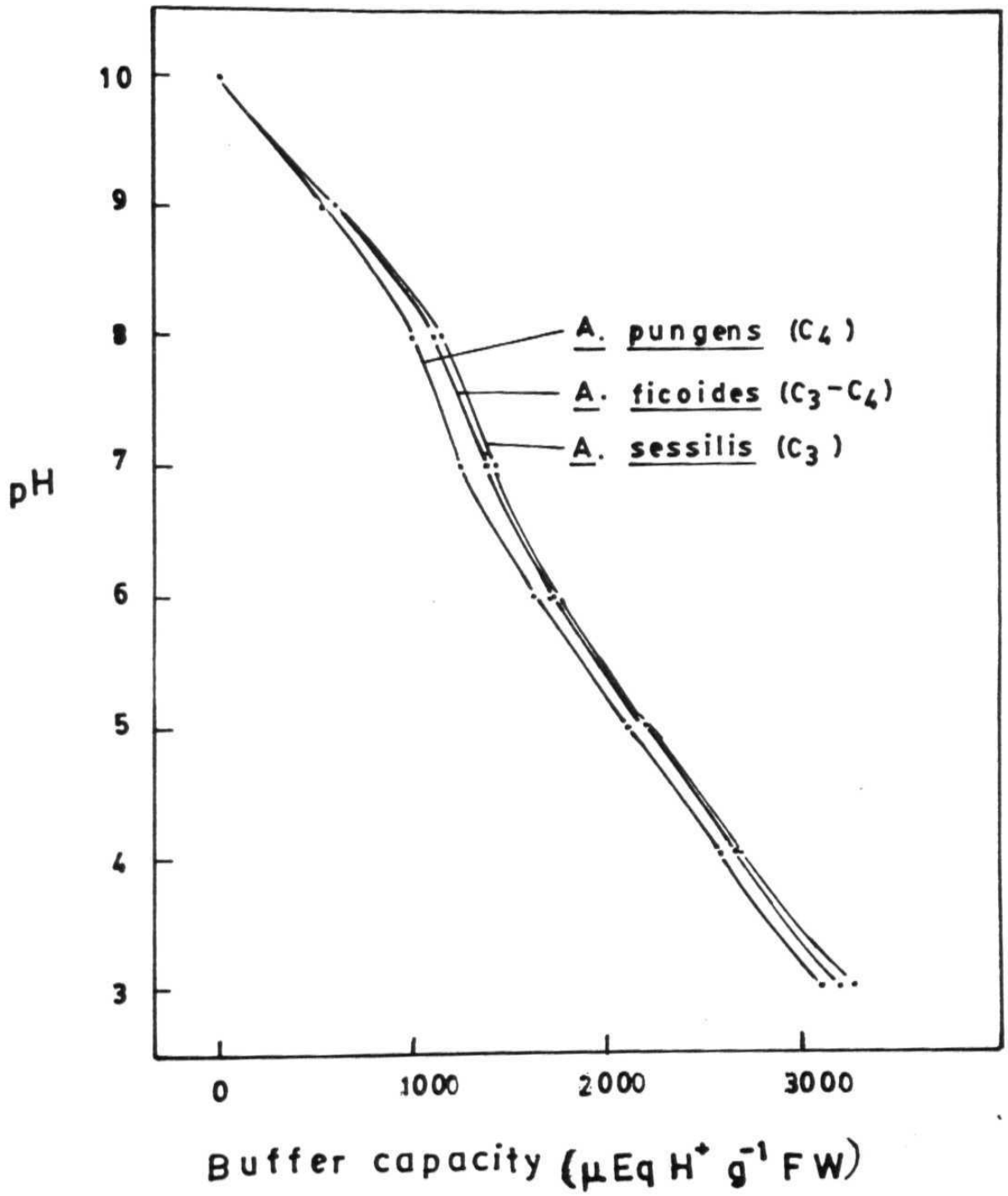

Figure 7.3. Buffer capacity of the cell sap extracted from predarkened (2-h) stem tissue of C_3 (*Alternanthera sessilis*), C3-C4 intermediate (*A. ficoides)* and C_4 *(A pungens)* species. After the predark-treatment, the tissue was blotted dry and then then extracted with, using water. The extracts were cleared by centrifuging and the supernatant (after centrifugation) was used for measruing the pH as indicated in Materials and Methods..

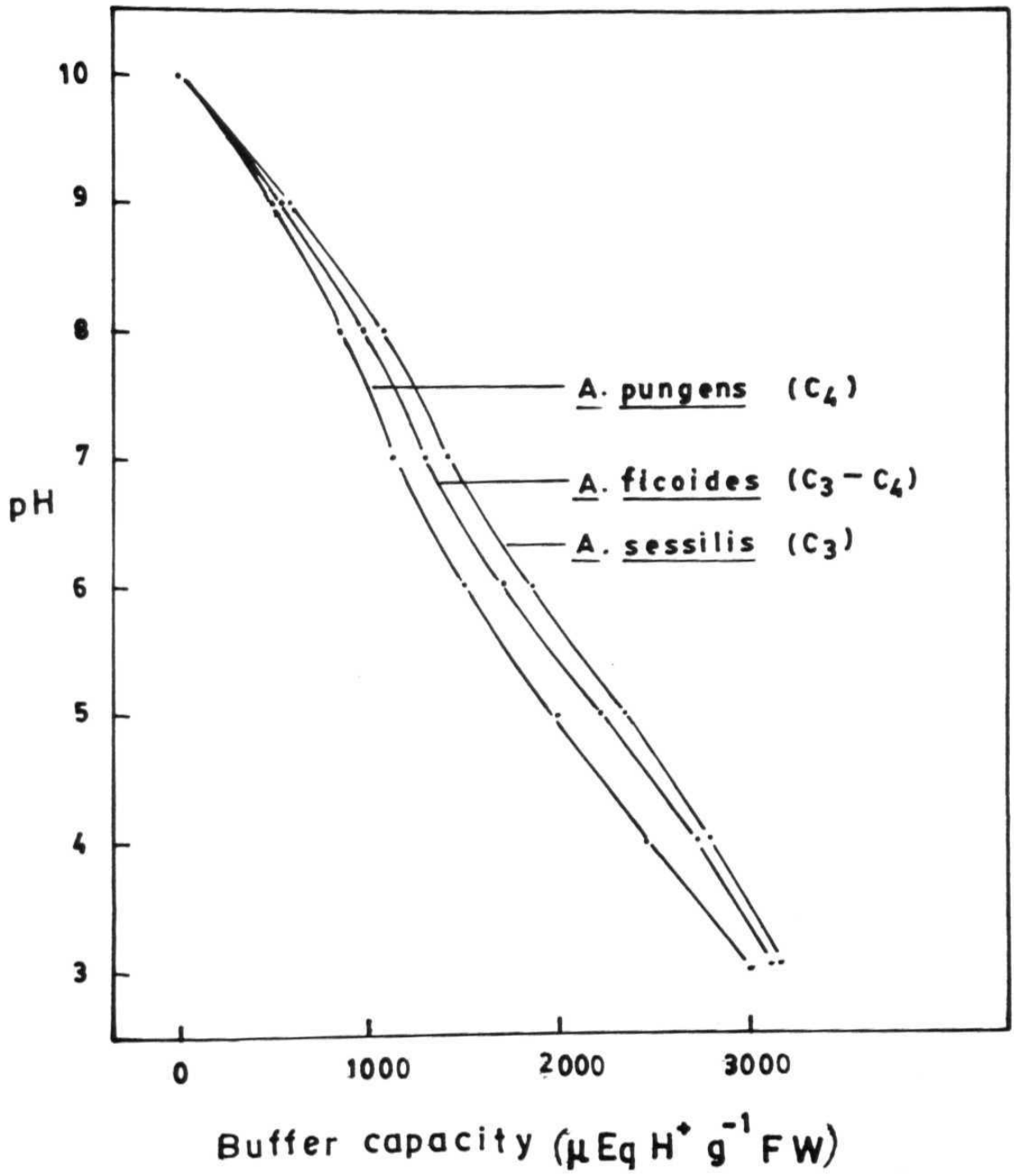

Figure 7.4. Buffer capacity of the cell sap extracted from iluminated stem tissue of C_3 *(Alternanthera sessilis),* C3-C4 intermediate (A. *ficoides)* and C_4 *(A. pungens)* species, using water. After the predark-treatment, the tissue was illuminated at 1000 μmol m^{-2} s^{-1} for 30 min at 30 °C and then extracts were prepared using distilled water and centrifuged at 15 000 *g* for 1 min to clear the debris. The supernatant (after centrifugation) was used for measuring the pH using a digital pH meter.

A comparison of physiological buffer capacity of extracts prepared from illuminated stem tissue, from different photosynthetic type plant species, indicates that the buffer capacity was minimum for the C_3 species, maximum for the C_4 species and intermediate for the C3-C4 intermediate species.

Root

The physiological buffer capacity of extracts prepared from root tissue (Fig. 7.5) from the three different photosynthetic type species was only about 35% of the values obtained with the leaf extracts (Fig. 7.1). The buffer capacity, at physiological pH range, of root extracts from the three different photosynthetic types was similar (Fig. 7.5). The total buffer capacity of the darkened extracts of the three different species was also similar.

Buffer Capacity of Cell Sap Prepared in 0.3 M Unbuffered Sorbitol

Leaves

The buffer capacity of leaf (darkened) extracts prepared from C_4 species, in unbuffered 0.3 M sorbitol, was about three-fourth of the physiological buffer capacity of C_3 species, while the total buffer capacity of darkened C4 leaf extracts was 87% of the C_3 (Fig. 7.6, Table 7.3).

Cellular buffering of the leaf extracts (prepared in sorbitol) was strong at the extremes of pH (9 to 10 or 3-4) for all the species examined, eventhough they belong to different photosynthetic types. Buffering was extremely low at pH values close to neutrality (pH 7 to 8; the working range of most of the cellular processes) (Table 7.4).

