Purification, Properties and Phosphorylation of PhosphoenolPyruvate Carboxylase from leaves of *Amaranthus hypochondriacus*, a NAD-ME type C_4 Plant

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

J. Gayathri

Supervisor: **Dr A.S. Raghavendra**

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500 046, INDIA

May 1996

DECLARATION

The candidate declares that this work has been carried out by her under the supervision of Dr A.S. Raghavendra, Associate Professor, 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.

Dr. A.S. Raghavendra 10/5/96
Associate Professor
(Supervisor)

J. Gayathri
(Candidate)
Enrol. No. 91LSPH07

University of Hyderabad
School of Life Sciences
Department of Plant Sciences

CERTIFICATE

This is to certify that the thesis entitled **"Purification, Properties and Phosphorylation of Phosphoeniolpyruvate Carboxylase from Leaves of *Amaranthus hypochondriacus*, a NAD-ME type C_4 Plant"** is based on the results of the work done by **Ms. J. Gayathri** 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 R.P.Sharma
Head
Department of Plant Sciences

Dr. A.S. Raghavendra
Associate Professor
(Supervisor)

Professor A.R. Reddy
Dean
School of Life Sciences

ACKNOWLEDGEMENTS

I express my deep sense of gratitude, with immense pleasure, to ***Dr A.S. Raghavendra,*** for his supervision, guidance and help in the preparation of the thesis. I also take this opportunity to express **my** appreciation towards his invaluable suggestions, and constant encouragement which helped me to develop my research career.

I thank **Prof R.P. Sharma, Head,** Dept. of Plant Sciences and **Prof A.R. Reddy.** Dean, School of Life Sciences and former Deans, **Prof P.R.K. Reddy** and **Prof N.C. Subramanyam** FNA, for allowing me to use the facilities of the School as well as the University.

I am thankful to **Prof A.R. Reddy, Prof V.S. Rama Das** FNA, **Prof R.P. Sharma, Dr M. Ramanadham, Dr R.V. Thampan, Dr K.V.A. Ramaiah, Dr Mohan C. Vemuri, Dr P. Reddanna** and other faculty members of the School of Life Sciences for their kind help and allowing me to use their laboratory facilities during my work.

I owe my thanks to **Dr Mohinder Pal,** National Botanical Research Institute, Lucknow for his generous gifts of seeds of ***Amaranthus hypochortdriacus.***

I am greatly indebted to my **parents** and my **husband** who encouraged me with all their efforts and patience during my Ph.D programme. It gives me immense pleasure to thank my **sister, brother** and my **mother-in-law** whose co-operation and encouragement made me to successfully complete the work for Ph.D. I thank my daughter for being co-operative during the course of my work.

I am thankful to my senior colleagues **Dr M. Tirumala Devi** and **Dr K. Sarada Devi** for their constant help and encouragement throughout my research work. I am very much thankful to my colleagues in the laboratory especially **Mrs K. Padma Sree, Ms K. Parvathi, Ms Manju** and **Mr A.V. Rajagopalan.**

I am happy to acknowledge the help of my friends **Ms Sarada Devi, Mrs Padma Sree, Ms Parvathi, Mrs Saigeetha, Mrs Radha Rani, Ms Rupali Datta, Mrs Shailaja** and **Ms Kavita,** for their timely suggestions and encouragement, at every stage of **my** work.

I wish to thank all my other colleagues of the School, especially, **Ms Padma, Mrs Janaki, Mr Krishna, Mr Haviryaji, Mr Ramachandra Reddy** and **Mr Imam** who extended their help.

I am also thankful to Mr Lalan Prasad, Mr Mishra, Mr Krishna and all other staff members of the School and University for their kind co-operation.

I am also grateful for the financial assistance from UGC (JRF/SRF).

J Gayathri

ABBREVIATIONS

AP_5A	=	P^1 P^5-di (adenosine-5')-pentaphosphate
BCIP	=	5-bromo-4-chloro-3-indolyl phosphate
ELISA	=	enzyme linked immunosorbent assay
G-6-P	=	glucose-6-phosphate
HAP	=	hydroxylapatite
L/D	=	light/dark
NBT	=	*p*-nitro blue tetrazolium chloride
NR	=	nitrate reductase
PEG	=	polyethylene glycol
PEPC	=	phospho*enol*pyruvate carboxylase
PEPC-PK	=	PEPC-protein kinase
PMSF	=	phenyl methyl sulphonvl fluoride
PPDK	=	pyruvate Pi dikinase
PVDF	=	polyvinylidene fluoride
SPS	-	sucrose phosphate synthase
TBS	=	tris-buffered saline
TCA	=	tricarboxylic acid
TFP	=	trifluoperazine

CONTENTS

Chapter 1

Introduction and Review of Literature

Chapter 1

Introduction and Review of Literature

C_4 photosynthesis (C_4 pathway) is one of the three types of photosynthetic carbon metabolism operating in higher plants, the other two being Calvin cycle (C_3 pathway) and Crassulacean Acid Metabolism (CAM) (Edwards and Walker, 1983; Leegood, 1993; Raghavendra and Das, 1993). C_4 pathway requires a co-ordinated functioning of mesophyll and bundle sheath cells in leaves (Hatch, 1987; Leegood and Osmond, 1990). The operation of C_4 pathway of carbon fixation in mesophyll cells leads to a marked increase in the concentration of CO_2 in the bundle sheath cells (Furbank and Hatch, 1987; Furbank and Foyer, 1988), thereby optimizing the function of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Readers interested in further information on C_4 photosynthesis may consult the articles of Edwards and Huber (1981), Hatch (1987, 1992), Furbank and Foyer (1988), Leegood (1993) and Raghavendra and Das (1993).

The key enzyme involved during primary carboxylation in C_4 photosynthesis as well as CAM pathway is phosphoelpyruvate carboxylase (PEPC, EC 4.1.1.31). The existence of PEPC **in** *C_4* plants helps to build up **a** pool of dicarboxylic acids, which on decarboxylation raise the CO_2 concentration in bundle sheath cells and minimize the process of photorespiration. As a result, C_4 plants can achieve high growth rates under conditions of high temperatures, strong illumination and atmospheric oxygen levels, which are not optimal for C_3 plants (Edwards et al., 1985). Due to the importance of PEPC in not only C_4 and CAM but also C_3 plants (during **anaplerotic** carbon fixation), studies on PEPC were always of interest. The literature **on** PEPC has been reviewed frequently by several authors. Some of the recent reviews **on** PEPC are by Jiao and Chollet (1991), Lepiniec et al. (1994), Rajagopalan et al. (1994) and Toh et al. (1994). Earlier reviews **on**

PEPC include those of O'Leary (1982, 1983), Gadal (1983), Guern et al. (1983), Kluge (1983), Latzko and Kelly (1983) and Andreo et al. (1987).

Occurrence and Importance

PEPC occurs in all plants and is believed to be absent in animal tissues (Utter and Kolenbrander, 1972), yeast or fungi (O'Leary, 1982; Lepiniec et al., 1994). Green algae also possess PEPC activity, while its presence in Chromophytes and Rhodophytes is uncertain (Lepiniec et al., 1994).

The activities of PEPC levels in leaves of C_4 plants are about twenty fold higher on a chlorophyll basis than those in C_3 plants (Edwards and Walker, 1983). The ratio of PEPC : Rubisco is about 2 in leaves of C_4 plants, compared to the ratio of 0.1 in those of C_3 plants (Williams and Kennedy, 1978). PEPC constitutes about 15% of total soluble protein in maize (Hague and Sims, 1980; Hayakawa et al., 1981). The enzyme is confined to the cytoplasm of mesophyll cells in C_4 and CAM plants (Perrot-Rechenmann et al., 1982). There are reports that PEPC may be present in the chloroplasts of C_3 plants (Perrot-Rechenmann et al., 1982; Latzko and Kelly, 1983), but this is to be confirmed further.

Physiological Role

PEPC is a principal enzyme in C_4 and CAM plants, mediating the primary carbon assimilation (O'Leary, 1982). It catalyses β-carboxylation of oxalacetate to yield PEP and Pi. The reaction is irreversible and exergonic.

One of the characteristic features of C_4 pathway is the occurrence of Kranz-like anatomy, which results in division of labour and spatial separation of biochemical reactions. Most of C_4-pathway enzymes, involved in fixation of atmospheric CO_2 and C_4-acid formation, including PEPC and pyruvate Pi-dikinase (PPDK) are located in mesophyll cells, while bundle sheath cells lodge the enzymes of Calvin cycle, along with those of C_4-acid decarboxylation

system. In CAM plants, the function of PEPC is similar to C_4 plants. Primary carbon fixation by PEPC occurs during the night, followed by decarboxylation of C_4 acids and refixation of CO_2 by rubisco during day (Kluge, 1983). The operation of CAM minimizes the loss of water in these plants, as the stomata open in the night but are kept closed during most of the day.

PEPC plays an anaplerotic role in C_3 plants, while producing C_4 acids (i.e.,oxalacetate and malate), to provide carbon skeletons for amino acid biosynthesis, nitrogen assimilation, and replenishment of tricarboxylic acid (TCA) cycle intermediates (Gadal, 1983; Melzer and O'Leary, 1987). Further, PEPC plays an important role in generation of NADPH for fatty acid synthesis in developing seeds, fruit maturation (Latzko and Kelly, 1983) and maintenance of cytoplasmic pH and electroneutrality (Davis, 1979). PEPC is also known to be involved in regulating stomatal movement (Willmer, 1983; Outlaw, 1990).

Form and Structure

Four isoforms of PEPC have been reported in higher plants: C_4 photosynthetic form, C_3 photosynthetic form, CAM-form and dark or non-autotrophic PEPC (O'Leary, 1982; Andreo et al., 1987; Rajagopalan et al., 1994). These forms can be distinguished by chromatographic, immunological and kinetic properties.

Thomas et al. (1987) reported two isoforms of PEPC in sorghum leaves (E-PEPC and G-PEPC). The E-form occurred in etiolated leaves and exhibited C_3 characteristics, while the G-form was present in green leaves and had characteristics of C_4 photosynthetic form. Vidal and Gadal (1983) have reported that etiolated sorghum leaves contain only one form (C_3 form) of the enzyme and a new isoform of enzyme appears on illumination upon greening (C_4 form). The expression of PEPC-gene encoding the C_4 isozyme was not leaf specific, since high accumulation of its transcripts was found in also other

parts of maize plant i.e., inner leaf sheaths, tassels and husks (Hudspeth and Grula, 1989). On the other hand, Schaffner and Sheen (1992) reported that the expression of C_4-specific PEPC-gene occurred only in illuminated (greening) leaves of maize. No signal of C_4-specific PEPC genome was detected in roots or stems or etiolated leaves of maize. The major form in maize leaves is the C_4-type and is the most abundant protein in mesophyll cells. However, there is a possibility that a small amount of etiolated form of the enzyme may exist also in green tissue, which could explain the detection of the two major isozymes of PEPC in leaves of maize (Ting and Osmond, 1973a, b; Mukerji, 1977).

There is a lot of variation in the number of PEPC isoforms reported from the leaves of C_3 plants and CAM species. Four major isoforms of PEPC are reported in leaves of a C_3 plant *Flaveria conquistii*, C_3-C_4 intermediate *Flaveria floridana* and a C_3 performing *Mesembryanthemum crystallinum* (Adams et al., 1986; Slocombe et al., 1993). Three isoforms in leaves of *Gossypium hirsutum*, a C_3 species (Mukerji and Ting, 1971) and also two in C_3 performing *Kalanchoë blossfeldiana* (Brulfert et al., 1979). The four isoforms of PEPC are encoded by different genes in C_4 plants (Hudspeth et al., 1986; Hudspeth and Grula, 1989; Hermans and Westhoff, 1990). The occurrence of multiple forms of PEPC suggests that during the evolution of C_4 plants, a mechanism has developed for preferential expression of C_4 specific PEPC gene (Lepiniec et al., 1994; Stockhaus et al., 1994).

PEPC is a homotetramer (Andreo et al., 1987). Although it is suggested that the enzyme may exist in different oligomeric forms *in vivo* (Wu and Wedding, 1985; Walker et al., 1986) there are no convincing evidences. The quaternary structure of PEPC *in vitro*, depends on protein and effector concentrations (Jiao and Chollet, 1991). In *Crassula argentea*, the enzyme purified during night existed as tetramer while the day form existed in dimer (Wu and Wedding, 1985). In C_4 plants, dissociation of PEPC occurs on incubation with NaCl in a time- and protein-concentration dependent manner

(Wagner et al., 1987). The phenomenon of oligomerization is further reviewed in the following pages, under the section "Post-translational Modification".

Chemical modifications of the enzyme have shown that cysteine, arginine and lysine residues are essential for the catalytic activity of PEPC from corn and sorghum leaves (Andreo et al., 1987; Wagner et al., 1988; Willeford et al., 1990; Terada et al., 1991). Histidine and cysteine residues of enzyme may be involved in activation of PEPC by glucose-6-phosphate (G-6-P) (Manetas and Gavalas, 1982; Wedding et al., 1989).

The primary structure of PEPC from several C_4 plants, besides lower micro-organisms like, *Escherichia coli* or *Anabaena variabilis*, has been deduced through sequence analysis of cDNA (Lepiniec et al., 1994; Rajagopalan et al., 1994; Toh et al., 1994). A comparison on the amino acid sequences of PEPC from variety of these organisms shows that the enzyme molecule contains a conserved C-terminal half and a variable N-terminal one (Ishijima et al., 1985; Izui et al., 1986). C_4-PEPC from maize has 970 amino acids (Izui et al., 1986; Hudspeth and Grula, 1989) compared to 952 of sorghum (Cretin et al., 1990), 966 in *F. trinervia* (Poetsch et al., 1991) and 883 of *E. coli* (Fujita et al., 1984; Ishijima et al., 1985). There is 88% sequence homology of sorghum PEPC with that of maize but only 40-50% with *E. coli* and *Anacystis nidulans* (Ishijima et al., 1985; Izui et al., 1986; Cretin et al., 1990).

Phylogenetic trees, constructed from amino acid sequences of as many as 26 different forms of PEPC reveal that enzyme from C_4 dicot *(F. trinervia)* is closer to the C_3 (tobacco and soybean) and C3/CAM isoform than to monocotyledonous C_4 PEPC (Koizumi et al., 1991; Poetsch et al., 1991; Kawamura et al., 1992; Sugimoto et al., 1992; Lepiniec et al., 1993). This observation suggests that monocot and dicotyledonous C_4 plants have evolved separately during the course of evolution.

Purification

One of the first attempts to purify PEPC from plant tissues was by Bandurski and Grciner (1953), who attempted to purify partially the enzyme from spinach leaves. Partial purifications have been made from leaves of cotton, *Pennisetum purpureum*, sorghum, maize, lupin root nodules, soybean nodules, maize root tips, guard cells of *Vicia faba* and epidermis of *Commelina communis* (O'Leary, 1982).

Purification of PEPC to homogeneity has been done using peanut cotyledons (Maruyama and Lane, 1962), spinach (Miziorko et al., 1974), maize (Uedan and Sugiyama, 1976), *Bryophyllum fedtschenkoi* (Jones et al., 1978), *Amaranthus viridis* (Iglesias et al., 1986), sugarcane (Iglesias and Andreo, 1989), sorghum leaves (Arrio-Dupont et al., 1992) and soybean nodules (Schuller and Werner, 1993).

The components of purification-protocol in most of these cases include ammonium sulphate fractionation of crude leaf extracts, followed by dialysis, and gel-filtration through DEAE-cellulose, hydroxylapatite (HAP) and Sephadex column (Uedan and Sugiyama, 1976; Iglesias et al., 1986; Iglesias and Andreo, 1989; Schuller and Werner, 1993). However, in recent reports, FPLC (Jiao and Chollet, 1988, 1989; Jiao et al., 1991) or HPLC (Cretin et al., 1984) have also been used for purification of PEPC. During purification by gel filtration, the enzyme is eluted by high concentration of salt, often chloride (Uedan and Sugiyama, 1976; Mukerji, 1977; Hatch and Heldt, 1985; Iglesias et al., 1986) and occasionally phosphate (Hague and Sims, 1980) or acetate (Smith and Woolhouse, 1984). Chloride, however, can affect the activity of the enzyme (Manetas et al., 1986; Wagner et al., 1987), presumably by inducing dissociation of the tetrameric form into dimers or monomers (Wagner et al., 1987; Manetas, 1990). On the other hand, phosphate is considered to be a stabilizer of the enzyme (Yancey et al., 1982). Instead of ammonium sulphate, polyethylene glycol (PEG) had been used to precipitate the enzyme (Selinioti et

al., 1987; Angelopoulos and Gavalas, 1991), since the yield of PEPC by using PEG was reportedly much better than that with ammonium sulphate. Techniques are now available for rapid purification of enzyme from leaves by immunoadsorbant columns (Vidal et al., 1980; Arrio-Dupont et al., 1992).

During purification, the enzyme is highly susceptible to proteolysis at the N-terminal end. The loss of N-terminal is reflected in the decrease in the sensitivity of enzyme to malate. As a result, the enzyme becomes less prone for phosphorylation and not so sensitive to malate. Therefore, addition of proteolytic inhibitors like phenylmethylsulphonylfluoride (PMSF) or chymostatin is essential during purification (McNaughton et al., 1989).

The specific activity of purified PEPC, reported in literature, varied from a very low average value of 4-10 U mg^{-1} protein (Coombs et al., 1973; Hayakawa et al., 1981; O'Leary et al., 1981; Sugiyama et al., 1984; Wedding and Black, 1986) and to as high values as 180-220 U mg^{-1} protein (Mukerji, 1977; Reibach and Benedict, 1977). The latter high values are presumably in error. In case of CAM plants, the best specific activity reported in *Kalanchoë daigremontiana* is around 35 U mg^{-1} protein. However, the specific activity from other CAM plants varied from 17-300 U mg^{-1} protein (Nott and Osmond, 1982). Reviewing the available literature, O'Leary (1982) commented that the specific activity of purified PEPC could be around 25 U mg^{-1} protein. Similar range of specific activities have been obtained by several authors (Hatch and Heldt, 1985; Iglesias et al., 1986; Wedding et al.. 1988; McNaughton et al., 1989). Recently a specific activity of 7.68 U mg^{-1} protein was reported for PEPC purified from developing seeds of *Brassica campestris* (Mehta et al., 1995).

***Stability** of **PEP** carboxylase*

Cytosolic enzymes such as PEPC are present *in vivo* at far higher concentrations than that are used during *in vitro* assays. The enzyme is

unstable and may dissociate into inactive di- or monomer on dilution (Wu et al., 1990). The addition of solutes (such as glycerol or PEG) during extraction and storage helps to maintain the tetrameric state of several enzymes (Rhodes and Hanson, 1993). Natural solutes, like betaine and proline can protect enzymes against heat denaturation (Paleg et al., 1981; Nash et al., 1982). Similarly, synthetic polymers like PEG are used often for protein stabilization (Reinhart, 1980).

PEPC is affected by compatible solutes in several ways: stabilization of enzyme during storage (Selinioti et al., 1987), protection of the enzyme against NaCl inhibition (Pollard and Wyn Jones, 1979; Manetas et al., 1986; Manetas, 1990) and improvement of catalytic efficiency (Stamatakis et al., 1988; Podesta and Andreo, 1989). Karabourniotis et al. (1983) and Medina et al. (1985) have used glycerol and other solutes as stabilizers of activity and regulatory properties of PEPC during and after extraction. The presence of glycerol stabilized the maize PEPC activity by promoting the tetrameric form and enhancing the V_{max} of the enzyme (Uedan and Sugiyama, 1976). However, glycerol was unable to prevent the dissociation of PEPC or shift the equilibrium of enzyme to active tetrameric form at pH 8.0 (Podesta and Andreo, 1989).

*Regulation of **PEP carboxy lase***

PEPC is an allosteric enzyme which is regulated by several internal and external factors (e.g. metabolic regulation by **effectors,** light, temperature and pH). The influence of these factors varies depending on the enzyme source and other interacting factors.

Metabolites (Inhibitors/Activators):

PEPC is highly regulated by feed-back inhibition by dicarboxylic acids oxalacetate and allosteric activation by metabolites, particularly phosphate-compounds (Raghavendra and Das, 1976; Gonzalez et al., 1984; Andreo et al.,

1987). Malate and aspartate (besides oxalacetate) are among the typical feed-back inhibitors. G-6-P is a powerful activator of PEPC, particularly in C_4 plants.

L-malate, which is a product of C_4-pathway, is a competitive inhibitor of the PEPC (Huber and Edwards, 1975). Malate is a known inhibitor not only of C_4 PEPC, but with different effectiveness also in C_3 and CAM forms (Kluge et al., 1988; Echevarria et al., 1990; Jiao and Chollet, 1990). Aspartate also inhibits the enzyme, quite strongly in some C_4 plants (Huber and Edwards, 1975; Iglesias et al., 1986). Aspartate may protect the enzyme against thermal inactivation (Rathnam, 1978; Mares and Leblova, 1980). Organic acids and analogues of PEP/pyruvate are powerful inhibitors of the C_4 enzyme (Rajagopalan et al.. 1994). Based on this property, several analogues of PEP are employed to study the reaction mechanism of the enzyme (Gonzalez and Andreo 1989; Janc et al., 1992 a, b). The extent of malate inhibition depends on various factors like assay pH, phosphorylation status of enzyme, proteolytic loss of N-terminal region and presence of activators, e.g. G-6-P (McNaughton et al., 1989, 1991; Jiao and Chollet, 1991; Ausenhus and O'Leary, 1992; Wang et al., 1992). Willeford et al. (1990) postulated that malate causes changes in the oligomeric structure of the enzyme, enhancing the formation of less active dimer. In contrast, as an adaptive feature, PEPC from C_4 and CAM plants can change their sensitivity to malate inhibition, by modification of enzyme-protein. Such change in malate sensitivity is achieved through a regulatory seryl-phosphorylation (Nimmo et al., 1984; Kluge et al., 1988; Jiao and Chollet, 1990). The dephosphorylated form of PEPC is extremely sensitive to malate, while the phosphorylated form is not so sensitive.

Besides G-6-P, a typical allosteric activator of the C_4 enzyme (Andreo et al., 1987), PEPC is activated by many phosphate-esters (Podesta et al., 1990). Walker et al. (1988) suggested that phosphatase activity of PEPC may be related to the activation process by phosphate compounds. However, in

maize, the activation of PEPC occurred without dephosphorylation of the activator (Bandarian et al., 1992).

The activation of PEPC by glycine is reported only in C_4 monocots such as maize (Nishikido and Takanashi, 1973; Doncaster and Leegood, 1987; Bandarian et al., 1992). Glycine exerted no effect on PEPC from dicotyledonous C_4 plants or from C_3 plants (Nishikido and Takanashi, 1973). This observation correlates well with the structural information, now available, on PEPC which suggests the C_4 enzyme of the monocots (e.g. maize and sorghum) may have evolved separately from other C_4 dicots, CAM and C_3 plants (Lepiniec et al., 1993).

Apart from the above, other known activators of PEPC that could be physiologically important, are: fructose-2,6-bisphosphate (Doncaster and Leegood, 1987), Pi (Podesta et al., 1990), dihydroxyacetone phosphate, fructose-6-phosphate (Doncaster and Leegood, 1987), AMP (Rustin et al., 1988), carbamyl phosphate (Gonzalez et al., 1987) and ribulose-1,5-bisphosphate (Leblova et al., 1991).

Light:

Illumination enhances, by 2-3 fold, the activity of PEPC. particularly in leaves of C_4 plants. Light-activation is a feature of key photosynthetic enzymes in C_3 plants (Buchanan, 1992). A two-fold activation of PEPC was first observed in *Amaranthus palmeri* leaves by Slack (1968). After a preliminary confirmation of light activation in *Atriplex tatarica* (Gavalas et al., 1981). light activation of PEPC has been reported in leaves of several C_4 species (Karabourniotis et al., 1983). The light activation of PEPC is distinct from light-induced synthesis of PEPC-protein, which is observed typically during greening of sorghum or maize leaves (Sims and Hague, 1981; Vidal and Gadal, 1983). Sims and Hague (1981) have reported an increase in level of mesophyll cell mRNA and PEPC-protein synthesis during leaf development and greening of etiolated maize leaves.

Besides the change in activity, the kinetic and regulatory properties of PEPC are markedly modulated by light/dark (L/D) transitions *in vivo* (Andreo et al., 1987; Jiao and Chollet, 1991). The enzyme exhibits two or three fold more activity on illumination at sub-optimal but physiological levels of PEP and pH (Jiao and Chollet, 1988). The light-form is less sensitive to feedback inhibition by malate and exhibits a marked stimulation by G-6-P. On the other hand, the dark-form is quiet sensitive to malate and is less activated by G-6-P. The response of PEPC in C_3-leaves to light is much less than in C_4 plants. The increase in PEPC on exposure to light is marginal (about 10-15%) in C_3 species (Rajagopalan et al., 1993). Further, the addition of an allosteric positive effector (G-6-P) provided much greater protection against malate inhibition of the enzyme from C_4 species than that from C_3 species (Gupta et al., 1994).

Marginal increase in light activation was reported in mesophyll protoplasts of maize (Devi and Raghavendra, 1992). Pierre et al. (1992) have demonstrated light induced phosphorylation of PEPC in mesophyll protoplasts in sorghum and its dependence on calcium and pH. Light induced phosphorylation also was observed in guard cell protoplasts of *Vicia faba* L. (Schnabl et al., 1992), although no light activation of PEPC could be detected in guard cell protoplasts of *Commelina communis* L. (Willmer et al., 1990). Using an artificial photosensitive dye in a reconstituted system, marked photo-activation of purified PEPC was demonstrated by Maheswari and Bharadwaj (1991), but this phenomenon needs to be confirmed.

The phenomenon of light activation of photosynthetic enzymes can be due to changes in the thiol groups (Iglesias and Andreo, 1984) or by phosphorylation-dephosphorylation of amino acid residues like serine (Jiao and Chollet, 1991). Buchanan (1991, 1992) have reported the involvement of thiol groups in modulation of several C_3 enzymes, but this phenomenon may not be well applicable to cytosolic enzymes like C_4-PEPC (Jiao and Chollet, 1991).

On the other hand, pH may be an important factor during light activation of PEPC, located in cytosol of mesophyll cells. Light induces alkalization of cytosol and can lead to activation of PEPC and PEPC-protein kinase (PEPC-PK) or both. This has been shown in "cytosol enriched" cell sap of *Alternanthera pungens,* a NAD-ME type plant (Rajagopalan et al., 1993). Light is also known to induce marked alkalization of cytosol in mesophyll cells of C_4 plants, as documented by the use of pH-dependent fluorescent probes (Raghavendra et al., 1993; Yin et al., 1993).

Temperature:

The effects of temperature on growth are often correlated to corresponding changes in activity of several enzymes, including PEPC in case of C_4 plants (Selinioti et al.. 1986). Attempts have been made to correlate the poor rate of C_4 photosynthesis at low temperature with cold lability of PPDK (Shirahashi et al., 1978) and thermal response of PEPC (Selinioti et al., 1986). However, there is no clear correlation in response of PEPC in cold-exposed leaves with behaviour of the enzyme *in vitro* (Petropoulou et al., 1990; Krall and Edwards, 1993).

Yet, the activity of PEPC in C_4 as well as CAM plants is known to be regulated by temperature. At higher temperature, there is an increase in V_{max} and a decrease in apparent K_m (PEP) of the C_4 isoform. Cold inactivation of PEPC was observed at higher pH in *Cynodon dactylon. Atriplex halinus* and *Zea mays* (Angelopoulos et al., 1990). The oligomeric status of the PEPC-enzyme may vary depending on the temperature. Above 25 °C, there is an aggregation of PEPC in case of C_4 or dissociation in case of CAM (Wu and Wedding, 1987). A change may occur on exposure to cold/chilling in form of PEPC: from active tetrameric to less-active dimers or monomers (Shi et al., 1981; Walker et al., 1986).

Krall and Edwards (1993) reported that the PEPC enzyme was very stable at even low temperatures in *Panicum miliaceum,* while the enzyme from *Panicum maximum,* lost 50% of its activity on incubation for 60 min at 0 °C. A

temperature dependent increase in PEPC activity, in presence of solutes, was reported in *Cynodon dactylon* (Drilias et al., 1994). The effect was more pronounced at temperature above 30 °C. PEPC is protected against cold inactivation by addition of compatible solutes like proline or betaine (Krall and Edwards, 1993). Temperature can affect oligomeric/aggregation status of enzymes. However, this is not well corroborated in case of C_4-PEPC (Shi et al., 1981; Walker et al., 1986).

pH:

PEPC is highly regulated by cytosolic pH (Davis, 1973, 1979; Andreo et al., 1987; Rajagopalan et al., 1993). The enzyme is active at pH 8.0 and becomes inactive at acidic pH, thereby slowing down carboxylation. The enzyme shows competitive inhibition at pH 8.0 but non-competition at pH 7.0 with malate (Gonzalez et al., 1984).

Besides the regulation by pH, PEPC has been proposed to be involved in the regulation of intracellular pH, and thus forms an important part of the biochemical pH-stat, particularly in plant cells (Davis. 1973, 1979). Recent reports by Raghavendra et al. (1993), Yin et al. (1993) using pH-dependent fluorescent dyes, have demonstrated the marked changes in the intracellular pH on illumination. Light induces an alkalization of cytosol and acidification of vacuole within the leaves. The extent of cytosolic alkalization in mesophyll cells of C_4 plants was much greater than that in C_3 leaves. The changes in pH could modulate the intracellular calcium, as in sorghum mesophyll protoplasts (Pierre et al., 1992). The response to pH may affect the catalytic activity of PEPC or PEPC-PK or both (Rajagopalan et al.. 1993).

Salt/Water stress:

Water stress, increases the activity of PEPC in leaves of *Salsola soda*, similiar to the effects of warm-temperatures on PEPC in C_4 plants. In contrast, PEPC in *Cynodon dactylon* is activated by betaine, while proline is a competitive inhibitor of PEPC (Manetas et al., 1986). However, the effect of salt stress on

PEPC is not manifested in C_4 plants as strongly as in CAM plants. For e.g. in an inducible-CAM plant, *Mesembryanthemum crystallinum,* PEPC-activity rises remarkably on exposure to salt or water stress, due to enhanced transcription of the ppc gene (Mc Elwain et al., 1992). Photoperiodism or water stress can shift young leaves of *Kalanchoe blossfeldiana* from C_3-type photosynthesis to CAM. It has been shown that endogenous levels of ABA preceded PEPC increase, independent of CAM induction in isolated leaves of *Kalanchoe blossfeldiana* (Taybi et al., 1995). Dai et al. (1994) provided evidence that in *Mesembryanthemum crystallinum,* the increase in PEPC activity upon ABA treatment was due to increased levels of CAM-specific isoform of the enzyme.