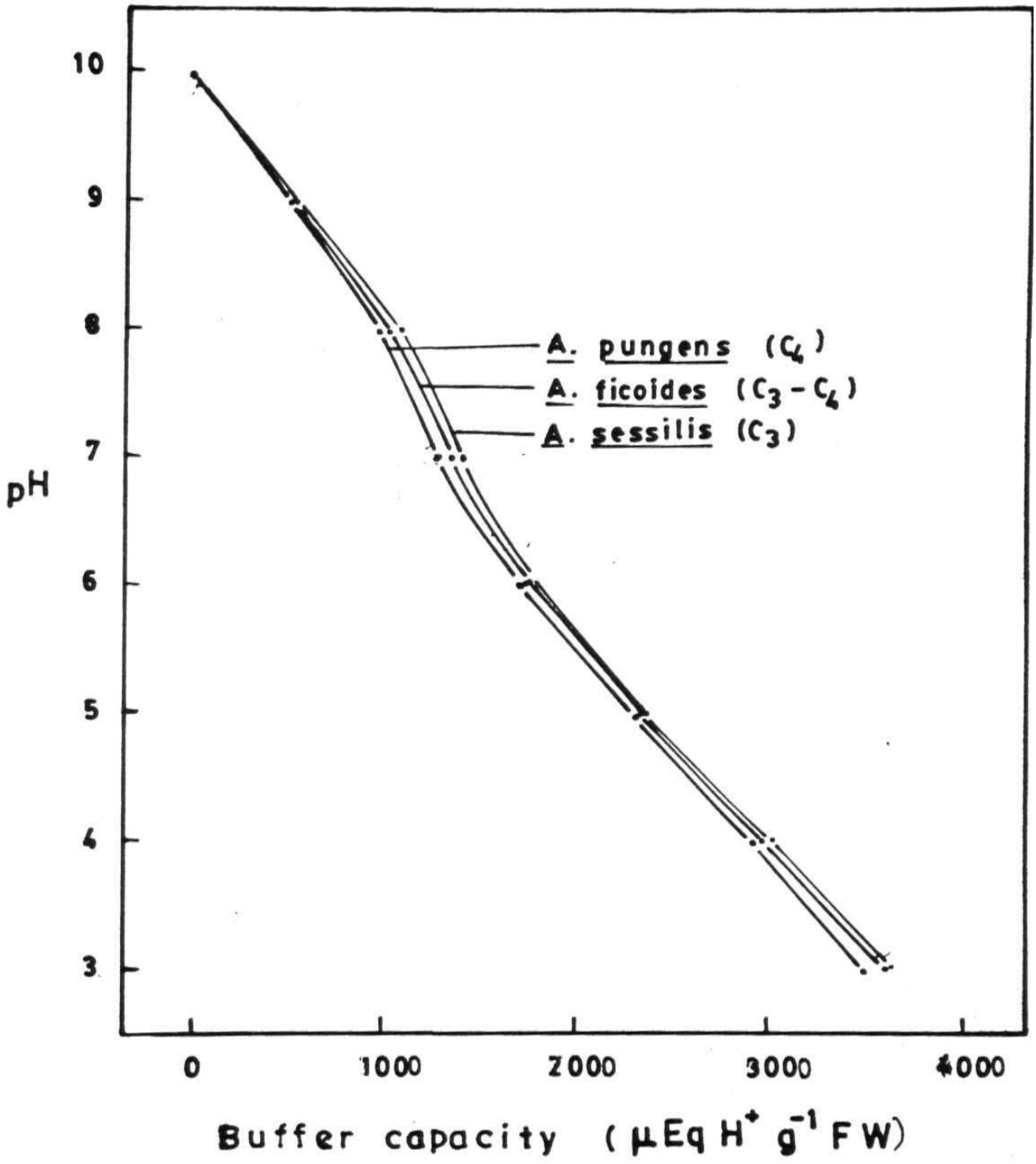

Figure 7.5. Buffer capacity of the cell sap extracted from predarkened (2-h) root tissue of C_3 (*Alternanthera sessilis),* C3-C4 intermediate *(A. ficoides)* and C_4 *(A. pungens)* species. After the predark-treatment, the root tissue was blotted dry and then extracts were prepared using distilled water and centrifuged at 15 000 *g* for 1 min to clear the debris. The supernatant (after centrifugation) was used for measuring the pH using a digital pH meter.

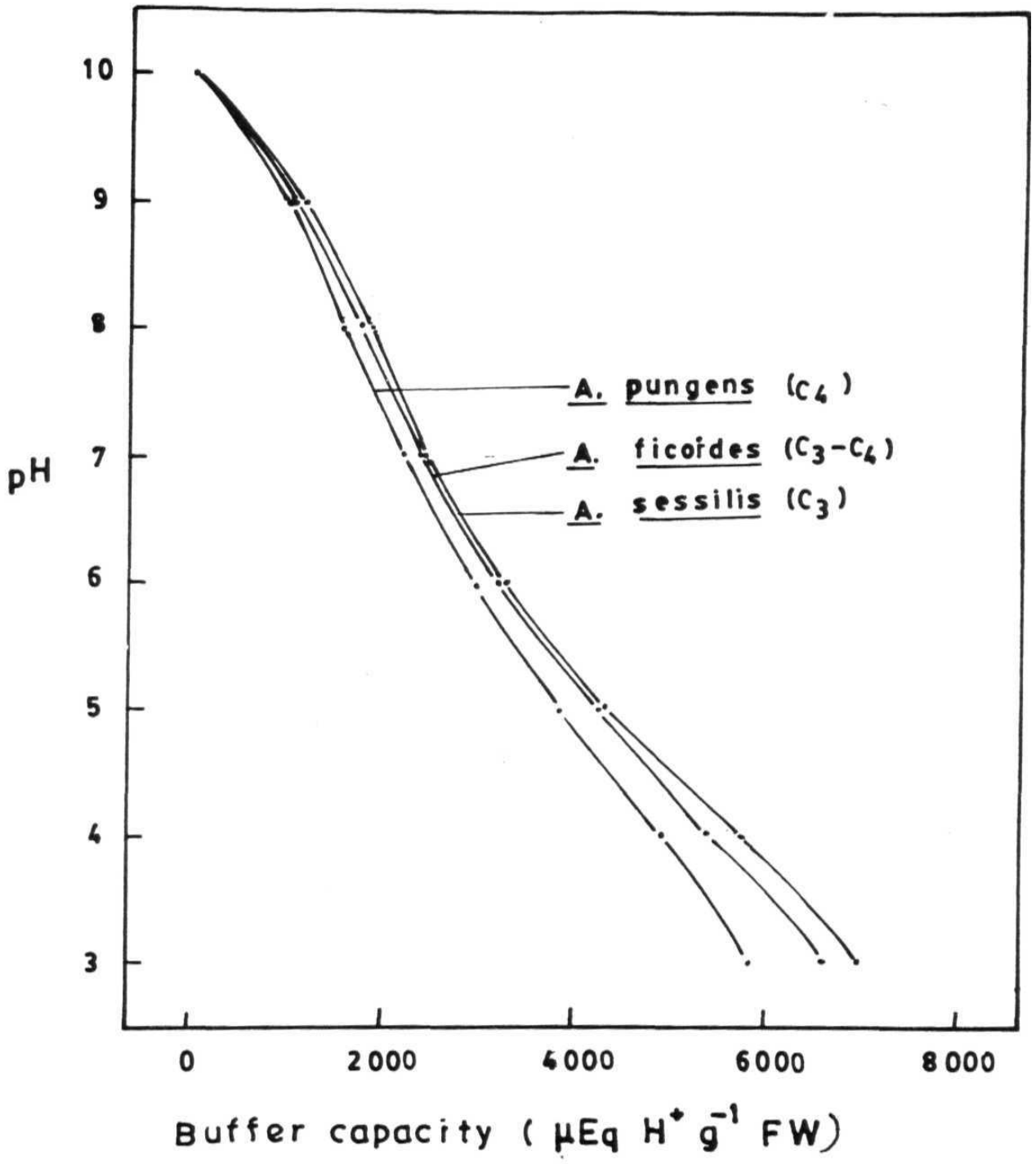

Figure 7.6. Buffer capacity of the cell sap extracted from predarkened (2-h) leaf discs of C_3 (*Alternanthera sessilis),* C3-C4 intermediate *(A. ficoides)* and C4 *(A. pungens)* species. After the predark-treatment, the leaf discs were blotted dry and then then extracted with, unbuffered 0.3 M sorbitol.. The extracts were cleared by centrifuging and the supernatant (after centrifugation) was used for measuring the pH as indicated in Materials and Methods..

Table 7.3. Buffer capacity of the cell sap prepared from darkened leaf discs of various plant species belonging to C_3, C4, and C3-C4 intermediate photosynthetic types. The extracts were prepared in unbuffered 0.3 sorbitol.

Photosynthetic type/ Species	Buffer capacity	
	Physiological (pH 7-8)	Total (pH 3-10)
	µEq H^+ pH $unit^{-1}$ g^{-1} FW	µEq H^+ g^{-1} FW
C_3 Species		
Alternanthera sessilis	620	7610
Arachis hypogea	620	7510
Commelina communis	620	7730
Lycopersicon esculentum	620	7870
Pisum sativum	630	7860
Tagetes erecta	610	8170
Tridax procumbens	620	8000
Average	620 (100)	7820 (100)
C3-C4 Intermediate Species		
Alternanthera ficoides	580	6970
Alternanthera tenella	580	7090
Mollugo nudicaulis	570	7100
Parthenium hysterophorus	560	7410
Average	570 (92)[a]	7140 (91)[a]
C_4 Species		
Alternanthera pungens	440	6780
Amaranthus hypochondriacus	470	7300
Amaranthus viridis	470	6220
Gomphrena globosa	470	6860
Portulaca oleracea	490	6980
Sorghum bicolor	490	6730
Zea mays	500	6590
Average	480 (77)"	6780 (87)[a]

[a] Values in parentheses are % of C3 species.

Table 7.4 . Average buffer capacity of cell sap prepared in unbuffered 0.3 M sorbitol from pre-darkened leaf discs of different photosynthetic types.

Buffer capacity/ pH range	Average buffer capacity for cell sap from		
	C_3 species	C3-C4 species	C4 species
	μ Eq H^+ pH unit^{-1} g^{-1} FW		
10-9	1289	1211	1200
9-8	1024	944	944
8-7	620	570	480
7-6	1025	906	867
6-5	1151	1089	978
5-4	1287	1111	1059
4-3	1424	1309	1252

The total buffer capacity of the C_4 leaf extracts was 87% of the C3 one (averages of 7820, 7140 and 6780 μEq H^+ pH unit^{-1} g^{-1} FW for C_3, C3-C4 intermediate and C_4 species, respectively; Table 7.3).

There was no significant change in the buffer capacity of extracts prepared from illuminated leaves (Fig. 7.7), although the difference among the photosynthetic type persisted (Table 7.5).

Similar to the pattern of buffer capacity exhibited by the extracts prepared from leaf discs kept in darkness, the buffering of cell sap prepared from preilluminated leaf discs was higher at a pH of above 8 or below 7 than that at pH 7 to 8 (Table 7.6).

Table 7.5. Buffer capacity of the cell sap extracted from illuminated leaf discs of various plant species belonging to C_3, C_4, and C3-C4 intermediate photosynthetic types. The extraction medium used was unbuffered 0.3 M sorbitol.

Photosynthetic type/ Species	Buffer capacity	
	Physiological (pH 7-8)	Total (pH 3-10)
	µEq H^+ pH $unit^{-1}$ g^{-1} FW	µEq H^+ g^{-1} FW
C_3 Species		
Alternanthera sessilis	610	7510
Arachis hypogea	610	7700
Commelina communis	610	7500
Lycopersicon esculentum	620	7640
Pisum sativum	620	7330
Tagetes erecta	630	7030
Tridax procumbens	600	7110
Average	620 (100)	7400 (100)
C3-C4 Intermediate Species		
Alternanthera ficoides	540	6480
Alternanthera tenella	540	6860
Mollugo nudicaulis	570	6760
Parthenium hysterophorus	560	7080
Average	560 (90)[a]	7080 (96)[a]
C_4 Species		
Alternanthera pungens	480	5810
Amaranthus hypochondriacus	480	5370
Amaranthus viridis	470	5360
Gomphrena globosa	480	5110
Portulaca oleracea	460	5680
Sorghum bicolor	480	5880
Zea mays	470	5900
Average	470 (76)[a]	5900 (80)[a]

[a] Values in parentheses are % of C3 species.

Table 7.6. Average buffer capacity of cell sap prepared in unbuffered 0.3 M sorbitol from pre-illuminated leaf discs of different photosynthetic types.

Buffer capacity/ pH range	Average buffer capacity for cell sap from		
	C_3 species	C3-C4 species	C4 species
	μ Eq H^+ pH $unit^{-1}$ g^{-1} FW		
10-9	1211	1200	1089
9-8	1078	1066	844
8-7	620	560	470
7-6	967	922	644
6-5	1076	1000	867
5-4	1148	1089	908
4-3	1300	1244	1078

Stem

The physiological buffer capacity of cell saps prepared from darkened stem tissue of the C_4 species was slightly less than (82% of the C_3) that of C_3, and the C3-C4 intermediate species (Figs. 7.7, Table 7.7).

There was no significant difference in the total buffer capacity of extracts prepared from darkened stem tissue of C_4 plants was 74% of the C_3 species (Fig. 7.8, Table 7.7). Illumination caused no significant change in the physiological or total buffer capacity of stem extracts (Fig. 7.9).

Table 7.7. Buffer capacity of the cell sap (extracted in unbuffered 0.3 M sorbitol) from darkened stem tissue of plant species belonging to C3, C_4, and C3-C4 intermediate photosynthetic types.

Extraction medium/ Plant Species	Buffer capacity	
	Physiological (pH 7-8)	Total (pH 10-3)
	$\mu Eq\ H^+$ pH $unit^{-1}$ g^{-1} FW	$\mu Eq\ H^+\ g^{-1}$ FW
C_3 Species		
Alternanthera sessilis	315	3300
Arachis hypogea	310	3340
Commelina communis	300	3470
Lycopersicon esculentum	295	3250
Pisum sativum	300	3000
Tagetes erecta	305	3280
Tridax procumbens	305	3440
Average	305 (100)	3330 (100)
C3-C4 Intermediate Species		
Alternanthera ficoides	290	3200
Alternanthera tenella	305	3150
Mollugo nudicaulis	270	3260
Parthenium hysterophorus	280	3150
Average	285 (93)[a]	3210 (97)[a]
C4 Species		
Alternanthera pungens	240	3010
Amaranthus hypochondriacus	265	3180
Amaranthus viridis	260	2700
Gomphrena globosa	255	2400
Portulaca oleracea	265	2600
Sorghum bicolor	220	2500
Zea mays	245	2500
Average	250 (82)[a]	2450 (74)[a]

[a] Values in parentheses are % of C3 species.

Root

The physiological and total buffer capacity of cell saps prepared from darkened root tissues of C_4 plants was very much closer to that of C_3 or C3-C4 intermediate species (Table 7.8).

Table 7.8. Buffer capacity of the cell sap (extracted in unbuffered 0.3 M sorbitol) from darkened root tissue of plant species belonging to C3, C_4, and C3-C4 intermediate photosynthetic types.

Extraction medium/ Plant Species	Buffer capacity	
	Physiological (pH 7-8)	Total (pH 10-3)
	μEq H^+ pH unit^{-1} g^{-1} FW	μEq H^+ g^{-1} FW
C_3 Species		
Alternanthera sessilis	275	3900
Arachis hypogea	255	3350
Commelina communis	240	3200
Lycopersicon esculentum	255	3200
Pisum sativum	240	3000
Tagetes erecta	265	3200
Tridaxprocumbens	225	3140
Average	250 (100)	3250 (100)
C3-C4 Intermediate Species		
Alternanthera ficoides	240	3150
Alternanthera tenella	260	3130
Mollugo nudicaulis	240	3200
Parthenium hysterophorus	260	3200
Average	250 (100)[a]	3150 (97)[a]
C_4 Species		
Alternanthera pungens	230	3000
Amaranthus hypochondriacus	230	3200
Amaranthus viridis	270	3200
Gomphrena globosa	240	3010
Portulaca oleracea	260	3180
Sorghum bicolor	250	3180
Zea mays	250	3180
Average	245 (98)[a]	3110 (96)[a]

[a]Values in parentheses are % of C_3 species.

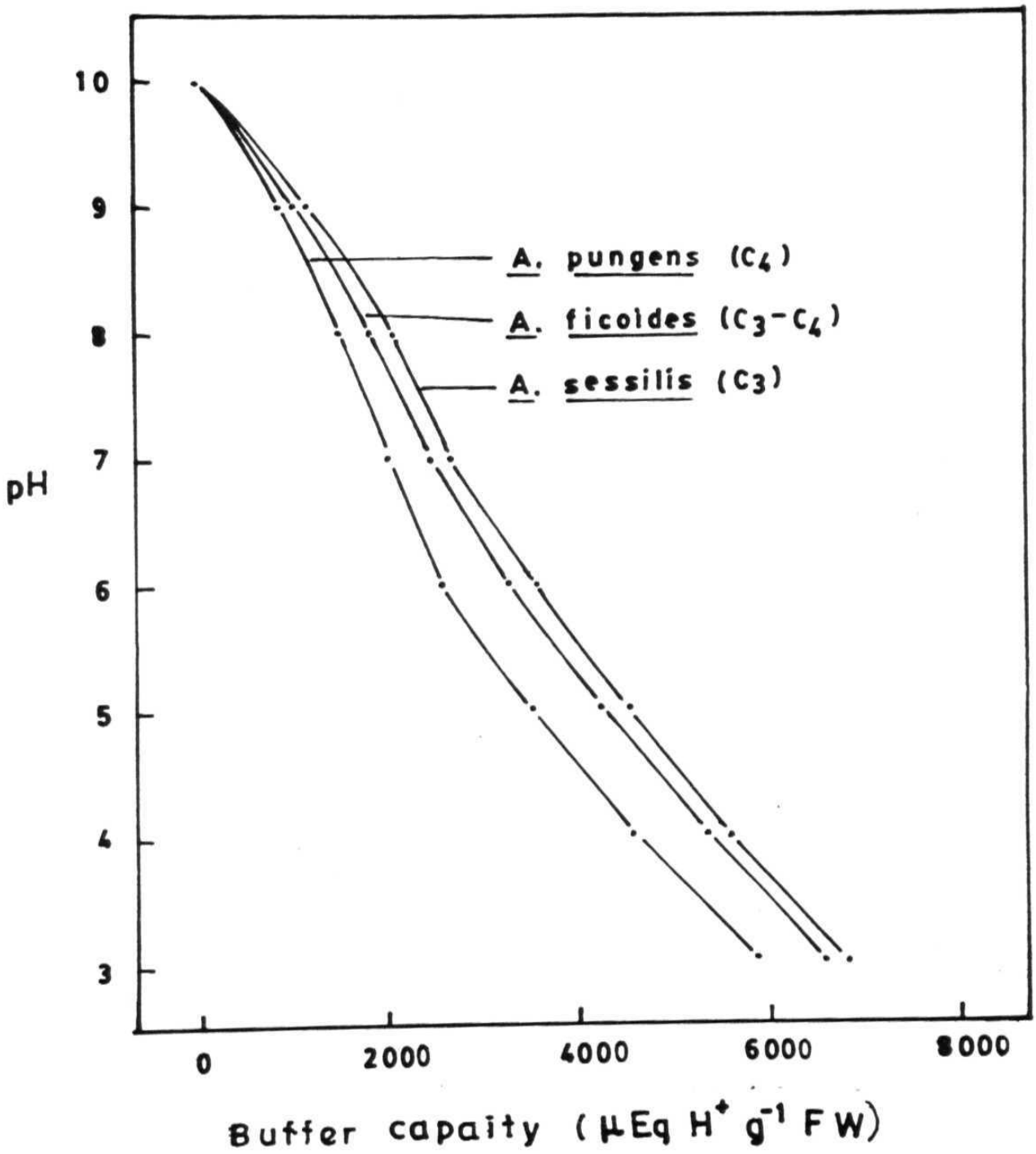

Figure 7.7. Buffer capacity of the cell sap extracted from lluminated leaf discs of C_3 *'{Alternanthera sessilis*), C3-C4 intermediate (A. *ficoides)* and C_4 (A. *pungens*) species, after the predark-treatment, the leaf discs were illuminated at 1000 μmol m^{-2} s^{-1} for 30 min at 30 °C and then extracts were prepared using unbuffered 0 3 M sorbitol and centrifuged at 15 000 *g* for 1 min to clear the debris. The supernatant (after centrifugation) was used for measruing the pH using a digital pH meter.

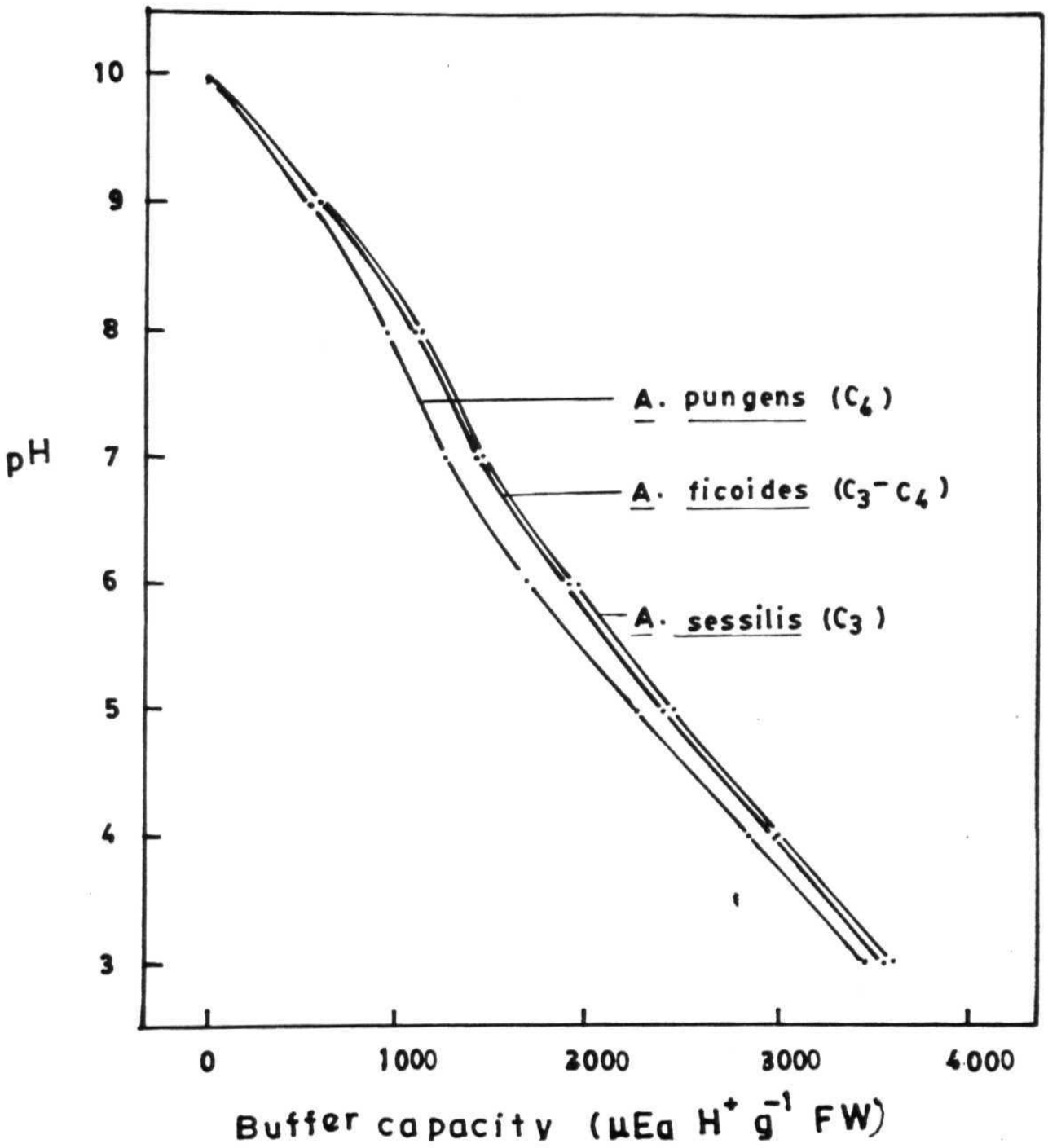

Figure 7.8. Buffer capacity of the cell sap extracted from predarkened (2-h) stem tissue of C3 (***Alternanthera*** *sessilis),* C3-C4 intermediate *(A. ficoides)* and C_4 *(A. pungens)* species. After the predark-treatment, the tissue was blotted dry and then then extracted with, using unbuffered 0.3 M sorbitol. The extracts were cleared by centrifuging and the supernatant (after centrifugation) was used for measuring the pH as indicated in Materials and Methods..