Effect of salts/inorganic ions

Stimulation of dark CO_2 fixation by ammonium is a well known phenomenon (e.g. Hammel et al., 1979; Miyachi and Miyachi, 1985). Ammonium ions enhance assimilation of carbon into C_4 acids in higher plants, algal cells and cyanobacteria (Ohmori et al., 1986; Müller et al., 1990; Vanlerberghe et al., 1990). Such stimulation was assumed to be due to the increase in the activity of PEPC in ammonia-treated cells. The rates of ammonia assimilation *in vivo* were well correlated with PEPC activity in a green alga, *Selanastrum minutum* (Vanlerberghe et al., 1990).

Ammonia is a toxic metabolite and inhibits several enzymes of plant and animal metabolism (WHO, 1986). Nevertheless, a few enzymes such as pyruvate kinase are stimulated by ammonium (Miller, 1970; Kanazawa et al., 1972; Peterson and Evans, 1978). Although the increase in PEPC activity has been correlated with elevated rates of dark carbon fixation, the direct effects of ammonium salts on PEPC activity *in vitro* were not assessed. Gayathri and Raghavendra (1994) have recently reported that ammonium ion can stimulate PEPC *in vitro*. The effect of ammonium on PEPC was at the regulatory

allosteric site on the enzyme. Another possibility is the modulation of intracellular pH by externally added NH_4^+. Ammonia diffusion into cells may cause alkalization of cytosol and the rise in pH could in turn stimulate PEPC.

Biosynthesis of PEPC in maize leaves was affected by the extent and form of nitrogen available to the plant. For e.g., the levels of PEPC are increased on feeding maize leaves with nitrate or ammonium (Sughiharto et al., 1990; Sughiharto and Sugiyama, 1992). Ammonium salt was two-fold more effective inducer of PEPC biosynthesis than that of nitrate (Sughiharto and Sugiyama, 1992). These effects can be termed as long-term ones.

Van Quy et al. (1991a, b) have reported in wheat leaves the light activation of PEPC is further enhanced in presence of nitrate. Van Quy and Champigny (1992) and Duff and Chollet (1995) suggested that presence of nitrate enhances the PEPC-PK activity which phosphorylates PEPC in wheat leaves in the light, leading to greater light activation. Thus the presence of nitrate/ammonia are of two kinds: long-term enhancement of PEPC biosynthesis and short-term: increase in the activity of PEPC/PEPC-PK.

Another important ion which is involved in regulation of PEPC is calcium. PEPC is one of the few plant proteins, photoregulated and controlled by a complex cascade of events, possibly involving calcium. Light dependent phosphorylation of PEPC in mesophyll protoplasts of sorghum was promoted by weak bases such as ammonium chloride and methylamine (Pierre et al., 1992). These ions were expected to increase the cytosolic pH. raise the levels of calcium and activate PEPC or PEPC-PK or both. However, the evidences on the involvement and regulation by calcium of PEPC-PK are contradictory. These are further discussed in the following pages. Further studies are needed to resolve the role of pH and calcium in regulating the activity of PEPC and its phosphorylation by PEPC-PK in C_4 and CAM plants.

Post-translational modification of PEP carboxylase

The post-translational modification of PEPC in plants involves two types of phenomena: Phosphorylation and oligomerization.

Phosphorylation-Dephosphorylation:

Regulation of enzyme activity in plants by reversible phosphorylation has been reviewed (Budde and Chollet, 1988; Huber et al., 1994). Regulatory phosphorylation can result in inactivation or activation and/or changes in the allosteric properties of the target enzyme. However, the extent of these changes can vary greatly among different target enzymes. For example, in case of PPDK, the difference in the catalytic activity between the phosphorylated and dephosphorylated forms can be so large, up to ten-fold, between the light- and dark-forms (Huber et al., 1994). On the other hand, alteration in phosphorylation status of some enzymes, may result only **in** two-three fold changes in the activity, as in case of PEPC. The phosphorylation of PEPC occurs at one or more seryl residues in C_4 and CAM plants (Nimmo, 1993). Regulatory phosphorylation of PEPC by a PEPC protein-serine kinase is established, both *in vitro* and *in vivo* (Jiao and Chollet. 1991). The phosphorylation of PEPC occurs on ser in sorghum (Jiao et al., 1991; Wang et al., 1992), ser^{15} in maize (Jiao and Chollet, 1990; Jiao et al.. 1991), ser" in tobacco (Wang and Chollet, 1993) ser" in soybean root nodule (Zhang et al., 1995). The phosphorylation occurs on ser (day) in *Mesembryanthemum crystallinum*(Jiao and Chollet, 1991; Baur et al., 1992; Nimmo. 1993).

Comparison of amino acid sequences around N-terminal region reveals that the important structural amino acid motif of "Lys/Arg-X-X-Ser", which appears to interact with PEPC-PK, is present in all C_3, C_3-C_4, and C_4 forms of PEPC but not in those of prokaryotes: *E. coli ox A. nidulans* (Cretin et al., 1991; Poetsch et al., 1991; Pathirana et al., 1992; Schäffner and Sheen, 1992). This observation suggests that PEPC of only higher plants possesses marked regulatory properties. It is now known that PEPC of also C_3 plants undergoes

reversible phosphorylation and changes its allosteric properties, like that of C_4 and CAM-PEPC (Gupta et al., 1994).

Limited information is available on post-translational regulation of the non photosynthetic C_3 enzyme, although it too is a subject of allosteric control. Phosphorylation of PEPC has been reported with purified PEPC of maize and tobacco (Wang and Chollet, 1993), and also in leaves of C_3 plants (Van Quy et al., 1991 a, b; Duff and Chollet, 1995), stomatal guard cells (Schnabl et al., 1992; Zhang et al., 1994), in legume root nodule extracts *in vitro* (Vance and Gantt, 1992; Schuller and Werner, 1993) and *in vivo* (Zhang et al., 1995). Phosphorylation of alfalfa and soybean root nodules in crude extracts by endogenous protein kinase *in vitro* has also been reported (Pathirana et al., 1992). The phosphorylation site is accessible to both homologous and heterologous protein kinases, e.g. mammalian protein-kinase A (Jiao and Chollet, 1990; Terada et al., 1990).

The post-translational modification by phosphorylation of PEPC is promoted further in light by virtue of reversibly light-activated nature of PEPC-PK (Echevarria et al., 1990; Jiao and Chollet, 1991; McNaughton et al., 1991; Bakrim et al., 1992; Jiao and Chollet, 1992). The activity of PEPC-PK is increased, possibly due to *de novo* synthesis of the enzyme. McNaughton et al. (1991) speculated that a signal generated in ehloroplast may initiate a sequence of events that leads to a significant increase in activity of PEPC-PK and phosphorylation of PEPC in the cytosol. On feeding with photosynthetic inhibitors like DCMU or DL-glyceraldehyde, phosphorylation of PEPC decreased indicating the co-ordination of both mesophyll and bundle sheath cells (Jiao and Chollet, 1992). However. Pierre et al. (1992) have shown that maize mesophyll protoplasts possess the intrinsic ability, similiar to whole leaf, in performing PEPC phosphorylation.

While phosphorylation of PEPC is catalyzed by PEPC-PK, the dephosphorylation is brought about by type 2A protein phosphatase(s) (Carter

et al., 1991; McNaughton et al., 1991). Although PEPC has been purified from leaves of several C_4 plants (maize and sorghum), CAM species (*Bryophyllum fedtschenkoi)* and C_3 species (tobacco), attempts to purify PEPC-PK from C_4 or CAM plants are still very limited.

Calcium-dependent and calcium-independent protein kinases were purified from sorghum leaves (Bakrim et al., 1992). Ogawa and Izui (1992) also have shown that phosphorylation of PEPC is by a calcium-dependent protein kinase in maize leaves. PEPC from sorghum was phosphorylated in a calcium-calmodulin dependent manner (Echevarria et al., 1988). However, Jiao and Chollet (1988, 1990, 1991) have reported that phosphorylation of PEPC in maize occurs in a calcium independent manner. Such discrepancy in the reports on the role of calcium during phosphorylation of PEPC could be due to presence of multiple kinases in cytosol (Ogawa and Izui, 1992).

The information about PEPC-phosphatase(s) from C_4 plants is even scarce. On treating with alkaline phosphatase, the malate inhibition of PEPC increases and enzyme functions in a manner similar to that of dark-form (Jiao and Chollet, 1988; Arrio-Dupont et al., 1992). The co-ordination of both PEPC-PK and PEPC-phosphatase(s) may determine the net phosphorylation and its sensitivity to malate during L/D transitions.

Oligomerization:

PEPC is a homotetramer (Andreo et al., 1987) and exists as dimer or monomer, depending on several factors: pH, ionic strength (Walker et al., 1986; Wagner et al., 1987), temperature (Wu and Wedding, 1987) and PEPC concentration (Willeford and Wedding, 1992). Presence of NaCl causes dissociation of enzyme into dimer at pH 7.0, and into dimers/monomers at pH 8.0. Presence of PEP, magnesium or G-6-P prevented dissociation of the enzyme (Wagner et al., 1987). Effectors such as G-6-P and malate, or presence of solutes (PEG, glycerol) can effect the aggregation of the enzyme (Podesta and Andreo, 1989; Manetas, 1990; Wedding et al., 1994). G-6-P increases the aggregation of **the**

enzyme (Wu and Wedding, 1994). Wang et al. (1992) have reported that phosphorylation has no effect on G-6-P and the activation of the enzyme by G-6-P occurs by a more complex mechanism than the activation by PEP or inhibition by malate.

Glycerol and high PEPC concentration shifts the enzyme to active tetrameric form (Podesta and Andreo, 1989). However, Weigend and Hincha (1992) have reported that there is no relation between the malate sensitivity and the oligomeric status of the enzyme. Dilution of the enzyme *in vitro* can change the oligomeric status of the enzyme (Wu et al., 1990). Most of these experiments on oligomerization of PEPC have been done *in vitro* and not much information is available on the form of PEPC under *in vivo* conditions. The physiological condition of the oligomerization of the enzyme *in vivo* is yet to be investigated critically and its relevance to phosphorylation would be of great interest.

A lot of work has been done on the physiology, biochemistry and molecular biology of PEPC. But there is still a large scope for further work, on PEPC particularly from C_4 plants and C_3-C_4 intermediates. For example, PEPC has been purified to homogeniety from leaves of several C_3- and -C_4 plant species like spinach, maize, sorghum (Rajagopalan et al.. 1994), and even from *Amaranthus viridis* (Iglesias et al., 1986). Nevertheless, a method of rapid purification, along with long-term storage, is extremely useful for detailed studies. We have therefore, attempted to purify PEPC from *Amaranthus hypochondriacus* and evolve an acceptable method of storage.

Addition of glycerol stabilizes PEPC (Karabourniotis et al., 1983). since otherwise the enzyme is unstable, particularly on dilution (Selinioti et al.. 1987). Inclusion of solutes, like PEG, has been conventionally used for stabilization of proteins (Reinhart, 1980). The effects of different solutes like PEG on stabilization and properties of PEPC from C_4 plants, is not yet studied in detail.

Although the existence of at least four isozymic forms of PEPC have been described in a number of C_3, C_3-C_4 intermediate and C_4 plants (Ting and Osmond, 1973a, b; Peterson and Evans, 1979; Vidal and Gadal, 1983), there is ambiguity about their distinction. Kinetic and characterization of PEPC from *Amaranthus hypochondriacus*, a C_4 plant in comparison to that of other C_3 or C_4 dicots/monocots is naturally a topic of interest.

PEPC has become a classic example of enzyme regulation by post-translational modification by phosphorylation-dephosphorylation cascade (Jiao and Chollet, 1991; Huber et al., 1994). However there is a lot of debate about the nature of PEPC-PK regulation of PEPC by calcium. There are conflicting reports that PEPC-PK is Ca^{2+} dependent or Ca^{2+} independent.

In the present investigation, PEPC was purified from the leaves of *Amaranthus hypochondriacus*, a NAD-malic enzyme type C_4 plant and used to answer some of those questions. The approach and objectives are further elaborated in the next chapter.

Chapter 2

Approach and Objectives

Chapter 2

Approach and Objectives

Our major objective is to study the biochemical and immunological properties of PEPC from leaves of a C_4-dicot plant. PEPC is located in the cytosol of mesophyll cells of C_4 plants, and this property make it easy to use crude extracts of a C_4 plant as the source of enzyme. Further, the leaf tissue of a C_4 dicot is soft and easy to extract the cell-sap. *Amaranthus hypochondriacus*, a NAD-ME type of C_4 plant, is an important grain crop/leafy vegetable, which grows vigorously in our semi-arid sub-tropical region. We have therefore used the leaves of *Amaranthus hypochondriacus* for studies on purification and characterization of PEPC. Emphasis was given to the methods of purification and storage of the enzyme, besides the interaction of PEPC with organic solutes like PEG, and regulation by cations, particularly ammonium and calcium.

The first set of experiments were designed to purify PEPC from leaves of *Amaranthus hypochondriacus* and to identify the best way of storage. Purification of the PEPC was done through conventional techniques of 40-60% ammonium sulphate fractionation, followed by DEAE-Sepharose, HAP chromatography and finally through Seralose 6-B column and the enzyme was concentrated with PEG-20,000. The purified PEPC is unstable, and if proper precautions are not taken, the enzyme loses frequently its N-terminal end and becomes malate-insensitive (McNaughton et al., 1989; Duff et al., 1995). Therefore, we have studied the properties of the purified enzyme by storing it in the presence and absence of 50% (v/v) glycerol and at different temperatures (i.e., room temperature, 4 °C, -20 "C, and liquid nitrogen). The kinetic parameters, such as V_{max}, K_m for PEP. K_i for malate, and K_A for G-6-P of the purified enzyme, were also studied after storing the preparation for 24 h al different temperatures.

Polyclonal antibodies were raised in rabbits against the purified PEPC from *Amaranthus hypochondriacus* and also against commerically available PEPC from maize (Sigma, Chemical CO., USA) using the procedure of Nimmo et al. (1986). Immunoprecipitation using the antibody confirmed that the PEPC was precipitated effectively in the crude extracts from leaves. Ouchterlony double diffusion method was employed to check the antibody titer value and to assess the cross-reactivity between the PEPC of C_4 dicots and C_4 monocots. Single radial immunodiffusion was performed to quantitate the amount of PEPC protein in total leaf crude extracts, after calibration using purified **enzyme** as the standard-protein. Further analysis was done through Western blots to examine the relationship between C_3, C3-C4, C_4 (monocot and dicots) species, using **anti-PEPC** antiserum from *Amaranthus hypochondriacus* and *Zea mays.* This was done to check the specificity of the antiserum prepared. **The** technique of Enzyme Linked Immunosorbent Assay (ELISA) using alkaline phosphatase (Gillina and Greally, 1993), was used to detect and quantitate very low levels of **PEPC in** crude extracts of C_4 species. Our observations suggest that C_4 dicot PEPC is distinct from that of C_3, C_3-C_4 and C_4 monocots, while the PEPC of C_4 monocot is closer to C_3 monocot than to C_4 dicot. This is similar to the recent assessment of PEPC-evolution by Lepiniec **et al. (1993,** 1994).

Organic solutes play an important role in maintaining the integrity of enzymes, even during adverse conditions of extraction (Rhodes and Hanson, 1993; Drilias et al., 1994). Addition of PEG, **ethylene** glycol, or glycerol, helps the enzymes to maintain their homologous interaction and thus improve their stability (Selinioti et al., 1987; Drilias et al., 1994). Interactions between the enzymes that exist at a high protein concentrations *in vivo* can be mimicked ***in vitro*** by addition of compatible solutes (e.g. PEG) to the reaction mixture. According to Timasheff (1992), the structure stabilizing solutes are excluded from the domain molecules which are thus confined to a small fraction of the

total volume and forced to a low-volume conformation. Under such a high protein concentration during extraction and storage, a situation, closer to the physiological situation, the stability of oligomeric enzymes is enhanced.