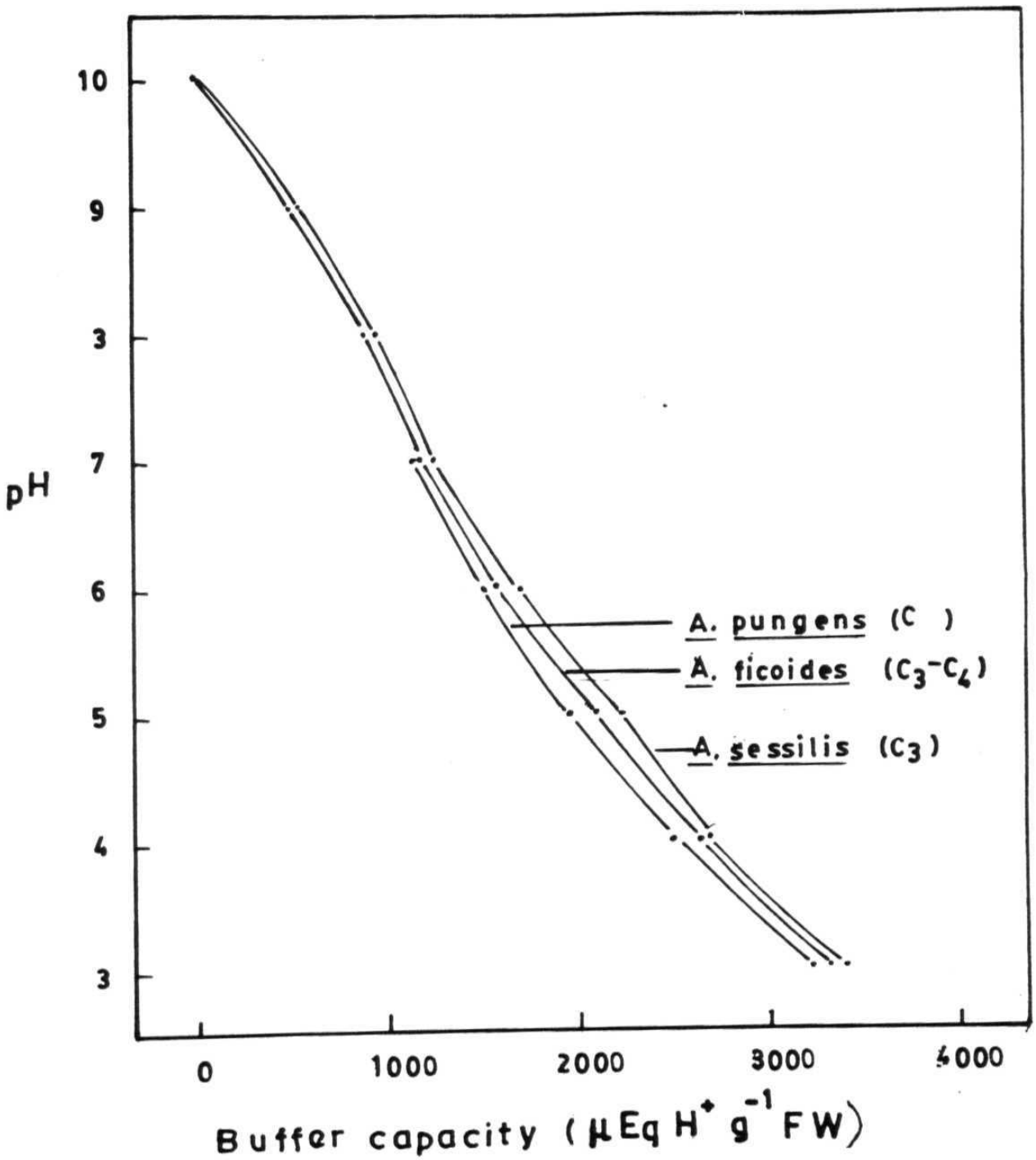

Figure 7.9 Buffer capacity of the cell sap extracted from illuminated stem tissue of C_3 (*Alternanthera sessilis*), C3-C4 intermediate (A. *ficoides*) and C_4 (A. *pungens*) species. After the predark-treatment, the tissue was blotted dry and illuminated at 1000 μmol m^2 s^{-1} for 30 min period and then extracts were prepared using unbuffered 0.3 M sorbitol and centrifuged at 15 000 *g* for 1 min to clear the debris. The supernatant (after centrifugation) was used for measuring the pH using a digital pH meter.

The physiological buffer capacity of cell saps prepared from roots of C_4 plants was only 51% of the buffer capacity of leaf tissue. Similarly, the total buffer capacity of root cell saps was only 48% of the root value in C_4 species (Tables 7.3, 7.8).

The physiological buffer capacity of root extracts was 40 and 44% of the leaves of the C_3 plants and C_3-C_4 intermediate species, respectively, while the total buffer capacity was 42 and 40% of the leaves of the C_3 and C3-C4 intermediate species, respectively (Figs. 7.3, 7.8).

Discussion

The cytoplasmic pH and its regulation are important during not only metabolism but also development and differentiation of cells (Guern *et al.* 1991). The cytosolic pH is an important secondary messenger during signal transduction (Felle 1989). It is therefore essential that the cellular pH is kept constant with minimum fluctuations, by efficient regulatory mechanism.

During metabolic reactions, the production and consumption of protons/hydroxyl ions is not uncommon. For example., the transport and metabolism of nutrients, which involve production as well as consumption of H^+, protons are transferred not only between intracellular compartments but also to and from surroundings. In bacteria and animal cells, regulation of cytoplasmic pH includes control over the H^+ permeability of the plasma membrane (Madshus 1988).

The sensitivity of biological processes to pH is a common biological response and can be due to several reasons (Guern *et al.* 1991, Gout *et al* 1992). The process may be catalyzed by H^+ or may involve a hydrogen ion as a reactant or product.

Alternatively, a pH change may alter the distribution of a compound or ion across a membrane by altering the permeability of the membrane.

The buffer capacity of the cytoplasm of a few plant species has been estimated from either acid-loading experiments (Sanders and Slayman 1982, Felle and Bertl 1986, Guern *et al.* 1986, Felle 1987), or from titrations of tissue homogenates or organelles (Pfanz and Heber 1986, 1989), or from theoretical calculations based on estimated concentrations of the buffering components in the cytoplasm (Raven and Smith 1976).

The results of this chapter point out that buffering is strong at the extremes of pH (Tables 7.4, 7.6), apparently owing to the presence of carboxyl groups (pK_a values between 2 and 5) and amino or guanidino groups (pK_a values between 8 and 12). Buffering at physiological pH range, *i.e.*, pH 7 to 8, is weak compared to the buffering at values near pH 3 or 9 (Figs. 7.6 to 7.9). However, the pH range of 7 to 8 is of physiological importance. This is the working range of cytosolic and chloroplastic metabolism, both of which are known to be pH sensitive (Guern *et al.* 1991).

The physiological buffer capacity of extracts (prepared in water) from dark-adapted leaves of C_3 or C_3-C_4 intermediate species similar to those of C4-leaf extracts (Table 7.1). However, the buffer capacity of extracts prepared (in water) from illuminated leaf discs of C_4 species were only 76% of the C_3 species or 92% in case of the C_3-C_4 intermediate species (Table 7.2). When the extracts were prepared in unbuffered 0.3 M sorbitol from darkened leaf discs, the buffer capacity of C_4 species was 77% of the C_3 or 84% of the C_3-C_4 intermediate species (Table 7.3). The buffer capacity of leaf extracts, when prepared in unbuffered 0.3 M sorbitol, did not change much upon illumination (Table 7.5).

At pH values close to neutrality phosphate and phosphate esters contribute significantly to buffering (Roos and Boron 1981, Raven 1985, Felle 1988). Homeostatic mechanisms maintain levels of phosphate/phosphate esters in cytosol and chloroplasts. Chloroplast/cytoplasmic proteins are assumed to be highly variable in different plant species (Guern *et al.* 1991). Therefore, we speculate that the variation caused in the buffering by proteins can lead to differences in the buffer capacity of extracts from different photosynthetic types. However, further studies are needed to support this highly speculative hypothesis.

The buffer capacity of the cytoplasm is low compared to the intensity of changes in proton concentration. Studies by Raven (1985) also demonstrated that internal cytoplasmic buffers are not efficient enough to compensate for the H^+ imbalances plant cells face. Furthermore, the buffer capacity has no significance for long-term pH regulation (Smith and Raven 1979) because the buffer components are themselves synthesized by H^+-generating pathways (Kurkdjian and Guern 1989).

The results presented in this chapter indicate that the buffer capacity of leaf extracts prepared in 0.3 M sorbitol from the C_4 plants is less than those of the C_3 species (Table 7.5, 7.6). This can be one of the reasons for the marked differences in the extent of light-induced cytosolic alkalinization between these species (Rajagopalan *et al.* 1993). In addition, our results also suggest that illumination of leaf tissues has no marked effect on the buffer capacity of the extracts (Figs. 7.6, 7.7, Tables 7.3, 7.4). The possibility that the observed changes upon illumination in the cytosolic pH in leaves of C_4 species, as a result of altered buffer capacity, can therefore be ruled out.

Chapter Eight

General Discussion

PEPC catalyzes the irreversible β-carboxylation of PEP in the presence of bicarbonate and Me^{2+} (for e.g., Mg^{2+}, Mn^{2+}) to yield OAA and Pi, a reaction that is responsible for primary carboxylation in C_4 plants (Rajagopalan *et al.* (1994). PEPC, considered as a marker enzyme for C_4 mesophyll cells, is located in the cytosol (Meister *et* al. 1996). PEPC activity is markedly regulated by light/dark transitions in C_4 and CAM species. Light can be expected to modulate the cytoplasmic levels of pH, Ca^{2+}, metabolic effectors (Glc-6-P, Triose-P or L-malate). However, attention has mainly been focused on the diurnal regulation of this enzyme's activity in C_4 and CAM species by posttranslational modifications (Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

Light induces a reversible two- to three-fold enhancement in catalytic activity, while reducing the L-malate sensitivity of PEPC from a variety of C_4 plants, when the enzyme is assayed at suboptimal but physiological levels of pH and PEP (Rajagopalan *et al.* 1994, Chollet *et al.* 1996). A related, physiologically important feature of CAM-PEPC is its day/night fluctuation in enzymatic properties; the rapidly prepared, desalted enzyme extracted from night leaf tissue has lower K_M(PEP) and higher K_I (L-malate) values than those from the corresponding day leaf tissue. These day/night changes in the properties of PEPC lead to a higher enzymatic activity at night and a much lower activity during the day which are paralleled by the classical physiological changes in the activity of CAM (Griffiths 1988).

The present results demonstrate that the light activation pattern of PEPC in the leaves of C_4 plants is quite pronounced and distinct from that in leaves of the C_3 plants or the C3-C4 intermediate species, in terms of both the extent and the pace of

light-activation/dark-deactivation (Tables 3.1, 3.2). Illumination enhances PEPC activity in leaves of C_4 plants by 2-3 fold. However, the level of PEPC light-activation, in the extracts prepared from leaf discs of C_3-C_4 intermediate and C_3 species, was low, with an average value of about 35%. (Table 3.1). Chastain and Chollet (1989) reported slight activation of PEPC from the C_3-C_4 intermediate species of *Flaveria* genus, while no light activation was observed in the C_3 species. We conclude that leaf-PEPC from the C_3-C_4 intermediate and C_3 species of *Alternanthera* undergoes (and other examined species) limited activation by light, even though the extent is low compared to the C_4 species. Gupta *et al.* (1994) also reported similar results.

Studies using intact leaves indicated a requirement of 40 to 120 min to reach maximum light activation (Jiao and Chollet 1988, Van Quy *et al.* 1991, Van Quy and Champigny 1992). Doncaster and Leegood (1987) using leaf slices reported an illumination period of 15 min for the activation of maize-PEPC. Our experiments with leaf discs from various C_4 plant species confirm that a minimum period of 20 min illumination is necessary to activate PEPC (Fig. 3.3; Rajagopalan *et al.* 1993).

The slow pace of light-activation/dark-deactivation of PEPC in C_4 plants is strikingly similar to the slow but steep rate of light-induced cytosolic alkalinization/dark-acidification in leaves of C_4 species, compared to the rapid but small changes in cytosolic pH of C_3 species (Yin *et al* 1990, 1993, 1996, Raghavendra *et al* 1993). The present results suggest that the pattern of light-activation can be taken as an additional criterion to distinguish PEPC from C_3 and C_4 plant species (Rajagopalan *et al* 1993). Comparative studies on C_3, C_4, and

C3-C4 intermediate plant species of *Flaveria* genus indicated a distinct isoform of PEPC in the leaves of C_4 species (Nakamoto *et al.* 1983, Adams *et al* 1986, Bauwe and Chollet 1986).