PEPC is likely to be in diluted state in crude leaf extracts. This would definitely change in the conformation and properties of PEPC. Since the solutes act as protein concentrating agents, there can be change in the oligomeric status of PEPC in the presence or absence of PEG. Therefore, we have examined the effect of three different types of PEG on the activity and kinetic characteristics of the enzyme (i.e., response to PEP, malate sensitivity and G-6-P). The effect of sorbitol was also checked on the enzyme to see if the enzyme-interaction with these solutes was due to changes in the osmotic strength. PEPC is known to get activated in light, with marked changes in the characteristics of the enzyme. Hence the effect of solutes was studied also on dark/light form of PEPC. The enzyme was assayed at sub-optimal or optimal substrate concentration.

The expression of PEPC in leaves is affected by the nitrogen availability to the plant. The levels of PEPC in nitrogen starved leaves of maize increase markedly on feeding with nitrate or ammonium (Sugiharto et a!., 1990; Sugiharto and Sugiyama, 1992). These reports demonstrated that the induction of PEPC biosynthesis by ammonium ions was two-fold stronger than that by nitrate. The effect of several inorganic salts (of monovalent and divalent ions) on PEPC was evaluated in leaf crude extracts of *Amaranthus hypochondriacus*. Among a range of different salts tested, PEPC in the leaf extracts of *A. hypochondriacus* was stimulated markedly (180% at 50 μM) by ammonium salts and to some extent by potassium ions. Acetate and sulphate salts of ammonium, besides chloride, were used to ensure the effect of NH_4 on PEPC. On the other hand, salts of monovalent ions like lithium, sodium and rubidium were also tested, besides those of ammonium or potassium and divalent cations such as calcium. Magnesium, any way, is essential for PEPC-enzyme reaction.

PEPC is subjected to phosphorylation-dephosphorylation cascade during L/D transitions *in vivo* (Jiao and Chollet, 1991; Nimmo, 1993; Rajagopalan et al., 1994). A protein-serine kinase catalyses the phosphorylation of PEPC, while a type 2A protein phosphatase dephosphorylates the enzyme. Recent reports on the PEPC kinase in C_4 plants indicate that *de novo* cytoplasmic protein synthesis is an important component. Experiments with mesophyll protoplasts of maize (Devi and Raghavendra, 1992) and sorghum (Pierre et al., 1992) have shown that pH and calcium are important factors during light activation and phosphorylation of PEPC. However, the mechanism of action of calcium and its interaction with pH during PEPC phosphorylation is under debate.

Experiments were therefore designed to investigate if calcium modulates PEPC or PEPC-PK or both. Phosphorylation of PEPC can be studied either directly or indirectly. An indirect way is to incubate crude extracts with ATP and determining the extent of activation of PEPC after incubation. On the other hand, the direct way would be to monitor and assess Pi incorporation into PEPC in crude leaf extracts. Experiments were therefore conducted to examine PEPC-phosphorylation using both direct and indirect methods. Incubation with ATP stimulated PEPC activity and decreased the malate sensitivity particularly in extracts from illuminated leaves. Pretreatment of leaves with EGTA *in vivo,* decreased the extent of stimulation by ATP of PEPC activity (a measure of PEPC-PK). particularly in leaves exposed to light. Direct evidence of PEPC-phosphorylation was obtained by either incubation of leaf extracts with AT P *(in vitro)* or feeding intact leaves with "Pi (*in vivo).* The leaves were extracted, proteins separated on 10% SDS-PAGE and autoradiographed. *In vivo* labelling of leaves with Pi showed that the phosphorylation of PEPC was more in light than that in dark treated leaves. Most of the experiments were therefore conducted with illuminated leaves.

Further experiments were designed to evaluate the involvement of calcium and calmodulin as an additional components involved in the transduction of light signal to activate PEPC or PEPC-PK or both. Leaves were fed through petiole, a calmodulin antagonist trifluoperazine (TFP) and diltiazem, verapamil and lanthanum (calcium channel blockers) in light. The phosphorylation pattern of PEPC from these leaves was analysed, using AT **P.**

Thus the objectives of present work are to:

1. Purify PEPC from leaves *of Amaranthus hypochondriacus* and to identify the best way of storage.

2. Examine the immunological properties of PEPC from *A. hypochondriacus* in comparison with those of a few other C_3 or C_4 monocot/dicot species.

3. Investigate the effect of polyethylene glycol (PEG) and its interaction with activity, kinetics and regulation of PEPC in leaf extracts and in purified enzyme preparations.

4. Study the effect of inorganic cations (including ammonium and potassium) *in vitro* on the activity and kinetics of PEPC extracted from leaves of *A. hypochondriacus.*

5. Examine and compare the effect of calcium on the activities of PEPC and PEPC-PK and involvement of calmodulin during the phosphorylation of PEPC.

Some of the experiments were conducted with crude leaf extracts of *A. hypochondriacus,* while the others were done with purified PEPC from these leaves. A few experiments, for comparitive purposes, involves PEPC in crude leaf extracts of a few C_3- and C_4 plants and C_3-C_4 intermediates.

Chapter 3

Materials and Methods

Chapter 3

Materials and Methods

Plant Material

Plants of *Amaranthus hypochondriacus* L. (cv. AG-67) were raised from seeds, supplied from cytogenetics division, National Botanical Research Institute, Lucknow. The plants were grown on soil supplemented with farm-yard manure, in 25-cm diameter earthen pots kept outdoors in the field (approximate photoperiod 12 h and temperature of 30-40 °C day and 25-30 °C night). The upper fully expanded leaves of 4 to 6 week old plants (Plate 3.1) were harvested, about 2-3 h after sunrise.

For some of the studies, plants belonging to C_3- or C_4- or C_3-C_4 intermediate types were used for ascertaining the comparative nature of PEPC. The plants used for these studies, which were grown in the field, are as follows:

C_3 species:

Alternanthera sessiles L.

Commelina benghalensis L.

Pisum sativum L. (cv. Arkel)

C_4 plants:

Amaranthus hypochondriacus L. (cv. AG-67)

Alternanthera pungens (L.) H. B. & K.

Amaranthus viridis L.

Pennisetum glaucum (L.) R. Br. (cv. IP-8166)

Sorghum bicolor (L.) M. (cv. CHS-11)

C3-C4 intermediates:

Alternanthera ficoides (L.) R. Br. R.

Alternanthera tenella Colla.

Parthenium hysterophorus L.

Plate 3.1. Plants of 4 to 6 week old *Amaranthus hypochondriacus* showing fully expanded leaves.

Experimental Methods

Extraction and Assay of PEPC

Leaves were excised from plants between 9.00 to 9.30 A.M., approximately 3 h after sunrise. After a period of darkness or illumination (as described in text), the leaves were cut into small pieces and used for extraction of PEPC.

For some of the experiments leaf discs were used. Discs of *ca.* 0.2 cm^2 were cut from leaves under water with help of a sharp paper puncher. Twenty discs were kept in a Petri dish (5-cm diameter) containing 10 ml of water and left in darkness for 2 h. If, necessary, the discs were illuminated at 1000 μE m^{-2} s^{-1} for 20 min (after pre-darkening).

Leaves/leaf discs were extracted with 100 mM Hepes-KOH pH 7.3, containing 10 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM **EDTA, 20% (v/v) glycerol,** 2 mM PMSF and 10 mM 2-mercaptoethanol. The extract was filtered through 4 layers of cheese cloth and rapidly centrifuged at 15,000 g for 5 min (Hermle Z-320 K centrifuge). An aliquot was kept aside, prior to centrifugation, for chlorophyll estimation.

PEPC activity was assayed by coupling to NAD-malic dehydrogenase. Enzyme activity was determined at 30 °C by monitoring NADH oxidation at 340 nm in a dual beam UV-Vis Spectrophotometer (Shimadzu UV-160A).

The reaction mixture (1 ml) contained 50 mM Hepes-KOH (pH 7.3), 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 units of NAD-MDH. 0.2 mM NADH and leaf extract (equivalent 1 μg of chlorophyll). The extract or the purified enzyme was incubated in the assay medium for 30 s and reaction was started by addition of 50 μl of 50 mM PEP (stock solution of PEP prepared in 50 mM Hepes-KOH pH 7.3). The reaction was linear for at least 8 min with crude extracts and 5 min **with** purified enzyme.

One unit of enzyme activity is the capacity of the enzyme to catalyze the formation of 1 μmol of oxalacetate min^{-1}.

Protein and Chlorophyll Estimation

Total protein was determined by using either Folin-phenol reagent (Lowry et al., 1951) or the protein-binding dye, Coomassie brilliant blue G (Bradford, 1976) with bovine serum albumin as a standard.

Chlorophyll was estimated after extraction with 80% (v/v) acetone and using the formula of Arnon (1949). 12.5 µl of crude leaf extract was added to 5 ml of 80% (v/v) acetone and the absorbance of solution was measured at 652 and 710 nm, using a Shimadzu Spectrophotometer UV-160A. The absorbance at 652 nm is due to chlorophyll while A_{710} reflects the turbity of solution. The concentration of chlorophyll was calculated using the following formula

$$\text{Chl (mg ml}^{-1}) = (A_{652} - A_{710}) \times 11.11$$

Kinetics of the Enzyme

The maximum velocity of the enzyme (V_{max}) and K_m for PEP on PEPC were examined in the presence or absence of solutes/effectors. The enzyme was allowed to incubate for 30 s in the assay medium and the reaction was started by addition of PEP (0.5 to 5 mM). K_m values were calculated from Lincweaver-Burk's plots.

Malate sensitivity was determined in a 1 ml assay mixture containing 50 mM Hepes-KOH pH 7.3, 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 units of NAD-MDH, 0.2 mM NADH, enzyme (extract) and ± malate (0 to 5 mM). The reaction was initiated by the addition of 2.5 mM PEP and the change in A_{340} was monitored. K_i values were calculated from linear inhibition-equation using "Enzkinet" computer program.

The activation of PEPC by G-6-P was also studied in a similar manner as described above, except that different concentrations of G-6-P (0 to 5 mM) was added instead of malate in the assay medium. K_A (G-6-P) values were calculated by activator equation, using "Enzkinet" computer program.

The stock solutions of PEP, malate and G-6-P were prepared in 50 mM Hepes-KOH pH 7.3.

Purification of PEPC from Amaranthus hypochondriacus

The purification protocol was similar to that described for the enzyme from *Amaranthus viridis* (Iglesias et al., 1986), but with slight modifications. The details are described below.

Extraction and ammonium sulphate fractionation

The leaves (40 g) of *Amaranthus hypochondriacus* picked from the field-grown plants (exposed to sunlight for 2-3 h) were washed, cut into small pieces and suspended in 200 ml of buffer containing 100 mM phosphate buffer, pH 7.2, 25% (v/v) glycerol, 5 mM DTT, 5 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 2 mM PMSF and 10 mM 2-mercaptoethanol. Solid polyvinylpyrrolidone (0.5 g/1 g of leaves) was added to the medium. The leaves were then homogenised using a Waring blendor (1.5 min; maximum speed). The homogenate was filtered through a cheese cloth and the filtrate was centrifuged at 15,000 g for 10 min. The above procedure was performed at 4 °C and the subsequent steps were carried out at room temperature, about 20-22 "C.

The supernatant (300 ml) was brought to 40% saturation with saturated ammonium sulphate solution. The suspension was stirred slowly for 30 min and then centrifuged at 15,000 g for 15 min. The precipitate was discarded, the supernatant was brought to 60% (v/v) saturation by further addition of saturated ammonium sulphate solution and the precipitate was collected by centrifugation at 15,000 g for 30 min.

DEAE-Sepharose chromatography

The above precipitate (after 60%, v/v. saturation of ammonium sulphate) was suspended in 5 ml of 200 mM potassium phosphate buffer (pH 7.2) plus 10% (v/v) glycerol. The suspension could be stored overnight at 4 °C, without any loss of enzyme activity. The next day, the solution was dialyzed against 20

mM potassium phosphate buffer (pH 7.2) and 10% (v/v) glycerol and then loaded, on to a DEAE-Sepharose CL-6B column (1 x 7 cm), equilibrated with 20 mM potassium phosphate buffer (pH 7.2) and 10% (v/v) glycerol. The column was washed with same buffer at a flow rate of 0.5 ml min^{-1} until A_{280} nm returned to baseline. A linear gradient of 40 to 200 mM potassium buffer (pH 7.2) containing 10% (v/v) glycerol was used to elute PEPC. PEPC eluted as a broad peak and eluted at around 70-80 mM Pi (Fig. 3.1). The active fractions containing maximum PEPC activity was pooled (20 ml) and the enzyme was precipitated with 60% (v/v) saturated ammonium sulphate solution.

Aliquots of the active fractions (Nos 8-15) were checked separately for the protein profiles on 10% SDS-PAGE (Plate 3.2).

HAP chromatography

The precipitate from the above step, after ammonium sulphate precipitation, was dissolved in 200 mM phosphate buffer pH 7.2 containing 10% (v/v) glycerol, and dialyzed against 20 mM phosphate buffer (pH 7.2) containing 10% (v/v) glycerol. The dialyzed sample was applied on to a 1 x 7 cm HAP column. HAP column was prepared as described by Oishi (1971). 25 ml each of 0.5 M calcium chloride and 0.5 M disodium hydrogen phosphate from separate burettes were mixed dropwise in a beaker containing 2.5 ml of 1 M NaCl. A flow rate of 4 ml/min was maintained from each burette. The brushite formed was allowed to settle and the supernatant was decanted. The precipitate was washed twice and boiled with simultaneous stirring for 1 h with 500 ml of double distilled water containing 1.25 ml of 1 M NaOH solution.. The precipitate was allowed to settle completely. The supernatant was decanted, the precipitate was washed twice with distilled water and was allowed to settle. The precipitate was taken out and added to 10 mM sodium phosphate buffer pH 6.8 and allowed to just boil (avoid boiling). The gel (HAP) was washed

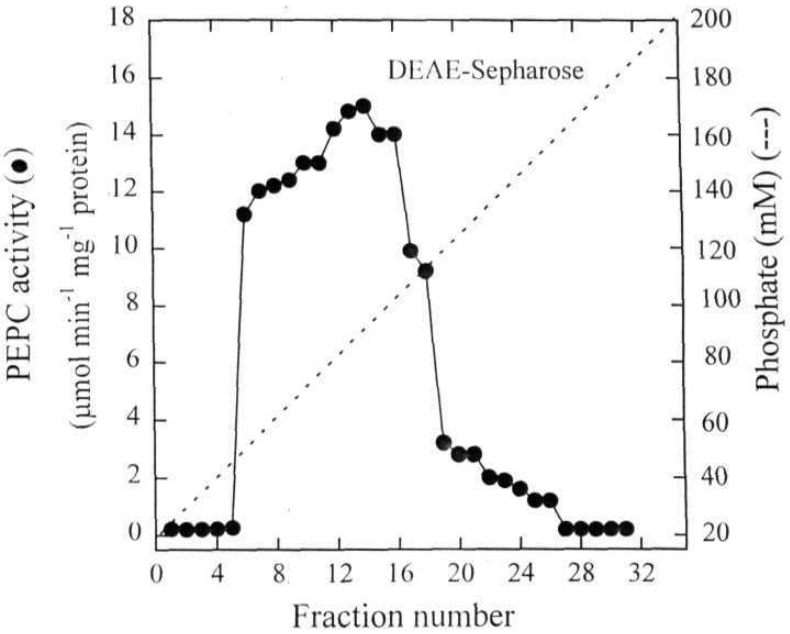

Figure 3.1. The pattern of PEPC from a DEAE-Sepharose column. The enzyme from 60% ammonium **sulfate** fraction of leaf extract was loaded, after dialysis, on a **DEAE-Sepharose** (1 x 7 cm) **column,** equilibrated with 20 mM potassium phosphate buffer (pH 7.2) containing 10% (v/v) glycerol. The column was washed with the same buffer and was **eluted** with a linear gradient from 40-200 mM phosphate buffer (7.2) containing 10% (v/v) glycerol. The activity of PEPC was assayed at pH 7.3 with 2.5 mM **PEP.** Further details are described in the text.

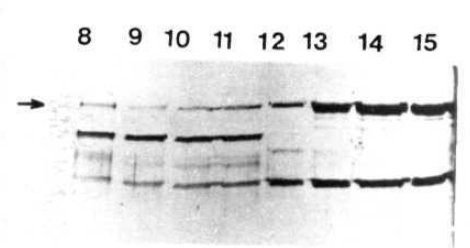

Plate 3.2. 10% SDS-PAGE of the active PEPC fractions (8 to15) eluted from the DEAE-Sepharose column. The pattern of elution and other details are described in Fig. 3.1.

with 20 mM phosphate buffer pH 7.2 and stored at room temperature until required. Later it was transferred on to a column of 1 x 7 cm and equilibrated with 20 mM phosphate buffer (pH 7.2) containing 10% (v/v) glycerol.

The dialysed eluate was applied slowly on the column and the eluate which passes out of the column is again recycled (5 to 6 times) into the column. This ensures complete binding of the enzyme to the column and removal of non-specific proteins from the column. PEPC was eluted with a linear gradient of 40-200 mM phosphate buffer (pH 7.2) plus 10% (v/v) glycerol. The PEPC was eluted in a single peak around 50-60 mM Pi (Fig. 3.2). The active fractions were pooled and then precipitated with saturated ammonium sulphate solution (60% v/v).

Seralose 6-B Chromatography

The precipitate was dissolved in 20 mM potassium buffer (1.5 to 2 ml) containing 10% (v/v) glycerol and applied on to a column of Seralose 6-B (1 x 25 cm) equilibrated with 20 mM potassium phosphate buffer pH 7.2 containing 10% (v/v) glycerol. Elution was accomplished at a flow rate of 15 ml h^{-1}. The enzyme eluted as a single peak (Fig. 3.3). The fractions containing high activity were pooled.

Concentration and Storage

The pooled active fractions were transferred into a dialysed bag (2.1 x 3.3 cm) and concentrated by covering with solid PEG 20,000 (Sigma Chemical Co.. USA). As protein concentration progressed, PEG became sticky and fresh PEG was added. The concentrated purified PEPC was stored in multiple aliquots with 50% (v/v) glycerol in liquid nitrogen.

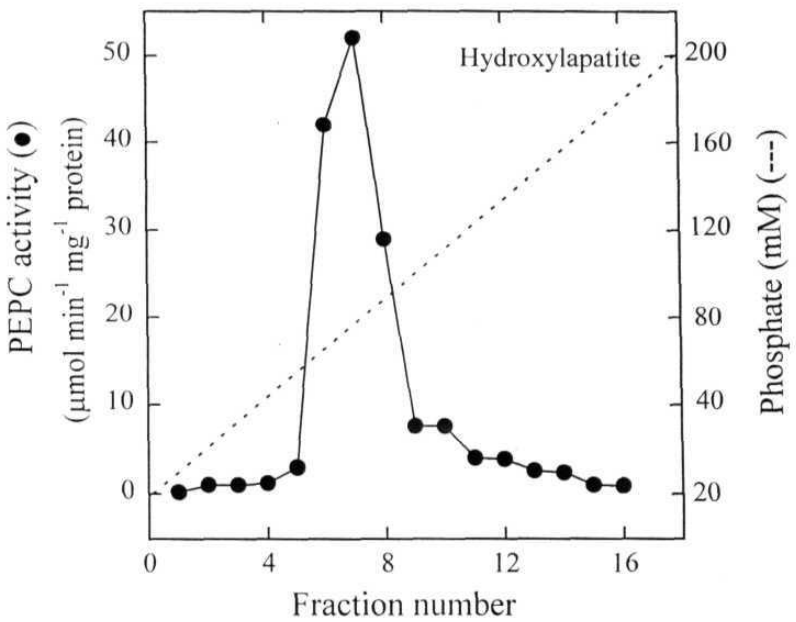

Figure 3.2. Elution profile of PEPC from a **hydroxylapatite** column. The active **fractions** obtained **through** DEAE-Sepharose column were pooled and **precipitated** by 60% ammonium sulphate. The precipitate was dissolved in 20 mM phosphate buffer (pH 7.2) and dialysed against the same buffer. The dialyscd enzyme was applied on to 1 x 7 cm hydroxylapatite column, equilibrated with the same above buffer. PEPC was eluted by employing a linear gradient **of** 40-200 mM phosphate plus 10% (v/v) glycerol. further details are as described in **the** text.

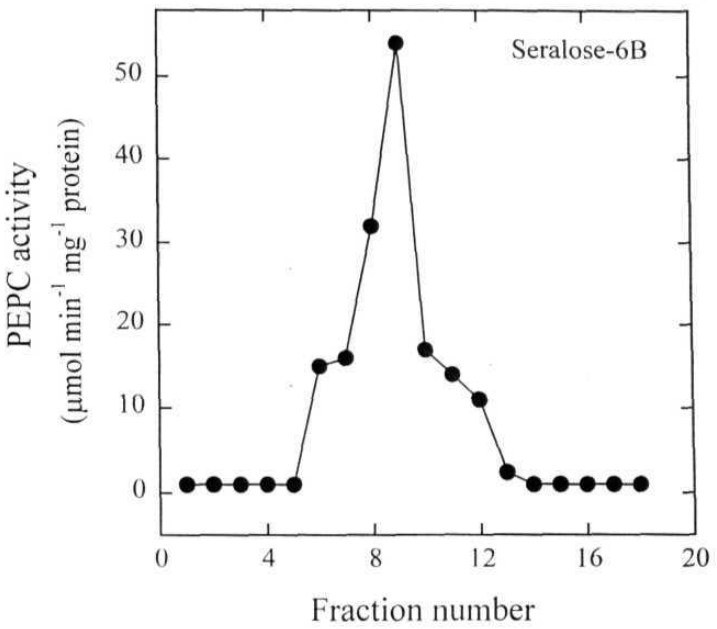

Figure 3.3. Elution profile of PEI'C from a **Seralosc 6-B** column, equilibrated with 20 mM potassium phosphate buffer (pH 7.2) containing 10% (v/v) **glycerol. Further** details as described in **the** text.

Electrophoresis

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Mini-gels (8 cm long x 8 cm wide) of 10% SDS-polyacrylamide were used and electrophoresis was performed, as per the principles of Laemmli (1970). The stacking gel (2 cm long x 8 cm wide) contained 0.5 mM Tris-HCl pH 6.7, 4% of acrylamide (from stock solution of 30: 0.8 of acrylamide and bisacrylamide), 0.1% (w/v) of SDS. The resolving gel (6 cm long x 8 cm wide) was polymerised using 1.5 M Tris-HCl buffer pH 8.8, 10% of acrylamide (from stock solution of 30: 0.8 of acrylamide and bisacrylamide) and 0.1% (w/v) of SDS.

The electrode buffer contained 25 mM Tris-HCl, 192 mM glycine, pH 8.3 and 1% (w/v) SDS. Proteins were dissolved in sample buffer containing 50 mM Tris-HCl, pH 8.8, 8% (w/v) SDS, 50% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.04% (w/v) bromophenol blue and boiled at 100 "C for 2 min and loaded onto 10% SDS-PAGE. Initially the gel was supplied with 75 volts until the dye migrated into the resolving gel and later the electrophoresis power supply was raised to 120 volts. Power was supplied through Atto Digi-Power (SJ-1081) for a total period of about 2 h. 40-60 |ig of protein was loaded in each of the well, unless otherwise specified. The gels after the electrophoresis were fixed for 1 h with fixative solution containing 40% (v/v) methanol and 7% (v/v) glacial acetic acid.

Some of the gels were stained with Coomassie blue-staining solution (0.25%, w/v, Coomassie brilliant blue R-250 in 50%, v/v, methanol and 12.5%, v/v, acetic acid) and some by silver stain.

Silver staining was done according to the procedure of Blum et al. (1987). After electrophoresis, the gel was fixed in fixative-solution containing 50% (v/v) methanol, 12.5% (v/v) ethyl alcohol and 0.5 ml of 37% (v/v) formaldehyde/litre for 1 h. Later the gel was washed thrice with 50% (v/v)

ethanol for 20 min each. The gel was pretreated with 0.02% (w/v) sodium thiosulphate solution for 1 min and rinsed with water for 1 min. The gel was impregnated with 0.2% (w/v) silver nitrate and 0.75 ml of 37% (v/v) formaldehyde/litre for 20 min. The gel was again washed with water for 1 min and developed with 6% (w/v) sodium carbonate and 0.5 ml of 37% (v/v) formaldehyde/litre for 10 min. The reaction was stopped with mixture containing 50% (v/v) methanol and 12.5% (v/v) acetic acid for 10 min after washing the gel with water for 4 min. Finally, the gel was washed well with 50% (v/v) methanol (for more than 20 min). After staining, the gel was destained with destaining solution containing 50% (v/v) methanol and 12.5% (v/v) acetic acid.

A set of molecular weight markers (29 to 116 kD, from Bio-Rad) was used as standard, for assessing molecular weight of proteins on SDS gels.

Non-denaturing PAGE

Native gel was run as described by Davis (1964). A 10% polyacrylamide gel (15 cm long x 13 cm wide) was polymerised without SDS, using 1.5 M Tris-HCl buffer pH 8.8, 10% of acrylamide (from stock solution of 30: 0.8 of acrylamide and bisacrylamide).

After the resolving gel is polymerised, 1 cm-wide wells were cut directly on the resolving gel with help of a comb. The polymerised gel was cooled at 4 °C before loading the protein. The electrode buffer contained 25 mM Tris-HCl, 192 mM glycine pH 8.3 and electrophoresis performed at 4 °C. The run was started with 75 volts for 2 h and the electrophoresis power supply of 120 volts was supplied through Atto Digi-Power (SJ-1081) for about 10 h. 20 µg of purified PEPC was loaded into each of the well.

Staining the gels for PEPC activity

Activity staining for PEPC was carried out at 30 °C as described by

Nimmo and Nimmo (1982).

After the native gel was run, glycine (which may inhibit PEPC) was removed from the gels by presoaking them in 50 mM Tris-HCl (pH 8.0), 0.1% (v/v) 2-mercaptoethanol,10 mM $CaCl_2$, as it inhibited PEPC for 10 min. The staining mixture contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 2 mM PEP, 10 mM $NaHCO_3$, 0.1% (v/v) 2-mercaptoethanol and 10 mM $CaCl_2$ at 30 °C. The gel was incubated for about 30 min at room temperature with the staining mixture. On reaction with PEPC, white bands appear on the gel. The PEPC (active) present in the gel on reaction with PEP and $NaHCO_3$ catalyses the formation of oxalacetate and Pi. The released Pi reacts with $CaCl_2$ present in the medium and forms calcium phosphate which appears as white bands on the gel. Control sets were run without PEP in the staining mixture.

2-D Electrophoresis

A two-dimensional electrophoresis system was performed to confirm the electrophoretic behaviour and subunit composition of PEPC (Vance and Stade, 1984).

The protein samples were run on (1 mm thick) native polyacrylamide gel (15 cm long x 13 cm wide) in first dimension as described above. After electrophoresis, the gel was cut into 12 cm long x 1 cm wide segments. One gel segment (12 cm long x 1 cm wide) was stained for PEPC activity and other segment was stained with Coomassie brilliant blue R-250. The remaining gel segments were frozen and stored overnight. Next day, gel-segments were thawed and incubated individually in three changes of an equilibration buffer [0.2 M Tris-HCl (pH 6.7), 22% (v/v) glycerol, 10 mM 2-mercaptoethanol and 4% (w/v) SDS in total volume of 120 ml] for 15 min each. The gel-segment (12 cm long x 1 cm wide) was then placed on top of a 1.5 mm thick SDS slab gel (15 cm long x 13 cm wide) composed of a 10% running gel (12 cm long x 13 cm wide) and a 3% spacer gel (3 cm long x 13 cm wide). The gel-

segment was recemented in with warm 1% agarose. Electrophoresis and staining/destaining were already described above.

A set of molecular weight standards (29-205 kD) were included during the running of SDS-PAGE in second dimension.

Gel Filtration on Sephadex G-200

Gel filtration of leaf extracts on a Sephadex G-200 (1 x 30 cm) column, was performed to assess the molecular size of PEPC. Leaf crude extracts were prepared with or without solutes (20% (v/v) glycerol or 5% (w/v) PEG or both) in 100 mM Hepes-KOH, pH 7.3, containing 10 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 2 mM PMSF and 10 mM 2-mercaptoethanol.

The standard buffer used for all gel chromatographic experiments was 50 mM Hepes/NaOH (pH 7.3) with or without PEG and/or glycerol, as mentioned in text. The column was washed and equilibrated with 50 mM Hepes/NaOH (pH 7.3) with additions as described. A standard quantity of 500 µl of protein containing 450-500 µg of protein or the leaf extract was applied to the column. All runs were made at a flow rate of 10 ml/h at 30 °C. Fractions of 1 ml were collected and the elution pattern of PEPC was checked by assaying the enzyme activity.

The column was calibrated by using four protein-standards: thyroglobulin (669 kD), apoferritin (443 kD), alcohol dehydrogenase (150 kD), BSA (66 kD). The void volume V_o, was determined with blue dextran (2,000 kD).

Immunological Characteristics of PEPC

Preparation of anti-PEPC antiserum

Anti-PEPC antiserum was raised in 6 month-old white rabbits, as per the principles described by Nimmo et al. (1986).

Pre-immune serum was collected from ear-vein of the rabbit. Then, 0.5 mg of purified PEPC in 500 µl, emulsified in equal volume (500 µl) of 50% Freund's complete adjuvant, was injected subcutaneously at about 10 sites. Four weeks later, the animal was given (through subcutaneous injections) a booster dose of 0.25 mg in 250 ul of purified enzyme, emulsified with equal volume (250 ul) of 50% Freund's incomplete adjuvant. After 2 weeks, blood was collected from the ear vein. The blood was allowed to coagulate and the antiserum was collected by centrifugation at 10,000 g for 30 min. The antiserum was split into several small aliquots and stored at -20 °C.

The animal was again inoculated with a further 0.25 mg in 250 µl of enzyme emulsified with equal volume (250 µl) of 50% Freund's incomplete adjuvant. Blood was collected after a further period of 6 to 8 weeks. Anti-PEPC antiserum was collected as described above by centrifugation and stored in multiple aliquots.

Ouchterlony double-dimensional diffusion

The specificity of PEPC antiserum was checked by using 1% (w/v) agarose gels prepared on glass microscope slides. Five wells (0.5-cm diameter) were punched in agarose gels, with the help of a sharp gel-puncher. One well was in the center and was surrounded by four wells located symmetrically in the outer ring. A graph sheet was placed below the glass slide, so as to achieve symmetry and precise distance between wells.

In the center-well, the purified PEPC or leaf extract from *A. hypochondriacus* (or other plants) was loaded. Different dilutions of crude anti-PEPC antiserum (1/10 to 1/100) was loaded into other four wells in the outer ring. The precipitin band was observed within 24 h of incubation at 10-12 °C. The reaction was stopped by washing the gel several times with 0.9% (w/v) NaCl, to remove unbound proteins. Non-immunized serum showed no cross-reaction (i.e., no precipitation) with either purified PEPC

or leaf extracts of *A. hypochondriacus*. The cross reactivity of PEPC from *A. hypochondriacus* with other species was examined by loading purified enzyme/crude extracts of various C_3, C_3-C_4 and C_4 dicot and C_4 monocot species in the outer wells and center well was filled with anti-PEPC antiserum.