The sensitivity of PEPC to feedback inhibition by L-malate changes markedly on illumination, particularly in C4 species, while no significant change was observed with PEPC from C_3 or C3-C4 intermediate species. As a result, there was only slight alterations in K_I (malate) due to illumination of leaf discs from C3 or C3-C4 intermediates, whereas K_I (malate) increased by nearly four-fold in C4 species as a result of illumination. When 1 mM L-malate was included in the assay mixture, the light/dark PEPC activity ratios ranged from 8 to 15 in the case of C4 species, compared to 1.5 to 1.9 in the C_3 or 1.7 to 2.2 in the C3-C4 intermediate species Table 3.3). These results again demonstrate that light exerts a differential effect on PEPC from leaves of the C3 or C3-C4 intermediate and C_4 species.

PEPC from the C4 plant species was activated by about 80% by 5 mM Glc-6-P, while only 60% activation was observed with the enzyme from C3 or C3-C4 intermediate species (Table 3.4). These results are consistent with the earlier reports that Glc-6-P activates C4-PEPC to a larger extent than that the enzyme from C3 or C3-C4 intermediate species (Nakamoto *et al.* 1983, Gupta *et al.* 1994).

PEPC is a cytosolic enzyme in C4-mesophyll tissue and it is known that light causes marked alkalinization of cytosol in mesophyll cells of C_4 plants (Raghavendra *et al* 1993). Since PEPC is an extremely pH-sensitive enzyme, the pH of mesophyll cytosol could be an important factor in regulating the activity of PEPC. Our observations illustrate that illumination elevates concurrently the pH of cell sap and

PEPC activity (Fig. 3.5, Table 3.4). Since the dark-form of the enzyme is very sensitive to pH of medium, *in vitro*, we hypothesize that light-induced cytosolic alkalinization will have a marked effect on the dark-form of the enzyme *in vivo*. Cytosolic pH affects the phosphorylation status of PEPC from mesophyll protoplasts of *Sorghum* (Pierre *et al.* 1992), *Digitaria sanguinalis* (Giglioli-Guivarc'h *et al.* 1995, 1996, Duff *et al.* 1996) and barley (Smith *et al.* 1996, Lillo *et al.* 1996). Our results provide further evidence for the regulation of PEPC in leaves of *Alternanthera pungens* by cytosolic pH.

From the results obtained in this study we suggest that 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 status of the enzyme, (b) change in the oligomeric state of the enzyme, and (c) an unknown change in the conformation state of the enzyme. Nevertheless, all these three factors could be interrelated (Rajagopalan *et al.* 1993).

The possible relationship between the light-induced changes in the properties of the C_4 enzyme and its reversible phosphorylation status was suggested from experiments with an *in vitro* ^{32}P-labeled phosphorylation system (Budde and Chollet 1986). This work demonstrated that PEPC from maize and sugarcane can be phosphorylated *in vitro* exclusively on serine residue by an ATP-dependent, soluble protein kinase(s) in desalted extracts prepared from illuminated green leaf tissue. Subsequent *in vitro* ^{32}P-labeling studies with maize (Nimmo *et al.* 1987, Jiao and Chollet 1988) and *Sorghum* (Vidal *et al.* 1990, Jiao *et al.* 1991b) leaf tissues have indicated that C_4-photosynthetic-PEPC is phosphorylated on serine residues near its

N-terminus in the light and the enzyme becomes less sensitive to L-malate sensitive than that in darkness. Using rapidly purified PEPC from maize leaves, Jiao and Chollet (1988) reported that the enzyme isolated from light-adapted plants has a higher catalytic activity and lower L-malate sensitivity than the corresponding dark-form of PEPC. Although both light and dark enzymes contained phosphorylated serine, the degree of phosphorylation in the light-form was 50% greater than that in the dark enzyme (Jiao and Chollet 1988).

Base-loading of leaf discs from the C_4 species *Alternanthera pungens* not only elevated the cell sap pH but also the activity of PEPC (Figs. 4.1 to 4.3, Table 4.1). The enhancement of PEPC activity upon base-loading can therefore be attributed to the elevation of cytosolic pH. A significant increase in cytosolic pH after exposure to NH_4^+ was reported in maize root tips (Kurkdjian *et al.* 1978), *Acer pseudoplatanus* root cells (Roberts *et al.* 1982) and very recently in mesophyll protoplasts and cells of *Digitaria sanguinalis* (Duff *et al.* 1996, Giglioli-Guivarc'h *et al.* 1996). Felle and Bertl (1986) showed that the cytoplasmic pH in *Riccia fluitans* cells increased from 7.5 to 8.1 on preincubation with 0.2 mM procaine. On the other hand, acid-loading of leaf discs resulted in acidification of the cytosol and decreased PEPC activity by 50% compared to those of control (Figs. 4.4, 4.5). There are reports on induction of cytosolic acidification by weak acids (Reid *et al.* 1985, Basso and Ulrich-Eberius 1987).

While the activity of PEPC (from the C_4 species, *A. pungens)* increased by almost two-fold due to cytosolic alkalinization the sensitivity of the enzyme to L-malate decreased by about 14%, compared to the control. The K_I (malate) increased

by 25% as a result of cytosolic alkalinization. In illuminated leaves, L-malate sensitivity of PEPC decreased by 34% and the K_i (malate) increased by about four-fold. Thus, the changes observed in the kinetic and regulatory properties of PEPC due to alkalinization were not exactly comparable to those induced by light (Table 4.1). However, the rate and extent of light-activation even increased (marginally) due to cytosolic alkalinization (Fig. 4.7).

Studies using isolated protoplasts from *Sorghum* (Pierre *et al.* 1992), *Digitaria sanguinalis* (Duff *et al.* 1996, Gigliolic-Guivarc'h *et al.* 1996), and barley (Smith *et al.* 1996, Lillo *et al.* 1996) have shown that cytosolic pH is an important factor in regulating the light-induced changes in activity and kinetic/regulatory properties of PEPC. However, cytosolic pH may modulate either PEPC or PEPC-PK or both (Rajagopalan *et al.* 1993, 1994). Recent findings by Chollet's group that PEPC-PK from maize (Wang and Chollet 1993b) as well as *Mesembryanthemum crystallinum* (Li and Chollet 1994) require alkaline pH for maximal activity further supports this hypothesis.

We also propose that activation/reduction in PEPC activity as a result of base-acid-modulation is not due to the limited proteolysis. Inclusion of PMSF or benzamidine or chymostatin (protease inhibitors) in the extraction medium had no effect on the increase in PEPC activity due to base-loading. Western blotting using anti-PEPC antiserum confirmed that there was no detectable change in PEPC protein during acid-/base-modulation of PEPC in leaf discs (Fig. 4.9). Our results indicate that the rise in cytosolic pH enhances the activity of PEPC. Further, it is possible that

changes in cytosolic pH would affect free cytoplasmic Ca^{2+} levels, and subsequent light-activation of C_4-PEPC-PK, as suggested recently (Duff *et al.* 1996, Giglioli-Guivarc'h *et al.* 1996).

Another important observation of the present study is the marked increase in cytosolic pH and PEPC activity in leaf discs of several C_4 plants, upon incubation in NH_4Cl (base-loading), with only marginal effects in leaf tissue from the C_3 or the C_3-C_4 intermediate species on base-loading (Table 4.3).

The kinetic and regulatory properties of PEPC from the acid- or base-loaded leaf discs (Table 4.1) indicate a relatively stable alteration in the enzyme. Marked modulation of PEPC activity *in vitro* by pH (or metabolites or protein concentration) has been recorded and related to changes in association/dissociation state of maize PEPC (Kruger and Kluge 1987, Wedding *et al.* 1992, Willeford and Wedding 1992). The importance of such changes in oligomeric status in regulation of PEPC *in vivo* is yet to be experimentally confirmed. The effect of pH modulation on PEPC activity reported in this study may be relevant under other conditions besides exposure to light/darkness. For example, the pH of cytosol is known to decrease under anoxia or gaseous (e.g., SO_2) pollution and increase after nitrate feeding (Pfanz and Heber 1989, Wagner *et al.* 1990, Heber *et al.* 1994).

Regulatory phosphorylation can lead to either activation or inactivation of the target enzyme. However, the extent of change in activity varies greatly among different enzymes. As a result of phosphorylation several fold activation of the enzymes can occur, so that one of the two forms is less active compared to the other.

In addition, protein phosphorylation can also alter regulatory properties of the target enzyme (Huber *et al.* 1994, Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

To assess critically the effects of phosphorylation on the properties of C_4-photosynthetic-PEPC, experiments were performed by incubating the light- or dark-form of PEPC, extracted from leaf discs of *A. pungens* with Mg^{2+}-ATP *in vitro.* Such incubation with Mg^{2+}-ATP, resulted in a marked increase in the activity of the enzyme besides alterations in the sensitivity to L-malate. The extent of PEPC activation, in the presence of Mg^{2+}-ATP, measured in the absence of 1 mM L-malate, enhanced dramatically, during the initial 30 min of preincubation in the phosphorylation medium (Fig. 5.1 A) and L-malate sensitivity decreased (Fig. 5.4b). The increase in PEPC activity along with the decrease in malate sensitivity indicates that PEPC is being phosphorylated on incubation with Mg^{2+}-ATP *in vitro.* Such approach of indirect measurement of PEPC phophosrylation has been used with the extracts of *Zea mays* (Jiao and Chollet 1988).

The activity of PEPC extracted from leaf discs, incubated in NH_4Cl, increased as a result of *in vitro* phosphorylation. PEPC activity enhanced by more than two-fold and L-malate sensitivity decreased from 75% to 54% (Figs. 5.4A, b). This clearly indicates that PEPC extracted from base-loaded leaf discs is more phosphorylatable compared to the control enzyme and further indicates that cytosolic alkalinization plays an important role in the phosphorylation of PEPC by making the enzyme more responsive to phosphorylation (Rajagopalan *et al.* 1993).

Typically, the ratios of PEPC activity following preincubation with the endogenous protein kinase and Mg^{2+}-ATP to those in the absence of ATP (control) were about two, when PEPC was assayed under standard conditions (pH 7.3, 1.5 mM PEP). The presence of 1 mM L-malate, during assay, increased this ratio to four. From these *in vitro* phosphorylation studies, it appears that the changes in L-malate sensitivity of PEPC are associated with an increase in catalytic activity.

The phosphorylation status of PEPC *in vitro* was further examined by dephosphorylation of the enzyme with AP. Exogenous AP has shown to affect the property of a variety of phosphorylated enzymes *in vitro* (e.g., Nimmo *et al.* 1987, Jiao and Chollet 1988, Arrio-Dupont *et al.* 1992). Preincubation of the light-form PEPC with exogenous AP converted the more active, less malate-sensitive PEPC to a less active, more malate-sensitive enzyme with properties similar to those of the control or phosphatase-preincubated dark enzyme. It is interesting to note that exogenous AP had little effect on the properties of dark-form PEPC even though it is partially phosphorylated (Jiao and Chollet 1988, Vidal *et al.* 1990). In the present study, the *in vitro* dephosphorylation, induced by AP on PEPC, and thus change in activity and L-malate sensitivity (Figs. 5.2, 5.5), was similar to those reported by Jiao and Chollet and Arrio-Dupont *et al.* (1992). The decrease in activity due to AP was rapid in the initial phase, and the activity decreased to 50% within the first 30 min (Fig. 5.2A).