Immunoprecipitation of PEPC in leaf extracts

The efficacy of anti-PEPC antiserum was checked by performing immunoprecipitation, as described by Vidal et al. (1983).

The leaves of *Amaranthus hypochondriacus* were extracted as described above for PEPC assay. The leaf extracts were cleared by centrifugation for 5 min at 15,000 g and the supernatants were assayed for PEPC. Extract containing 0.2 Units of PEPC-activity (approximately 300 ul) was mixed with different volumes of anti-PEPC antiserum (0 to 100 ul) solution and left overnight at 4 °C. The mixture was later centrifuged for 5 min at 15,000 g. The supernatant was assayed for PEPC activity, while the pellet was checked for PEPC-protein by SDS-PAGE. The control sets were run with the prc-immune serum from the same rabbits.

The pellet was washed twice with 0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl and 1% Triton X-100, and once with 0.1 M Tris-HCl. pH 8.0. The pellet was finally suspended in SDS-PAGE sample buffer [50 mM Tris-HCl, pH 8.8, 8% (w/v) SDS. 50% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.04% (w/v) bromophenol blue] and boiled at 100 °C for 2 min and loaded onto 10% SDS-PAGE (Laemmli. 1970).

Single radial immunodiffusion

10 ml of 1% (w/v) agarose was prepared in 10 mM phosphate buffered saline, pH 7.5 by boiling. When the solution was cooled, 50 ul of the anti-PEPC antiserum was added. This agarose-gel mixture was used for polymerisation on glass microscope slides. Five circular wells, each of 0.5-cm in diameter,

were punched in a row on the 1% (w/v) agarose gel containing anti-PEPC antiserum. Purified PEPC (1 to 5 μg), crude extracts *of A. hypochondriacus,* of different volumes (15, 30, 40 μg of protein) were added to the wells. The slides were incubated overnight at 4 °C for 24 h. After the precipitin ring was formed the gels were washed several times with 0.9% (w/v) NaCl, to remove any unbound proteins.

The amount of PEPC in crude extracts of *A. hypochondriacus,* can be quantified by measuring the diameters of outer ring of immunoprecipitate and correcting these values with the diameter of inner well. The amount of PEPC in leaf extracts can be easily extrapolated from the standard graph obtained by plotting the diameter of immunoprecipitate of purified PEPC (known antigen) in each well.

Western Blotting

Cross reactivity of PEPC between C_3, C_3-C_4 and C_4 plants was evaluated through Western blots (Betz and Dietz, 1991), after transferring electrophoretically the proteins from the gel on to the polyvinylidene difluoride (PVDF) membranes (Towbin et al., 1979).

Leaf extracts were prepared and subjected to SDS-PAGE, as described above. The proteins were transferred on to PVDF membranes (Immobilon-PC, from Millipore, procured from Sigma Chemical Co., USA). The gel, PVDF membranes and chromatography papers were soaked in transfer buffer containing 25 mM Tris-HCl/192 mM glycine, pH 8.3 and 20% (v/v) methanol for 30 min. The gel and membranes were sandwiched between the filter papers (three on each side) saturated with the buffer and blotted using a semi-dry blotter (LKB 2117 Multiphor) for 3 h. A constant power of 90 volts was supplied (through Atto Digi-Power SJ-1081).

The transfer of proteins was confirmed by Ponceau's staining [0.2% (w/v) Ponceau's stain and 3% (w/v) TCA]. Ponceau's stain was removed by

repeated washing with distilled water. The membranes were blocked with 5% non-fat milk powder in Tris-buffered saline (TBS) containing 25 mM Tris-HC1, pH 7.5 and 150 mM NaCl. Blocking was necessary to saturate the non-specific binding sites. The blocking was allowed for 1 h at room temperature with constant shaking.

The blocked membranes were probed for 1 h with antiserum of PEPC from *A. hypochondriacus* or *Zea mays,* diluted 1:500 and 1:400 respectively, in blocking solution. The blotted membranes were washed three times (15 min each wash) with TBS and incubated with anti-IgG-alkaline phosphatase conjugate (1:7500) for one hour and washed for three times. The washed blot was developed with 33 ul 5-bromo-4-chloro-3-indolylphosphate (BCIP) (50 mg ml^{-1} stock solution) and 60 ul of *p*-nitro blue tetrazolium chloride (NBT) (50 mg ml^{-1} stock solution) in 10 **ml of** 16 mM Tris-HCl (pH 9.5), **4** mM NaCl and 0.2 mM $MgCl_2$.

Two types of blots were made for comparison: with equal amounts of either specific activity or equal amounts of PEPC-protcin (in leaf extracts of different plants) on SDS gels. Similarly, blots were developed by probing with antiserum against PEPC of either *A. hypochondriacus* or *Zea mays.*

Enzyme Linked Immunosorbent Assay (ELISA)

Attempts were made to estimate PEPC-protein in crude extracts of *P. sativum* and *A. hypochondriacus,* by the technique of ELISA, as per the procedure of Gillina and Greally (1993).

Leaf extracts were prepared from leaves of *P. sativum* or *A. hypochondriacus*, as described above. The leaf extract (antigen) was diluted (µg or ng level) in coating buffer (0.05 M carbonate buffer. pH 9.6). The ELISA plates (96 flat bottomed wells, Tarsons Products) were coated with 100 µl of the above leaf extracts in coating buffer in each well (in triplicates) and were left overnight at 4 °C. Triplicate wells were left uncoated to be later

used as enzyme and substrate blanks. The unbound antigen from each well, after overnight incubation, was discarded. The plates were washed four times (250 u1 per well per wash) with TBS. Then 200 ul of blocking solution (containing 5% non-fat milk powder in TBS) was added and then left for 1 h at room temperature. Later the wells were washed 5 times with TBS and incubated with 100 u1 containing 1: 3000 of primary antibody in coating buffer (antiserum against PEPC from *A. hypochondriacus*) in each well for 2 h. The wells were washed again 5 to 6 times and incubated for 1 h in secondary antibody conjugated to alkaline phosphatase (1: 7500 in 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 0.1% BSA). The wells were thoroughly washed (5 or 6 times) with 200 ul of TBS containing Tween-20 and developed by adding 150 ul *p*-nitrophenylphosphate to each well (1 mg/ml stock in coating buffer containing 5 mM $MgCl_2$). The plate was left at 37 °C until the color development and the reaction was stopped with 50 ul 3 M NaOH.

The color was read at 405 nm using an ELISA-Plate Reader (Molecular Devices, USA, Model UVT-06050).

Light Activation of PEPC in leaves/leaf discs

Leaves were excised from the plant, with petiole cut under water. Leaf discs of *ca.* 0.2 cm^2 were punched under water (with a sharp paper-punch). One leaf was kept in each of 100 ml conical flasks containing 100 ml water. Alternatively, 20 leaf discs *{ca.* 80 mg) were floated on 10 ml of water in a 5-cm diameter Petri dishes. After pre-darkening for 2 h. the leaves/leaf discs were illuminated (white light; Philips Comptalux R95 flood bulbs) at an intensity of 1000 uE m^{-2} s^{-1} (after passing through a water filter of 10-cm thickness). The 10-cm thick water filter helped to dissipate the heat and to maintain an optimal temperature, during illumination.

Interaction of PEPC with PEG (or other solutes) during** assay **and/or extraction

The effect of PEG or other compatible solutes on PEPC was checked by incubating with 2 $\mu g\ ml^{-1}$ of purified PEPC (unless otherwise specified) with different concentrations of six types of solutes: PEG-6000, PEG-8000, PEG-20,000, ethylene glycol, glycerol or sorbitol.

The stock solutions (50%, w/v) of these solutes were prepared in distilled water. The purified enzyme or the extract was left in 1 ml of the assay medium (± solutes) for 30 s and reaction was started by the addition of 50 μl of 50 mM PEP. The reaction was linear up to 5 min with purified enzyme. To ascertain the response of PEG in relation to PEPC concentration in the medium, 1 to 5 μg of PEPC- protein ml^{-1} was used in 1 ml of the assay medium..

In a further extension of these experiments, leaf extracts were prepared without or with PEG. For this 20 leaf discs were extracted with extraction buffer in presence of different concentrations (0-10%, w/v) of PEG-6000. Thus, PEPC activity was examined by including PEG-6000 during extraction alone, assay alone or during both extraction and assay.

*Effect **of NH_4Cl and other in** organic salts **on** PEPC* **in vitro**

Activity of PEPC was examined in presence of several test salts, for e.g. acetate, chloride or sulphate salts of ammonium, K^+, Rb^+, Na^+, Li^+, and Ca^{2+}. These salts were included during the assay of PEPC. The concentration of these salts, in the standard assay medium (already described), ranged from 0-500 uM. The reaction was started by addition of 2.5 mM of PEP.

*Phosphorylation **of PEPC and** assay of PEPC-protein kinase*

Phosphorylation of PEPC in leaves or leaf extracts was studied both indirectly and directly.

When PEPC in leaf extracts is incubated with 1 mM ATP and 5 mM $MgCl_2$, the activity of PEPC increases while the inhibition of enzyme by malate decreases, both due to the phosphorylation of PEPC. Thus, the increase in PEPC activity/decrease in malate sensitivity after incubation with Mg-ATP reflects indirectly the phosphorylation of PEPC and can conveniently be monitored by assaying the PEPC activity, before and after incubating the leaf extracts with Mg-ATP.

On the other hand, PEPC can be phosphorylated directly by using ^{32}Pi. Such phosphorylation can be achieved by feeding the leaves *in vivo* with ^{32}Pi; or incubating the leaf extracts *in vitro* with $AT^{32}P$. In both these techniques, the extent of radioactive label in the PEPC-protein is determined after extraction, separation of proteins on SDS-PAGE and autoradiography.

ATP-dependent activation of PEPC in leaf extracts

The leaves were excised, with petiole cut under water, left in 100 ml beaker containing water and were stabilized for 30 min under light. The leaves were fed with 2 mM EGTA or water (control) (through the petiole) and left in darkness for 2 h followed by illumination (1000 $\mu E\ m^{-2}s$) for 30 min. The illuminated leaves were extracted for assaying PEPC activity before and after incubation with ATP and $MgCl_2$.

The crude extracts were incubated with 1 mM ATP and 5 mM $MgCl_2$ for 60 min. Control sets contained $MgCl_2$ (without ATP). Aliquots of incubated extracts were assayed for PEPC before and after the 60 min incubation. The stimulation of PEPC activity on incubation with ATP ± 1 mM represents the phosphorylation of PEPC and activity of PEPC-PK. The malate sensitivity of PEPC was also examined by including 1 mM malate during assay. The experiments were performed at both sub-optimal (pH 7.3) and optimal pH (pH 7.8).

The response to Ca^{2+} of PEPC and PEPC-PK activity was examined by including $CaCl_2$ (0 to 500 |iM) during either assay of PEPC or preincubation of leaf extracts with ATP.

In vivo *Labelling of PEPC with* ^{32}Pi·

Labelling of PEPC with ^{32}Pi *in vivo* was done by following procedure described by Bakrim et al. (1992).

Excised leaves were fed through petiole with 100 µl (60 µCi) of $KH_2{}^{32}PO_4$ (Specific activity of 10 mCi/ mmol) under moderate illumination (400 $\mu E\ m^{-2}\ s^{-1}$) for 1 to 2 h. The leaves were left in darkness for 2 h to ensure that PEPC is mostly dephosphorylated. A set of leaves were exposed to light (1000 $\mu E\ m^{-2}$ s') for 30 min, after the 2 h pre-darkening period, while the others were left in darkness for further period of 30 min.

The light or dark-adapted leaves (about 1 g fresh weight) were extracted in 5 ml of extraction medium described above. An aliquot was examined for PEPC activity. Another aliquot of leaf extract was subjected to immunoprecipitation by incubation with 100 µl anti-PEPC antiserum overnight at 4 °C. The precipitation was collected by centrifugation and the immunoprecipitates were dissolved in sample buffer containing 50 mM Tris-HC1, pH 8.8, 8% (w/v) SDS, 50% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.04% (w/v) bromophenol blue and was boiled for 2 min at 100 °C. The solublized proteins were separated by SDS-PAGE and autoradiographed. The details of autoradiography are described below.

In vitro *Phosphorylation of PEPC with* $AT^{32}P$

Phosphorylation of PEPC *in vitro* was performed for 45 min at 30 °C according to Jiao and Chollet (1992).

Assays (60 µl) were made with a reaction mixture containing 0.1 M Tris-HCl (pH 7.5), 20% (v/v) glycerol, 35 µl of leaf extract (60 µg of protein), 10 mM $MgCl_2$, 4 mM $MgCl_2$, 4 mM phosphocreatine, 10 µl creatine

phosphokinase, 0.25 mM P^1 P^5-di (adenosine-5')-pentaphosphate, 0.5 M DTT, 10 mM NaF and 20 µCi γ-$AT^{32}P$ (Specific activity of 3000 Ci/mmol). The mixtures were incubated at 30°C for 45 min. The reaction was stopped by addition of 20 µl of SDS sample buffer containing 0.25 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.04% (w/v) bromophenol blue. The mixture was boiled for 2 min at 100 °C.

Various concentrations of EGTA, Ca^{2+} or metabolite inhibitors were included in the reaction mixture, as required for the experiments.

SDS-PAGE and Autoradiography

Proteins in solubilized extracts were separated by SDS-PAGE, as already described in the previous pages. The gels were subjected to autoradiography to assess the incorporation of ^{2}Pi label (from ^{32}Pi or $AT^{32}P$) into PEPC-protein.

Gels were stained with Coomassie brilliant blue R-250 and destained thoroughly by using destaining solution. These gels were dried under vacuum. The X-ray film was cut to the gel-size and was placed on top of the gel. The gel and X-ray film were placed between two intensifiers, inside an X-ray cassette. The gel position was marked by cutting the corner of the X-ray film. The cassette was left in the deep freezer (-80 °C). After 20 to 25 days *(in vivo* labelling with ^{32}Pi) or 4 to 5 days (*in vitro* incorporation of $AT^{32}P$) the X-ray film was developed using X-ray film-developer and fixer. The developed and fixed X-ray film was washed thoroughly and was allowed to dry.

Pretreatment of leaves with EGTA or $CaCl_2$ or other metabolic inhibitors

Leaves were cut with petiole under water and were stabilised for 1/2 h under light (400 µE m^{-2} s'"). They were then fed through petiole with EGTA or $CaCl_2$ or test solutions and used for phosphorylation studies (both *in vivo* or *in vitro).*

The test solutions of diltiazem, lanthanum and TFP were prepared in distilled water, while verapamil was dissolved in ethyl alcohol.

Chemicals/Materials

BCIP, Coomassie brilliant blue R-250, Dialysis tubing, Diltiazem, DTT, EGTA, G-6-P (barium salt), L-Malic acid (sodium salt), MDH (from porcine heart, cytoplasmic), NBT, *p*-nitrophenylphosphate, PEG-8000, PEG-20,000, PEP (mono cyclohexylammonium salt), PMSF, Purified PEPC from maize, PVDF membranes, Secondary antibodies (goat anti-rabbit antibodies conjugated to alkaline phosphatase), DEAE-Sepharose CL-6B, Tris and Verapamil were procured from Sigma Chemical Company., U.S.A. Molecular weight markers (29-205 kD) were from Bio-rad, USA and Sephadex G-200 from Pharmacia, Sweden. 96-well ELISA plates (Flat bottom wells) were from Tarsons Products, Bombay. TFP was a gift from SKF. Bombay.