Our studies using α-D-Man (an analogue of Glc) demonstrated that millimolar concentrations of the Glc-analogue were sufficient to reduce the light activation of

PEPC . Man causes sequestration of Pi and decreases cellular ATP and is expected to restrict phosphorylation of cytosolic enzymes. The sensitivity to Man thus indicates the dependence of the concerned phenomenon to the phopshorylation of protein/enzyme. The activity of PEPC in illuminated leaves decreased by 70%, as a result of Man-feeding (Fig. 5.3A). In addition, L-malate sensitivity of PEPC decreased and reached the level of dark-control (Fig. 5.3B). There was no marked change due to Man-feeding in the activity of PEPC extracted from dark-Man fed leaves (Fig. 5.3A). There was a two-fold decrease in PEPC activity as a result of feeding 5 mM Man to base-loaded leaf discs. Further, the degree of light-activation decreased markedly compared to the control (Table 5.1). These results illustrate that the light activation of PEPC is an ATP-dependent process, *i.e.,* phosphorylation of enzyme.

Van Quy and Champigny (1992) reported that feeding of Man to low-NO_3^- leaves of wheat (a C_3 species) restricted the light-activation of PEPC, while causing deactivation of the previously light-activated PEPC of high-NO_3^- leaves and making the activity-levels similar to that of dark-adapted low-NO_3^- leaves.

The *in vivo* feeding of inorganic nitrate salts (of K^+, Na^+ and NH_4^+) are quite effective in enhancing extractable PEPC activity (Tables 6.1 to 6.3) from both the C3 and C4 species of *Alternanthera.* The order of activation observed by nitrate salts is: NH_4NO_3 > $NaNO_3$ > KNO3. This seems logical since NH4NO3 is a combined nitrogen source containing ammonium and nitrate ions, both are known to activate the enzyme. The greater effect of $NaNO_3$ compared to potassium salt is intriguing, but may be interpreted to be due to the beneficial effect of sodium as a micronutrient for C_4 species (Brownell 1979). The malate sensitivity of PEPC, which has been used to

assess the phosphorylation status of the enzyme, indicates that there is an enhanced phosphorylation of the enzyme on short-term feeding with various nitrate salts (Tables 6.4 to 6.7). Salts of SO_4^{2-} have not much effect on the L-malate inhibition of PEPC extracted from either darkened or illuminated leaves.

The cytoplasmic pH and its regulation are important during not only metabolism but also development and differentiation of cells (Guern *et al.* 1991). The cytosolic pH is an important secondary messenger during signal transduction (Felle 1989). It is therefore essential that the cellular pH is kept constant with minimum fluctuations, by cell's efficient regulatory mechanisms. The sensitivity of biological processes to pH is a common biological response and can be due to several reasons (Guern *et al.* 1991, Gout *et al.* 1992). The process may be catalyzed by H^+ or may involve a hydrogen ion as a reactant or product. Alternatively, a pH change may alter the distribution of a compound or ion across a membrane by altering the permeability of the membrane. In addition, several enzymes exhibit drastic changes in their kinetic/regulatory properties as a result of small changes in the proton concentration (Tifton and Dixon 1983).

The buffer capacity of the cytoplasm of plant species has been estimated by using various methods (Guern *et al.* 1991). We have employed one of the simplest ways of titrating the extracts of plant tissues for estimating the buffering capacities of tissues from C_3-, C4-plants and C_3-C_4 intermediates. The results of our study point out that buffering is strong at the extremes of pH (Table 7.4, 7.6), apparently owing to the presence of carboxyl groups (pK_a values between 2 and 5) and amino or guanidino groups (pK_a values between 8 and 12). Buffering at physiological pH range *i.e.*,

pH between 7 and 8, was weak compared to the buffering at values near pH 3 or 9 (Figs. 7.6 to 7.9). However, the pH range of 7 to 8 is of great physiological significance. This is the working range of cytosolic and chloroplastic metabolism, both of which are known to be pH sensitive (Edwards and Walker 1983, Guern *et al.* 1991).

When the extracts were prepared in unbuffered 0.3 M sorbitol from darkened leaf discs, the buffer capacity of C_4 species was 77% of the C_3 (Table 7.3). The buffer capacity of leaf extracts, when prepared in unbuffered 0.3 M sorbitol, did not change much upon illumination (Table 7.5).

At pH values close to neutrality, phosphate and phosphate esters contribute significantly to buffering (Roos and Boron 1981, Raven 1985, Felle 1988). Homeostatic mechanisms maintain levels of phosphate/phosphate esters in cytosol and chloroplasts. Chloroplast/cytoplasmic proteins are assumed to be highly variable in different plant species (Guern *et al.* 1991). Therefore, we speculate that the variation caused in the buffering by proteins can lead to differences in the buffer capacity of extracts from different photosynthetic types.

The buffer capacity of cell saps made from leaf extracts in unbuffered 0.3 M sorbitol from the C_4 plants was less than those of the C_3 species (Table 7.5, 7.6). This can be one of the reasons for the marked differences in the extent of light-induced cytosolic alkalinization between these species (Rajagopalan *et al.* 1993). In addition, our results also suggest that illumination of leaf tissues has no marked effect

on the buffer capacity of the extracts (Figs. 7.6, 7.7, Tables 7.3, 7.4). We also rule out the possibility that the observed changes upon illumination in the cytosolic pH in leaves of C_4 species are a result of altered buffer capacity.

The major contribution of the present work is to demonstrate and emphasize the role of cytosolic pH in regulating PEPC activity, particularly on illumination. Another factor could be the cytosolic calcium. The cytosolic pH/calcium can modulate the activity of either PEPC or PEPC-PK or both (Fig. 8.1). While indicating the possible effects of pH and calcium, Fig. 8.1 attempts to summarize the current thoughts on short-term regulation by light of this `multi-faceted' enzyme, particularly in mesophyll cells of C_4 plants.

Figure 8.1. A hypothetical scheme for the regulation of PEPC by various factors/phenomena. Two major mechanisms of posttranslational regulation of PEPC are known: The first mechanism is the reversible phosphorylation (common to other cytosolic enzymes such as nitrate reductase or sucrose-phosphate synthase) carried out by PEPC-protein kinase, while the second one is the transitions in the oligomeric status of the protein observed *in vitro* but not yet *in vivo.* In addition to light, hormonal or nutritional factors modulate the expression of PEPC gene. The activity of both PEPC-PK and PEPC are influenced by light-induced cytosolic alkalinization and/or inorganic ions (for example Ca^{2+} or Mg^{2+}). As a result of reversible *(in vitro* or *in vivo)* phosphorylation by PEPC-PK of PEPC, PEPC's kinetic and regulatory properties are changed. The enzyme become more active, less sensitive to malate, and to a limited extent increase in Glc-6-P. Compounds which are known to inhibit (-) any of these reactions are shown. The major area, yet to be exploited is to understand the possible interrelationship between phosphorylation/dephosphorylation and the oligomeric status of the enzyme.

Chapter Nine

Summary and Conclusions

Phospho*enol*pyruvate carboxylase (PEPC, Orthophosphate : oxaloacetate carboxylase [phosphorylating], EC 4.1.1.31) is a ubiquitous enzyme catalyzing the irreversible carboxylation of PEP to oxalacetate (OAA) and inorganic phosphate. The enzyme plays a key role in (photosynthetic) primary CO_2 fixation by C4 plants and during CAM. PEPC is a cytosolic enzyme located in mesophyll cells of C_4 species. In spite of its cytosolic location, PEPC is markedly activated by light, particularly in leaves of C_4 plants (Rajagopalan *et al.* 1994).

PEPC from C4 plants is an allosteric enzyme, which is sensitive to regulation by allosteric activators (for example, Glc-6-P, Triose-P) and feedback inhibitors (oxaolacetate, L-malate, aspartate). Besides, these effectors can also act as signaling elements during the light-activation of C4-photosynthetic-PEPC *in situ.* A major factor of this posttranslational modification is the regulatory reversible phosphorylation cycle mediated by a PEPC-protein serine/threonine kinase (Lepiniec *et al.* 1994, Rajagopalan *et al.* 1994, Chollet *et al* 1996).

An important factor in the microenvironment of mesophyll cell is the cytosolic pH. Using various mesophyll cell preparations (cell sap, protoplasts, and cells) from both C_4 and C3 plant leaf sources, it was found that the degree of PEPC-protein-serine kinase (PEPC-PK) activity, and thereby PEPC phosphorylation status, is dependent on the light-induced alkalinization (and also free Ca^{2+} levels) of the cytosol (Rajagopalan *et al.* 1993, Duff *et al.* 1996, Giglioli-Guivarc'h *et al.* 1996, Lillo *et al.* 1996, Smith *et al.* 1996).

The magnitude of light-induced changes in the activity of PEPC in C3 leaves are significantly less than those of C_4 species (Rajagopalan *et al.* 1994). At present, there are two different hypotheses on the light-activation/regulation of PEPC from the

leaves of C_3 plants: (a) regulation by mainly reversible protein phosphorylation, in a light- and/or N-modulated manner, as in leaves of N-deficient wheat seedlings (e.g. Duff and Chollet 1995), and/or (b) either only a minor or no role of phosphorylation and regulation of PEPC by primarily the metabolite levels (such as Glc-6-P, Triose-P, 3-PGA, L-malate, aspartate) and pH of cytosol (for example, Gupta *et al.* 1994, Leport *et al.* 1996). Light and darkness may also up- or downregulate phosphatases (though yet to be documented) and thereby modulating the activity and L-malate sensitivity of PEPC.

The present investigation is to study and analyze the regulation of PEPC in leaves of *Alternanthera pungens*, an NAD-ME type C4-dicot species, in relation to the pattern of response in leaves of a few other C3, C4, and C3-C4 intermediate species. Particular emphasis is made on light activation of PEPC, light-induced cytosolic alakalinization and related factors such as buffer capacities.

The following aspects were **examined:**

(1) Pattern of PEPC activity and pH of ` cytosol-enriched' cell sap during light activation and dark-deactivation,

(2) Modulation of ` cytosol-enriched' cell sap pH and PEPC activity after acid- or base-loading,

(3) Indirect assessment of the phosphorylation status of PEPC, by monitoring the stimulation of PEPC activity after incubation with Mg^{2+}-ATP and decrease after treatment with alkaline phosphatase,

(4) Modu'lation of PEPC activity (in leaves) by feeding inorganic salts *in vivo*,

(5) Buffer capacity of cell sap prepared from leaves, stems and roots of C3, C_4, and C3 -C4 intermediate species.

The first set of experiments were performed to evaluate the effects of light on PEPC in plant species belonging to three different photosynthetic groups, *viz.*, C3-, C4-, and C3-C4 intermediate species and to assess whether the light activation pattern can be used as a criterion to establish the C_4-form of PEPC. Attempts were made to evaluate the responses of `cytosol-enriched' cell sap pH to light, in relation to PEPC activity. `Cytosol-enriched' cell sap was prepared by extracting the leaves in unbuffered 0.3 M sorbitol, and PEPC activity was assayed, as described by Rajagopalan *et al.* (1993).

When the predarkened leaf discs were illuminated, the activity of PEPC increased by about 2- to 3-fold, similar to changes occurred in cytosolic pH, in *Alternanthera pungens* (C4 species), compared to the limited (50 to 60% above dark-level) light-activation in leaves of C3 or C3-C4 intermediate species, *A. sessilis* or *A. ficoides,* respectively. Similar to light-activation, the dark-deactivation of PEPC was also similar to the pattern of changes in cell sap pH. A survey of PEPC from several plants confirmed that the pace of dark-deactivation of PEPC is slow requiring 60 to 80 min in case of C3 or C3 -C4 intermediate species and about 2 h in C4 species.

L-malate is a feedback inhibitor of PEPC. The sensitivity of the C4-photosynthetic-PEPC to L-malate changes markedly upon illumination. However, the L-malate sensitivity of PEPC from leaf discs of the C_3 or the C3-C4 intermediate species, show no significant differences on exposure to light. Consequently, the K_I (L-malate) increased by nearly 4-fold as a result of illuminating C4 leaf discs, while there was only marginal change in the K_I (L-malate) of PEPC from the C3 or the C3-C4 intermediate species. We feel that the pattern of light-activation, dark-deactivation

and sensitivity to L-malate can be taken as additional criteria to distinguish C_4-photosynthetic-PEPC from the enzyme from the C_3 species.