γ[AT^{32}P] and [^{32}P]-labelled KH_2PO_4 were procured from Board of Radiation and Isotope Technology, Bombay. Indu X-ray film (QX16) from Hindustan photofilms Mfg. Co. Ltd. X-ray Developer and Fixer from Allied photographers India Limited.

All other chemicals were of analytical grade from either Sisco Research Laboratories E-Merck (India) Spectrochem, Loba Chemie, Himedia Laboratories all from, Bombay or Ranbaxy Laboratories from New Delhi, India.

Chapter 4

Purification, Storage and lmmunological Properties of PEPC

Chapter 4

Purification, Storage and Immunological Properties of PEPC

Purification

PEPC from *Amaranthus hypochondriacus* was purified to homogeneity using the steps of 40-60% ammonium sulfate fractionation, followed by DEAE-Sepharose column, HAP chromatography and finally through a Seralose 6-B column (Table 4.1). The enzyme eluted as a broad peak with a specific activity of 15.2 U mg^{-1} protein on DEAE-Sepharose. Later the enzyme, when loaded on a HAP column, appeared as a single peak with an activity of 52 U mg^{-1} protein. The purified enzyme (after elution from Seralose 6-B column) had a specific activity of 54 U mg^{-} protein. This is one of the highest specific activities reported for PEPC from C_4 plants. Further, the yield also was as high as 50%. Our method of purification of PEPC therefore appears to be one of the best.

The activity of the enzyme varies highly depending on the type of extraction, pH, ionic strength and salt used for elution. All the conventional purification methods include ammonium sulfate or PEG fractionation, ion exchange chromatography in which elution is performed with step wise or linear gradient of a salt, most often chloride or phosphate. Purification protocols are available for PEPC from leaves of several species like maize (Uedan and Sugiyama, 1976; Reibach and Benedict. 1977; Hague and Sims, 1980; O'Leary et al., 1981; Hatch and Heldt, 1985), cotton (Mukerji, 1977), *Amaranthus viridis* (Iglesias et al., 1986), sugarcane (Iglesias and Andreo, 1989), latex of *Hevea brasiliensis* (Jacob et al., 1979) and seeds of *Brassica campestris* (Mehta et al., 1995).

Phosphate is known to be stabilizer of purified PEPC from leaves of *Amaranthus viridis* (Podesta et al., 1990). Phosphate has also been used for elution of PEPC from soybean root nodule (Schuller and Werner, 1993) and

Table 4.1. *Purification of PEPC from leaves of* Amaranthus hypochondriacus

Step	Total Activity	Total Protein	Specific Activity	Purification	Yield
	(μmol min^{-1})	*(mg)*	*(μmol min^{-1} mg protein)*	*fold*	*(%)*
Crude extract	164	410	0.4	-	100
40-60% $(NH_4)_2SO_4$	152	117	1.3	3	93
DEAE-Sepharose	136	90	15.2	38	83
Hydroxyl-aptatite	88	17	52.0	130	54
Seralose 6-B	81	1.5	54.0	135	49

from leaves of maize (Reibach and Benedict, 1977; Hague and Sims, 1980). Apart from chloride and phosphate, acetate has been used to elute PEPC from genus of *Spartina* species (Smith and Woolhouse, 1984). Iglesias et al. (1986) reported that PEPC from *A. viridis* also eluted around 50-60 mM Pi. However, in sugarcane PEPC eluted at 120 mM Pi (Iglesias and Andreo, 1989). In our experiments, PEPC from leaves of *Amaranthus hypochondriacus* was eluted from HAP column, at a Pi concentration of 50-60 mM (Fig. 3.2).

PEPC can be purified, also by using affinity chromatography or HPLC or FPLC (Cretin et al., 1984; Jiao and Chollet, 1988, 1989; McNaughton et al., 1989; Jiao et al., 1991; Arrio-Dupont et al., 1992; Wang and Chollet, 1993; Zhang et al., 1995). Rapid purification of PEPC by immunoaffinity chromatography has been successful with leaves of sorghum (Vidal et al.. 1980; Bakrim et al., 1992; Arrio-Dupont et al., 1992).

A review of the literature, revealed that the specific activities of PEPC varied from low values of 4-10 U mg^{-1} protein (Coombs et al., 1973; Hayakawa et al., 1981; O'Leary et al., 1981; Sugiyama et al., 1984; Wedding and Black, 1986) to as high values as 180-220 U mg^{-1} protein (Mukerji, 1977; Reibach and Benedict, 1977). It is concluded however that a reasonable range of average specific activity of purified PEPC is about 20-25 U mg" protein (Uedan and Sugiyama, 1976; Hatch and Heldt, 1985: Iglesias et al., 1986; Wedding et al., 1988; McNaughton et al., 1989; Bandarian et al., 1992; Baur et al., 1992; Arrio-Dupont et al.,1992; Wang and Chollet. 1993). With specific activity of 54 U mg^{-1} protein and high yield of 50%, we feel that our method of PEPC-purification from leaves of *A. hypochondriacus* is quite good.

The enzyme PEPC appeared as a single band on 10% SDS-PAGE with a MW of about 100 kD protein (Plate 4.1). The enzyme appeared as two distinct bands on non-denaturing gel electrophoresis and both these bands stained for PEPC activity (Plate 4.2). Two-dimensional electrophoresis was therefore performed to check the nature of two bands. Both these bands merged as a

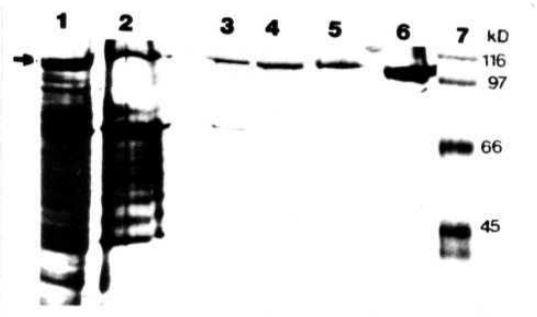

Plate 4.1. Silver stained gels after SDS-PAGE of PEPC at different steps of purification from the leaves of *Amaranthus hypochondriacus*. Lane 1: Crude extract, Lane 2: 40-60% saturated ammonium sulfate fraction, Lane 3: DEAE-Sepharose pool, Lane 4: Hydroxylapatite pool, Lane 5: Seralose 6-B pool, Lane 6: Purified PEPC, after concentration with PEG-20,000 and storage in presence of 50% (v/v) glycerol. Lane 7. Molecular weight markers (29-116 kD). Lanes 1 and 2 contained 10 µg protein, while lanes 3 to 6 contained 4 µg of protein.

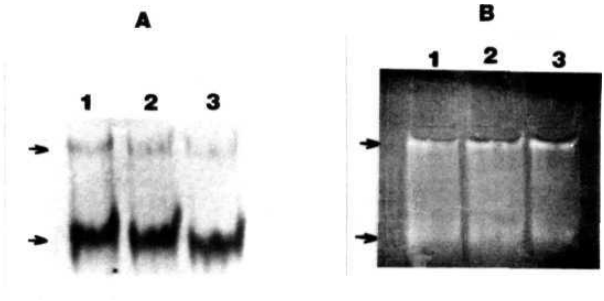

Plate 4.2. Electrophoresis of purified PEPC on a nondenaturing 10% polyacrylamide gel. (A) Gels stained with Coomassie brilliant blue R-250. (B) Gels stained for PEPC activity, showing the presence of two isoforms. 20 μg of purified PEPC was loaded in each of the lanes 1 to 3.

broad single band on 10% SDS-PAGE (Plate 4.3), indicating that two bands are identical subunits of the holoenzyme. We suggest that these two bands may be isozymes of homotetramer, since dimer or monomer are not expected to stain for PEPC activity. Further studies on amino acid sequence and corresponding mRNA patterns may be necessary to confirm the identity of these two bands on the native gel.

The first demonstration of the existence of three PEPC isozymes in cotton leaf tissue was by Mukerji and Ting (1971). Several reports appeared later indicating the existence of multiple forms of PEPC in the leaf tissue of higher plants. For example, Hatch et al. (1972) have separated three distinct types of PEPC proteins (two from C_4 plant species and one from C_3 plant species) by PAGE. Ting and Osmond (1973a) have observed two PEPC proteins (C_3 and C_4 types) by ion-exchange chromatography from *Zea mays*, while Goatly and Smith (1974) have demonstrated existence of two isozymes in etiolated and light-grown sugarcane leaves. Mukerji (1977) reported the presence of two isoforms in maize leaves. Besides higher plant-PEPCs, two isozymes of PEPC were reported in an unicellular green alga, *Chlamydomonas reinhardtii* (Chen and Jones, 1970).

However, there have also been conflicting reports on the presence of PEPC isozymes. In maize leaves, Uedan and Sugiyama (1976) have observed a single band of 100 kD protein after SDS-tube gel electrophoresis. But Hague and Sims (1980) have reported two bands of PEPC. again from maize leaves, on highly resolving SDS-PAGE slab gels. Budde and Chollet (1986) have reported that a doublet protein band is observed at 94 to 100 kD protein which may be PPDK and PEPC, and these polypeptides get merged on the gel, particularly at higher loads (>30 |ig of total protein).

In our experiments, a single band of PEPC was observed when the SDS gels were loaded with either low (>10 μg) or high (>30 ug) amounts of pure PEPC-protein. On the other hand, two bands of PEPC appeared on the native

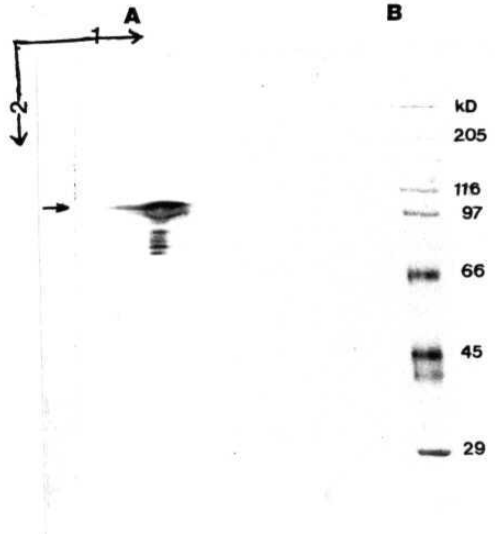

Plate 4.3. Two dimensional electrophoresis of purified PEPC. In the first dimension, purified PEPC was resolved on nondenaturing gel. A segment of this gel was resolved on SDS-PAGE in second dimension. Gel A: Purified PEPC (10 μg). Gel B: SDS-PAGE of molecular weight markers (29-205 kD). Further details are described in "Materials and Methods".

gel electrophoresis and both these bands stained for PEPC activity (Plate 4.2). The possibility of contamination with PPDK can be ruled out.

Ting and Osmond (1973a), while purifying PEPC from maize, speculated that one of the PEPC-isozymes might have originated from mesophyll and other from adjacent bundle sheath cells. However, there is now overwhelming evidence that PEPC is present exclusively in mesophyll cells of C_4 plants (Perrot-Rechenmann et al., 1982). We therefore suggest that these two bands of isozymes are present only in mesophyll cells. However, the function of these two isozymes of PEPC is not known, though they may have an anaplerotic function as suggested by a few workers (Coombs et al., 1973; Wong and Davies, 1973).

A detailed examination of the kinetics of our enzyme - PEPC purified from *Amaranthus hypochondriacus* leaves revealed a V_{max} of 54 U mg^{-1} protein and a K_m for PEP of 0.4 mM, at pH 7.3 (Table 4.2). The enzyme was activated by G-6-P with K_A of 0.3 mM and inhibited by malate with a K_i of 0.5 mM. These values of our enzyme are similar to the kinetic parameters of light-form of PEPC reported in the literature.

There is considerable variation in the kinetic characteristics of PEPC from the leaves of C_3- and C_4 plants (O'Leary, 1982; Andreo et al.. 1987; Rajagopalan et al., 1994). The V_{max} of PEPC was 20 to 40 fold higher and the K_m values for PEP were about 5 fold higher for the enzyme from C_4 plants than those of the enzyme from C_3 plants (Ting and Osmond, 1973a). PEPC in C_3 plants differ from that in C_4 species with respect to also the regulatory properties, as indicated by the response to metabolites such as malate or G-6-P. K_m values for PEP of PEPC from algae, bacteria and C_3 and CAM species varied from 0.1-1.6 mM (Utter and Kolenbrander, 1972).

The kinetic characteristics of PEPC are dependent markedly on pH. In C_4 plants, the PEPC exhibits sigmodial kinetics at pH 7.0 and hyperbolic kinetics at higher pH 8.0 (Nakamoto et al., 1983). Maize enzyme shows several

Table 4.2. *Kinetic characteristics of PEPC purified from leaves of* Amaranthus hypochondriacus

Parameter	Value
V_{max} (μmol min^{-} mg^{-} protein)	54 ±1.0
K_m PEP (mM)	0.4 ± 0.02
K_i malate (mM)	0.5 ±0.01
K_A G-6-P (mM)	0.3 ±0.06

fold higher K_m at pH 7.0 (with sigmoidal kinetics) than that at pH 8.0 (with hyperbolic kinetics). However, hyperbolic kinetics have also been reported for PEPC from several plants such as, cotton (Mukerji and Ting, 1971), C_3 and C_4 Atriplex species (Ting and Osmond, 1973a), *Pennisetum purpureum* (Coombs et al., 1973, 1974, 1975), *Digitaria sanguinalis* (Huber and Edwards, 1975) and *Bryophyllum fedtschenkoi* (Pays et al., 1980).

The K_m value for PEP of purified PEPC from maize was 2.6 mM (Uedan and Sugiyama, 1976). Iglesias et al. (1986) and Iglesias and Andreo (1989) reported a K_m value of 0.29 mM for PEP in *Amaranthus viridis* and 0.25 mM for PEP in sugarcane leaves. The K_m for PEP of purified PEPC from tobacco leaf was 95 μM (Wang and Chollet, 1993) and from germinating cotyledons of *Ricinus communis* was 62 μM (Podesta and Plaxton. 1994b). In case of PEPC purified from developing seeds of *Brassica* ***campestris,*** the K_m for PEP was 0.125 mM (Mehta et al., 1995).

O'Leary (1982) observed that a wide range of K_i values for malate were obtained for PEPC from various C_4 plants (0.04-10 mM) or CAM plants (0.006-6.2 mM). K_i of malate was 2.5 mM for PEPC from Amaranthus (Iglesias et al., 1986), 0.14 mM in purified PEPC from tobacco (Wang and Chollet, 1993), 1.25 mM in developing seeds of *Brassica campestris* (Mehta et al., 1995). Light-form PEPC in maize leaves had I_{50} for malate of 0.35 mM (Jiao and Chollet, 1988) and PEPC purified from soybean nodule had a I_{50} for malate of 0.5 mM at pH 7.0 (Schuller and Werner, 1993). The K_A for G-6-P of 0.6 mM was reported for PEPC from maize leaves (Uedan and Sugiyama, 1976) and 0.28 mM for PEPC from developing seeds *of Brassica campestris* (Mehta et al., 1995).

Storage

There is a vast amount of literature available on purification and the kinetic properties of C_4-PEPC, as described in three recent exhaustive reviews

(Lepiniec et al., 1994; Rajagopalan et al., 1994; Toh et al., 1994). However, the properties of PEPC can vary depending on the assay pH, presence or absence of glycerol and storage conditions of enzyme. We have therefore studied the kinetic properties of the enzyme under different conditions of storage at different temperatures and presence or absence of glycerol.