Illumination caused a measurable increase in pH of `cytosol-enirched' cell sap prepared from the leaves of *Alternanthera pungens,* a C4 species. The pH of `cytosol-enriched' cell sap increased during illumination and decreased after transfer to darkness. The extent of light-dependent alkalinization of `cytosol-enriched' cell sap was three-fold higher in leaves of the C4 species evaluated than in the C3 species. The degree of cell sap alkalinization in the C3-C4 intermediate species was more similar to the pattern of the C3 species.

Upon illumination, the degree of cytosolic alkalinization was much greater than the vacuolar acidification in both C3 and C4 plant species (Yin *et al.*. 1993, Raghavendra *et al.* 1993, Rajagopalan *et al.* 1993). In their studies, changes in cytosolic pH was enormous, particularly in mesophyll cells of C4 leaves, and unlike the weak acidification of the vacuole. This indicates that most of the light-induced changes in pH of the cell sap observed in this study (Figs. 3.6, 3.7) is probably the result of cytosolic alkalinization.

In the next set of experiments, the cell sap pH in leaf discs of the C4 species, *Alternanthera pungens,* was modulated by using exogenous compounds capable of either cytosolic acidification (e.g., propionic, butyric, or salicylic acid) or alkalinization (NH_4Cl or procaine). Base-loading of *A. pungens* (C4 species) leaf discs enhanced the activity of PEPC by two-fold and elevated the cytosolic pH by 0.2 unit. In contrast, the acid-loading resulted in 50% reduction in PEPC activity and a decrease of 0.3 unit in cytosolic pH. To our knowledge, this is the first report on acid-base-modulation of PEPC activity in leaf discs of C_4 species. Our results clearly

demonstrate the marked modulation of extractable PEPC activity in C_4 leaf discs in response to alteration in cytosolic pH by acid-base-loading. These observations form a nice complement of recent findings that cytosolic pH and Ca^{2+} levels play an important role in the transduction of light-signal during the activation of PEPC in mesophyll protoplasts of *Digitaria sanguinalis* (Duff *et al.* 1996, Giglioli-Guivarc'h *et al.* 1996) and barley (Lillo *et al* 1996, Smith *et al.* 1996).

The marked increase in cytosolic pH and PEPC activity, upon base-loading, were observed in only leaves of C4 plants, only marginal effect in leaf tissue from the C3 or the C3-C4 intermediate species. The reason for this difference is unknown. The kinetic and regulatory properties of PEPC extracted from acid-loaded or base-loaded leaf discs indicated a stable alteration in the enzyme. The acid-/base-induced changes were reversible. Marked modulation of PEPC activity *in vitro* by pH (or metabolites, protein concentration, ionic strength; see Rajagopalan *et al.* 1994) has been recorded and related to changes in association/dissociation state of C4 PEPC (Wedding *et al.* 1992, Willeford and Wedding 1992). We speculate that cytosolic alkalinization may induce conformational changes in PEPC, making this enzyme more phosphorylatable by protein kinases. Besides the physiological importance of this basic observation, acid-/base-modulation of PEPC *in vivo* could be an important tool to study the enzyme.

Reversible phosphorylation of protein is among the important control mechanisms on cellular processes in plants and animals (Smith and Walker 1996). The phosphorylation status of proteins is regulated by opposing activities of protein kinases and protein phosphatases. PEPC is also subjected to such posttranslational regulation by phosphorylation (by a PEPC-protein kinase) and dephosphorylation

(by a type 2A protein phosphatase) (Carter *et al.* 1991, Jiao and Chollet 1991, Rajagopalan *et al* 1994, Chollet *et al.* 1996).

In the next step, we have assessed indirectly the *in vitro* phosphorylation and dephosphorylation of PEPC, by using agents capable of phosphorylation (Mg^{2+}-ATP) or dephosphorylation (alkaline phosphatase; AP) of the enzyme. Further, we have used α-D-mannose (Man), a phosphate sequester in cells or organelles). Man, an analogue of Glc, sequesters Pi and reduces the availability of cellular ATP (Moore 1981, Kime *et al*. 1982).

Incubation of PEPC in crude extracts with Mg^{2+}-ATP resulted in an increase in the enzyme activity and a decrease in L-malate sensitivity suggesting that PEPC is phosphorylated by an endogenous protein-kinase in the presence of ATP and $MgCl_2$. Such indirect assessment of PEPC phosphorylation has earlier been made with extracts of maize and *Sorghum* (Nimmo *et al.* 1987, Jiao and Chollet 1988, Arrio-Dupont *et al.* 1992). The extent of phosphorylation was more in samples prepared from light-adapted leaves, while base-loading further enhanced the phosphorylation status of the enzyme.

Our studies also indicated that the cytosolic alkalinization, induced by base-loading, enhanced the extent of phosphorylation of PEPC. Wang and Chollet (1993a, b) reported that protein-kinase preparation from maize and tobacco is pH-sensitive, with higher values reaching at about pH 7.8.

Exogenous AP has been used to dephosphorylate a variety of phosphorylated enzymes *in vivo* including phosphorylated PEPC from a CAM plant (Nimmo *et al.* 1986), maize (Jiao and Chollet 1988) and *Sorghum* (Arrio-Dupont *et al.* 1992). Incubation of leaf extracts with AP (5 U mL^{-1}) rapidly decreased the PEPC activity, to

50% within the first 30 min. The enzyme preparation from dark-PEPC was not altered much as a result of AP-treatment. PEPC extracted from base-loaded leaf discs was more exogenous AP, suggesting that the enzyme is highly phosphorylated.

Feeding of NO_3^- to nitrate-starved leaves, of wheat and maize, enhances markedly the activity of PEPC (Van Quy *et al.* 1991, Gupta *et al.* 1994, Duff and Chollet 1995). However, the mechanism of such activation appears to be different in C3 and C4 plants. Reports are ambiguous since they indicate that either the synthesis of PEPC is stimulated (Sugiharto *et al.* 1992a) or the activity of PEPC-kinase is enhanced (Wang and Chollet 1995).

Experiments therefore were planned to study the effect of feeding inorganic salts to the leaves of *Alternanthera pungens* (C4 species) and *A. sessilis* (C3 species). Feeding *in vivo* of inorganic salts indicated that nitrate salts of K^+, Na^+, or NH_4^+ were quite effective in enhancing the extractable activity of PEPC, from both C_3 and C4 species of *Alternanthera* genus. The order of activation of PEPC due to feeding of nitrate was: NH_4NO_3 > $NaNO_3$ > KNO3. Salts of sulfate were not effective on both C3- and C4-PEPC. The sensitivity of PEPC to malate, which gives a measure of the phosphorylation status of the enzyme, indicated that feeding of leaves with NO_3 enhanced the phosphorylation status of the enzyme.

The buffer capacity of leaf extracts prepared in unbuffered 0.3 M sorbitol indicates that the buffer capacity was maximum at the extremes of pH (pH 3 to 4 or 9 to 10), while the buffer capacity was weak at physiological range of pH (pH 7 to 8). This may be important, since many of the metabolic processes occur at this pH range of 7 to 8.

The buffer capacity of leaf extracts from C_4 species was lower than that of the C3 or C3-C4 intermediate species. The buffer capacity did not change as a result of illumination.

In summary, the following conclusions can be made from the present study:

(1) Light activation of PEPC was two- to three-fold higher (over the dark-control) in the C_4 species compared to 10 to 60% in the C_3 or C3-C4 intermediate species.

(2) The sensitivity to L-malate, a feedback inhibitor, of PEPC was lowered by four fold, on illumination, in C4 leaves, while the changes in L-malate sensitivity of PEPC on illumination in leaves of C3 or C3-C4 intermediate species, were marginal.

(3) The light-induced cytosolic alkalinization was three-fold higher in the C4 species compared to that of the C_3 or C3-C4 intermediate species. There is a marked correlation exists between light-induced increase in cytosolic pH and PEPC activation due to illumination, in leaves of C4 leaves.

(4) Base-loading of leaf discs of a C4 species, *Alternanthera pungens*, enhanced the activity of PEPC by about two-fold compared to the control, while L-malate sensitivity of the enzyme decreased by 14%. As a result of the base-loading there was an increase of cytosolic pH about 0.20 unit.

(5) Acid-loading of leaf discs of *Alternanthera pungens* markedly decreased both the PEPC activity (by about 50% compared to that of the control) and the cytosolic pH (by about 0.30 pH unit). The changes in cytosolic pH and PEPC induced by acid-/base-loading were reversible. The extent and pace of light activation increased as a result of base-loading.

(6) The increase in cytosolic pH and PEPC activity in leaves of the C_4 species, upon base-loading, is quite high compared to only marginal effect in leaf tissues from the C_3 or C3-C4 intermediate species.

(7) Studies using CHX and Western blot analysis indicated that increase in PEPC activity due to base-loading and decrease due to acid-loading were due to neither synthesis of PEPC protein, nor decrease/degradation of the enzyme.

(8) Incubation of leaf extracts with Mg^{2+}-ATP enhanced the activity of PEPC while decreasing the inhibition by L-malate. Since the degree of sensitivity to malate is measure of phosphorylation state of the enzyme, these observations suggest that PEPC is presumably phosphorylated, by an endogenous protein kinase. Preincubation of the *Alternanthera pungens* light-form PEPC preparation with exogenous decreased the activity and increase the L-malate sensitivity of PEPC, indicating that PEPC is dephosphorylated by AP.

(9) Exogenous feeding of leaf discs of *Alternanthera pungens* with α-D-mannose, an analogue of Glc, which decreases the cytosolic Pi by sequestering Pi as Man-P, resulted in a two-fold decrease in the activity of light-form PEPC.

(10) The activation of PEPC by Mg^{2+}-ATP was more in the extracts from the base-loaded leaf discs of *Alternantherra pungens* than that of the enzyme from the control. This indicates that cytosolic alkalization possibly favours the phosphorylation of PEPC, besides the direct activation of the enzyme.

(11) *In vivo* nitrate-feeding of leaves enhanced significantly extractable PEPC activity ifrom the leaves of *Alternanthera pungens* (C4 species) and *A. sessilis* (C3 species), while the magnitude of enhancement being more in the C4 species. In addition, nitrate-feeding also significantly increased the

phosphorylation status of the light and dark-form enzyme, as revealed by L-malate inhibition test.

(12) Though protein synthesis/turnover is needed to enhance *in vivo* PEPC activity upon nitrate-feeding, the enhanced light-activation of PEPC in the C_3 or C_4 leaves appears to be also due to a direct effect (of activation) on the endogenous PEPC-protein kinase.

A model is proposed to integrate the present information on PEPC with the observations of present work. A major contribution of the present work is to demonstrate and emphasize the role of cellular/cytosolic pH in the light-activation and phosphorylation of PEPC.

Chapter Ten

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Appendix

Research Articles Published

1. **Rajagopalan AV**, Devi MT, Raghavendra AS (1992) Light activation and dark deactivation of phospho*enol*pyruvate carboxylase in leaves of C_4 plants. *Trans Malaysian Soc Plant Physiol* **3**: 115-117

2. Devi MT, **Rajagopalan AV,** Raghavendra AS (1992) Structure, regulation and biosynthesis of phospho*enol*pyruvate carboxylase from C_4 plants. *J Plant Biochem Biotechnol* 1: 73-80

***3. Rajagopalan AV**, Devi MT, Raghavendra AS (1993) Patterns of phospho*enol*-pyruvate carboxylase activity and cytosolic pH during light activation and dark deactivation in C3 and C_4 plants. *Photosynth Res* 38: 51-60

4. Devi MT, **Rajagopalan AV**, Raghavendra AS (1993) Photosynthetic and photorespiratory characteristics of C3-C4 intermediates of *Alternanthera* and *Parthenium*. *DAE Symp on Photosynth Plant Mol Biol.* BARC, Mumbai

***5. Rajagopalan AV**, Devi MT, Raghavendra AS (1994) Molecular biology of C_4 phospho*enol*pyruvate carboxylase: structure, regulation and genetic engineering. *Photosynth Res* 39: 115-135

*6. Devi MT, **Rajagopalan AV**, Raghavendra AS (1995) Predominant loclization of mitochondria enriched with glycine-decarboxylating enzymes in bundle sheath cells of *Alternanthera tenella,* a C3-C4 intermediate species. *Plant Cell Environ* 18: 589-594

*7. Devi MT, **Rajagopalan AV,** Raghavendra AS (1996) Purification and properties of glycolate oxidase with different photosynthetic pathways: Distinctness of C_4 enzyme from that of C_3 species and C3-C4 intermediate. *Photosynth Res* 47:231-238

*The first pages of these articles are enclosed.

Photosynthesis Research 38: 51–60. 1993.

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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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*pyruvate carboxylase (PEPC) in leaf discs of C_4 plants, the activity being three times greater than that in the dark 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 than 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-phenylmethylsulfonyl 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_3 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-

Photosynthesis Research 39: 115-135, 1994.

Review

Molecular biology of C_4 phospho*enol*pyruvate carboxylase: Structure, regulation and genetic engineering

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Key words: C_4 photosynthesis, gene expression, oligomerization, phosphorylation-dephosphorylation cascade, PEPC-protein kinase, site-directed mutagenesis

Abstract

Three to four families of nuclear genes encode different isoforms of phospho*enol*pyruvate (PEP) carboxylase (PEPC): C_4-specific, C_3 or etiolated, CAM and root forms. C_4 leaf PEPC is encoded by a single gene (*ppc*) in sorghum and maize, but multiple genes in the C_4-dicot *Flaveria trinervia.* Selective expression of *ppc* in only C_4-mesophyll cells is proposed to be due to nuclear factors, DNA methylation and a distinct gene promoter. Deduced amino acid sequences of C_4-PEPC pinpoint the phosphorylatable serine near the N-terminus, C_4-specific valine and serine residues near the C-terminus, conserved cysteine, lysine and histidine residues and PEP binding/catalytic sites. During the PEPC reaction, PEP and bicarbonate are first converted into carboxyphosphate and the enolate of pyruvate. Carboxyphosphate decomposes within the active site into Pi and CO_2, the latter combining with the enolate to form oxalacetate. Besides carboxylation, PEPC catalyzes a HCO_3^--dependent hydrolysis of PEP to yield pyruvate and Pi. Post-translational regulation of PEPC occurs by a phosphorylation/dephosphorylation cascade in vivo and by reversible enzyme oligomerization in vitro. The interrelation between phosphorylation and oligomerization of the enzyme is not clear. PEPC-protein kinase (PEPC-PK), the enzyme responsible for phosphorylation of PEPC, has been studied extensively while only limited information is available on the protein phosphatase 2A capable of dephosphorylating PEPC. The C_4 *ppc* was cloned and expressed in *Escherichia coli* as well as tobacco. The transformed *E. coli* produced a functional/phosphorylatable C_4 PEPC and the transgenic tobacco plants expressed both C_3 and C_4 isoforms. Site-directed mutagenesis of *ppc* indicates the importance of His^{138}, His^{579} and Arg^{587} in catalysis and/or substrate-binding by the *E. coli* enzyme, Ser^8 in the regulation of sorghum PEPC. Important areas for further research on C_4 PEPC are: mechanism of transduction of light signal during photoactivation of PEPC-PK and PEPC in leaves, extensive use of site-directed mutagenesis to precisely identify other key amino acid residues, changes in quarternary structure of PEPC in vivo, a high-resolution crystal structure, and hormonal regulation of PEPC expression.

Abbreviations: OA A - oxalacetate; PEP – phospho*enol*pyruvate; PEPC – PEP carboxylase; PEPC-PK - PEPC-protein kinase; PPDK – pyruvate, orthophosphate dikinase; Rubisco - ribulose 1,5-bis-phosphate carboxylase/oxygenase; CAM - Crassulacean acid metabolism

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Predominant localization of mitochondria enriched with glycine-decarboxylating enzymes in bundle sheath cells of *Alternanthera tenella*, a C_3–C_4 intermediate species

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ABSTRACT

Mesophyll protoplasts and bundle sheath cells were prepared by enzymatic digestion of leaves of *Alternanthera tenella*, a C_3–C_4 intermediate species. The intercellular distribution of selected photosynthetic, photorespiratory and respiratory (mitochondrial) enzymes in these mesophyll and bundle sheath cells was studied. The activity levels of photosynthetic enzymes such as PEP carboxylase (EC 4.1.1.31) or NAD-malic enzyme (EC 1.1.1.39) and photorespiratory enzymes such as glycolate oxidase (EC 1.1.3.1) or NADH-hydroxypyruvate reductase (EC 1.1.1.29) were similar in the two cell types. The activity levels of mitochondrial TC A cycle enzymes such as citrate synthase (EC 4.1.3.7) or fumarase (EC 4.2.1.2) were 2- to 3-fold higher in bundle sheath cells. On the other hand, the activity levels of mitochondrial photorespiratory enzymes, namely glycine decarboxyla.se (EC 2.1.2.10) and serine hydroxymethyltransferase (EC 2.1.2.1), were 6-9-fold higher in bundle sheath cells than in mesophyll protoplasts. Such preferential localization of mitochondria enriched with the glycine-decarboxylating system in the inner bundle sheath cells would result in efficient refixation of CO_2 from not only photorespiration but also dark respiration before its exit from the leaf. We propose that predominant localization of mitochondria specialized in glycine decarboxylation in bundle sheath cells may form the basis of reduced photorespiration in this C_3–C_4 intermediate species.

Keywords: glycine decarboxylase; photorespiration; serine hydroxymethyltransferase; TCA cycle enzymes.

Abbreviations: BSC, bundle sheath cells; Chl, chlorophyll: GDC, glycine decarboxylase; GO, glycolatc oxidase; HPR, hydroxypyruvate reductase; ME, malic enzyme; MP, mesophyll protoplasts; PEP, phospho*enol*pyruvate; SHMT, serine hydroxymethyltransferase.

INTRODUCTION

In C_3 plants, both photosynthetic and photorespiratory metabolisms operate in a single type of cell (mesophyll) (Edwards & Walker 1983). In contrast, photorespiratory glycolate metabolism and the Calvin cycle occur in the bundle sheath cells while primary carbon assimilation through the C_4 cycle is localized in the mesophyll cells of C_4 plants (for reviews, see Edwards & Walker 1983; Hatch 1987, 1992; Raghavendra & Das 1993). Several plants, identified as C_3–C_4 intermediates, have reduced photorespiration (Monson, Edwards & Ku 1984; Edwards & Ku 1987; Rawsthorne 1992; Raghavendra & Das 1993). The degree of Kranz anatomy in these C_3–C_4 intermediates varies from very poor to as good as that of a C_4 species (Brown & Hattersley 1989).

It has recently been shown that glycine decarboxylase, the key mitochondria] enzyme which releases CO_2 during photorespiration, is confined to bundle sheath cells in C_3–C_4 species in the genera *Panicum*, *Flaveria, Mollugo* and *Moricandia* (Hylton *et al.* 1988; Rawsthorne *et al.* 1988a; Moore *et al.* 1988; Rawsthorne & Hylton 1991; Morgan, Turner & Rawsthorne 1993). Such confinement of glycine decarboxylation to bundle sheath cells, along with the physical proximity of mitochondria and chloroplasts in these cells, has been proposed to form the basis for the C_3–C_4 intermediacy (for a review, see Rawsthorne 1992).

We have examined photorespiratory metabolism in three C_3–C_4 intermediate species: *Alternanthera tenella*, *A. ficoides* and *Parthenium hysterophorus* (Devi & Raghavendra 1993a,b). These intermediates have partial Kranz leaf anatomy, and reduced photorespiratory enzymic capacity/metabolites. The present work is an attempt to isolate mesophyll protoplasts and bundle sheath cells from the leaves of *Alternanthera tenella* and to determine in these tissues the maximum catalytic activities of some of the key enzymes involved in photosynthetic, photorespiratory and respiratory metabolism.

MATERIALS AND METHODS

Plant material

Plants of *Alternanthera tenella* Colla were grown in earthen pots in the field under a natural photoperiod of approximately 12 h and an average temperature of 30 °C/20 °C, day/night. The plants were watered daily with tap water. The third and fourth leaves were excised from 3-

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Regular paper

Purification and properties of glycolate oxidase from plants with different photosynthetic pathways: Distinctness of C_4 enzyme from that of a C_3 species and a C3-C4 intermediate

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Key words: C3, C3/C4 intermediate and C_4 species, glycolate, glycolate oxidase, glyoxylate, photorespiration

Abstract

Glycolate oxidase (GO; EC 1.1.3.1) was purified from the leaves of three plant species: *Amaranthus hypochondriacus* L.(NAD-ME type C_4 dicot), *Pisum sativum* L. (C3 species) and *Parthenium hysterophorus* L. (C3-C4. intermediate). A flavin moiety was present in the enzyme from all the three species. The enzyme from the C4 plant had a low specific activity, exhibited lower K_M for glycolate, and required a lower pH for maximal activity, compared to the C3 enzyme. The enzyme from the C4 species oxidized glyoxylate at <10% of the rate with glycolate, while the GO from the C3 plant oxidized glyoxylate at a rate of about 35 to 40% of that with glycolate. The sensitivity of GO from C4 plant to α-hydroxypyridinemethane sulfonate, 2-hydroxy-3-butynoate and other inhibitors was less than that of the enzyme from C_3 source. The properties of GO from *Parthenium hysterophorus*, were similar to those of the enzyme from *Pisum sativum.* The characteristics of glycolate oxidase from leaves of a C4 plant, *Amaranthus hypochondriacus* are different from those of the C_3 species or the C_3–C_4 intermediate.

Abbreviations: FMN - flavin mononucleotide; GO - glycolate oxidase; HBA - 2-hydroxy-3-butynoate; α-HPMS - a-hydroxypyridinemethane sulfonate; PEP – phospho*enol*pyruvate; PCMB – *p*-chloromercuribenzoate; PHMB - *p*-hydroxymecuribenzoate

Introduction

Glycolate oxidase (GO; Glycolate:oxygen oxidoreductase, EC 1.1.3.1) is a flavoprotein catalyzing the oxidation of glycolate to glyoxylate, an important step during photorespiration (Tolbert 1981; Huang et al. 1983; Ogren 1984; Canvin 1990; Zelitch 1992). The enzyme is located in peroxisomes of both C3 and C4 plants (Tolbert 1981; Huang et al. 1983; Canvin 1990). The enzyme can oxidize a-hydroxyacids (including L-lactate) and glyoxylate (which mimics a *a*-hydroxyacid in solution) (Richardson and Tolbert 1963; Tolbert 1981; Canvin 1990; Betsche et al. 1992; Iwamoto and Ikawa 1992), but glycolate is the preferred substrate.

Glycolate oxidase was purified to homogeneity from the leaves of C3 plants (Tolbert et al. 1949; Kerr and Groves 1975; Lindquist and Bränden 1979; Fendrich and Ghisla 1982; Davies and Asker 1983; Ernes and Erismann 1984; Betsche et al. 1992), algae (Stabenau and Saftel 1982; Gross and Beevers 1989; Betsche et al. 1992; Iwamoto and Ikawa 1992), and greening cotyledons of cucumber seedlings (Kindl 1982). The enzyme was crystallized from spinach (Frigerio and Harbury 1958; Lindquist and Bränden 1979, 1980, 1985) and pumpkin cotyledons (Nishimura et al. 1983). To our knowledge, there has been only one attempt to characterize in detail the GO in bundle sheath cells of a C4 plant, *Digitaria sanguinalis* (L.) Scop. (Liu and Black 1972). However, to date, the