PEPC is highly unstable and loses its N-terminal, due to proteolysis. The loss of N-terminal from PEPC during storage is a common problem, but can be detected easily by the extent of malate sensitivity of enzyme. The extent of malate inhibition of PEPC decreased as the enzyme lost its N-terminal and became smaller in its molecular mass (McNaughton et al., 1989). Proteolysis of PEPC occurs, particularly in absence of protease-inhibitors like PMSF or chymostatin during purification. According to McNaughton et al. (1989), addition of chymostatin is essential to prevent proteolysis of PEPC and to retain the high malate sensitivity of the enzyme. However, the external pH may have an additional role, since the effects of chymostatin are evident at pH 7.0, but not at pH 8.0 (Salahas et al., 1994). Chymostatin may affect also the oligomeric status of the enzyme (either directly or indirectly) and favors the equilibrium of enzyme towards tetrameric state (Salahas et al., 1994). We have used the protease inhibitor such as PMSF, so as to avoid proteolysis and to maintain the stability of the enzyme during extraction and purification.

Apart from the need of protease inhibitors for stability, there are several reports in literature that instability of the enzyme can be prevented by inclusion of glycerol under *in* ***vitro*** conditions (Uedan and Sugiyama, 1976; Manetas et al., 1986; Selinioti et al., 1987; Podesta and Andreo, 1989). Therefore we have examined the stability of the purified enzyme by storing in the absence or presence of glycerol and under different conditions of temperatures (room temperature, 4 °C, -20 °C, liquid nitrogen). The properties of purified PEPC

were examined after storage for either 24 h or a very long period of up to 3 months.

Table 4.3 shows the effect of temperature on the activity of purified PEPC after storage for 24 h. At ambient room temperature, in the absence of glycerol, the enzyme lost almost completely its activity within 24 h. The enzyme retained only about 16% and 33% of its initial activity at 4 °C and -20 °C, respectively, while in liquid N_2 the enzyme retained 50% of activity: all these in the absence of glycerol and after 24 h of storage. However, on the addition of glycerol, the enzyme retained only 27% of its initial activity at room temperature, and maintained > 77% of the initial activity at 4 °C, -20 °C and in liquid N_2. During the extended period of study for 3 months, the enzyme retained its activity only when stored at liquid N_2, in presence of 50% (v/v) glycerol (Fig. 4.1). The enzyme showed a marked decrease in activity by 2 months at cither -20 °C or 4 °C. The decrease in activity was associated with a decrease in malate sensitivity.

The kinetic characteristics of purified PEPC were studied after a 24 h-storage. The V_{max} of the enzyme was maintained only at liquid nitrogen in presence of 50% (v/v) glycerol. The affinity of PEPC for PEP was not much affected, when stored in liquid nitrogen, while the affinity decreased markedly at even -20 °C (Table 4.4). K_A for G-6-P increased at 4 °C and -20 °C in comparison to PEPC stored in liquid N_2. The high specific activity of the enzyme, malate sensitivity, response to G-6-P, and affinity towards PEP are maintained (close to the values of freshly purified enzyme) only in presence of glycerol. The malate sensitivity and the specific activity of the enzyme were quite stable when stored in liquid nitrogen with 50% (v/v) glycerol for 3-4 months.

Mukerji (1977) observed marked inactivation of purified PEPC from corn, when PEPC was left at 45 °C or 50 °C. In *Cynodon dactylon*, the

Table 4.3. *The stability of purified PEPC in leaf extracts as indicated by the activity of the preparation, after storage for 24 h.*

Storage temperature	Glycerol in the suspension medium	
	0 (control)	50% (v/v)
	PEPC activity ($\mu mol\ min^{-1}\ mg^{-1}$ protein)	
Before storage	53 ±0.8	54 ±0.1
After storage for 24 h		
Ambient (30 °C)	1.3 ±1.0	15 ±0.2
4°C	8.3 ±4.0	42 ± 0.8
-20 °C	16.0 ±2.3	49 ±1.3
Liquid nitrogen	27.0 ±1.2	54 ± 1.7

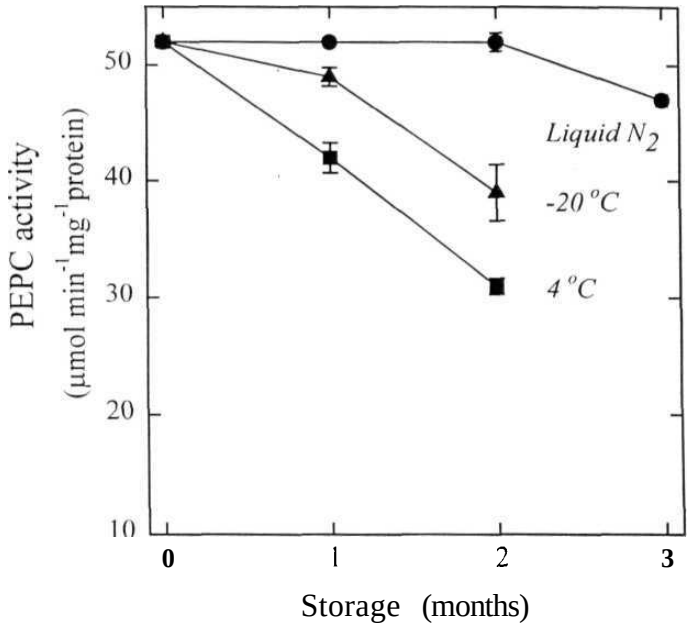

Figure 4.1. The **stability** of PEPC **purified** from leaves **of** *Amaranthus hypochondriacus*. The enzyme was stored in **either in** liquid nitrogen, or **freezer** (-20 °C), or a refrigerator (4 °C) **in** presence of 50% (v/v) glycerol. The **enzyme** activity was maintained fully for 2 months and later there was **a** marginal loss of **activity by** 3 months, when stored at liquid N_2. **PEPC** activity was assayed at pH 7.3 with 2.5 mM PEP.

Table 4.4. *Kinetic characteristics of purified PEPC after storage (with 50% (v/v) glycerol) for 24 h at different temperatures*

Storage temperature	Parameter			
	V	K_m PEP	K_i Malate	K_A G-6-P
	($\mu mol\ min^{-1}\ mg$ protein)	(mM)	(mM)	(mM)
Before storage	54 ± 0.2	0.44	0.5	0.3
After storage for 24 h				
4 °C	41 ±2.0	0.27	7.0	2.3
-20 °C	45 ± 3.2	0.24	2.5	1.8
Liquid nitrogen	54 ±1.0	0.40	0.5	0.3

desalted enzyme shows considerably a high activity only in presence of glycerol at 25 °C, compared to decline of activity in assays without glycerol (Drilias et al., 1994). Mehta et al. (1995) have shown that PEPC purified from developing seeds of *B. campestris* loses 60% of activity on incubation at 45 °C for 5 min in absence of glycerol. Glycerol has been added to stabilize the PEPC enzyme during storage (Manetas, 1982; Karabourniotis et al., 1983). The addition of a solute such as glycerol during storage helps to stabilize several enzymes including PEPC (Selinioti et al., 1987; Drilias et al., 1994), PPDK (Shirahashi et al., 1978) and cytosolic pyruvate kinase (Podesta and Plaxton, 1993). Glycerol appears to act as a cryo-protectant in maintaining the stability of PPDK, a chloroplastic enzyme of C_4 pathway (Shirahashi et al., 1978). The dissociation of PPDK from an active tetramcric form to a dimer at low temperature is prevented by glycerol. sucrose or sorbitol. On the other hand. the exact mechanism as how glycerol can stabilize the PEPC during storage, still remains to be elucidated.

Since the purified enzyme is unstable, it is essential to store the purified enzyme under proper conditions. so as to maintain not only the maximum activity, but also high malate sensitivity. Several authors attempted PEPC-storage in different ways. The enzyme is usually stored with 5 to 50% (v/v) glycerol at either 4 °C (Vidal et al.. 1980; Jiao and Chollet. 1988; Arrio-Dupont et al.,1992), or -15 °C (Nimmo et al., 1986), or -20 °C. (Coombs et al., 1973; Hatch and Heldt, 1985; Jiao and Chollet, 1988; Jawali. 1990; Willeford et al., 1990; Baur et al., 1992; Zhang et al., 1995). or -70 °C or liquid N_2 (Coombs et al., 1973; Iglesias et al.. 1986; Iglesias and Andreo. 1989; Bandarian et al., 1992; Schuller and Werner, 1993). Addition of glycerol, during storage of maize PEPC, helped to maintain N-terminal end and retention of the enzyme's malate sensitivity (McNaughton et al., 1989).

There are also reports of storing PEPC without glycerol. Mukerji (1977) has stored PEPC purified from corn leaves at -2 °C for several months. Jones et al. (1978) stored PEPC purified from *B. fedtschenkoi* as suspension (2mg/ml) at 4 °C. Sorghum PEPC purified through an immunoadsorbent column was stored directly at 4 °C in absence of glycerol for 15 days without loss of activity (Bakrim et al., 1992). PEPC from developing seeds of *B. campestris* was stored for one week at 4 °C (Mehta et al., 1995).

Thus, there is lot of variation in published reports describing the storage of PEPC from different sources. Our observations, while confirming the good stability of enzyme in presence of glycerol, provide a comprehensive information on the storage of PEPC at four different temperatures and for a long period of 3 months. The enzyme therefore appears to be best stored in presence of 50% (v/v) glycerol in liquid N_2. Addition of 5-10 mM malate or G-6-P has also been recommended (Nimmo et al., 1986; Willcford et al., 1990; Zhang et al., 1995). Protease inhibitors like PMSF or chymostatin have been included during storage by McNaughton et al.(1991) and Arrio-Dupont et al. (1992).

We conclude that purified PEPC can be easily stored, along with 50% (v/v) glycerol, in liquid N_2 at least for 3 months. In our hands, addition of malate or PMSF was not necessary.

Immunological Characteristics

In contrast to extensive literature on purification and storage of PEPC. the studies on immunological properties of PEPC are quite limited. An attempt has therefore been made to study comprehensively the immunological properties of PEPC purified from *A. hypochondriacus*, a NAD-ME type C_4-dicot plant and assessed its cross-reactivity with a few other C_3- and C_4-type dicots and C_4-monocots.

Antibodies were raised in rabbits against PEPC purified from the leaves of *Amaranthus hypochondriacus*. The antibody showed a titre value of 1/100 against purified PEPC from leaves of *A. hypochondriacus* (Plate 4.4 A) as well as PEPC in leaf extracts *of A. hypochondriacus* or *Alternanthera pungens*(Plate 4.4 B & C).

The monospecificity of the PEPC-antiserum was checked initially by immunoprecipitation. The leaf extracts were incubated with anti-PEPC antiserum and incubated overnight to precipitate down the PEPC-protein. There was a marked decrease in PEPC activity on treatment with anti-PEPC antiserum, presumably due to the precipitation of PEPC from the supernatants (Fig. 4.2). In controls, when leaf extracts were treated with non-immunised rabbit serum, there was no change in PEPC activity in the supernants, and obviously PEPC was not precipitated from the supernatants. The examination of protein precipitate on 10% SDS-PAGE confirmed the presence of PEPC in the precipitate. The levels of PEPC-protein in the precipitate increased as the amount of PEPC antiserum increased (Plate 4.5). This indicates the antisera prepared was fairly specific to PEPC protein even in crude leaf extracts. Sugiyama et al. (1984) have shown the specificity of the antiserum prepared against purified maize PEPC by immunotitration with crude extracts of maize PEPC.

The technique of single radial immunodiffusion was used to assess the quantitative relation between the amounts of PEPC-protein in crude leaf extracts and anti-PEPC antiserum. When purified PEPC was used for calibration, there was a linear relationship between the diameter of immunoprecipitate and the quantity of antigen-protein (PEPC) in the agarose-wells (Fig. 4.3 A & B). A similarly linear relationship was observed between the diameter of the immunoprecipitate-ring and leaf extracts of *A. hypochondriacus* containing 15, 30 or 45 µg of protein. Based on these

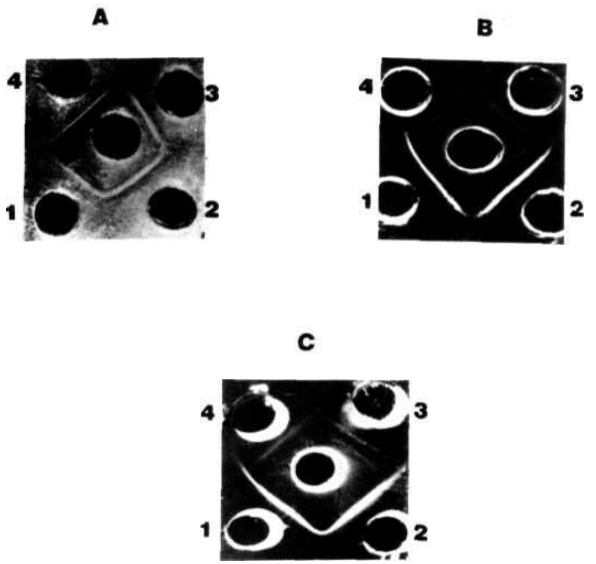

Plate 4.4. Ouchterlony double-diffusion to determine the titer value of anti-PEPC antiserum. Outer wells 1 to 4 contained the dilutions of anti-PEPC antiserum in the order of 1/10, 1/20, 1/50, and 1/100. The center well contained purified PEPC from *Amaranthus hypochondriacus.* (A) or leaf extracts of A. *hypochondriacus* (B) or *Alternanthera pungens* (C).

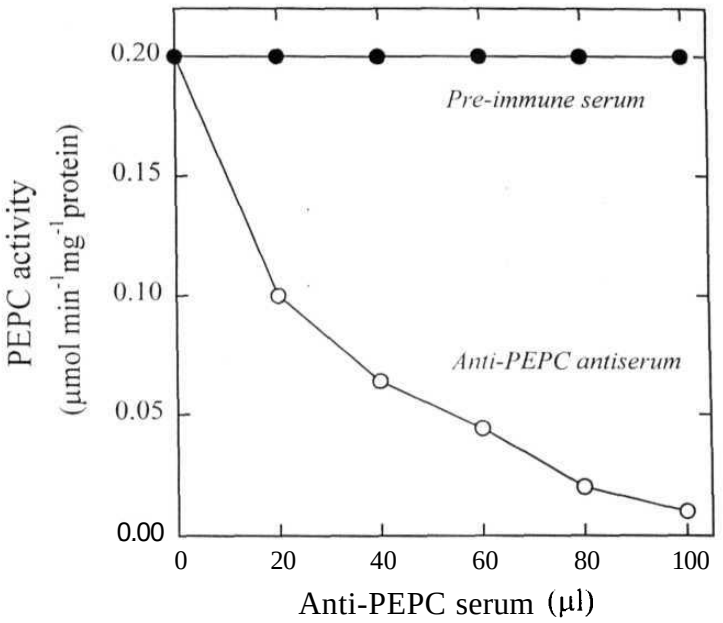

Figure 4.2. Immunoprecipitation of PEPC extracted from illuminated leaves. To a fixed volume of leaf extract, containing 0.2 units of PEPC, variable **volumes of anti-PEPC** antiserum or preimmune scrum (0-100 μl) were added and the mixtures were left overnight at 4 °C. After centrifugation, the **supernatant** was checked for **PEPC** activity. The pellet was washed and examined by **SDS-PAGE** for the presence **of PEPC** (see Plate 4.5). The control samples were treated **with** non-immunised serum. further details are as in 'Materials and Methods".

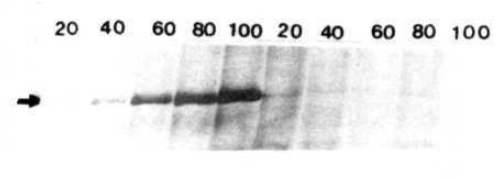

Plate 4.5. PEPC from leaf extracts of *Amaranthus hypochondriacus* was precipitated by addition of 0-100 µl of anti-PEPC antiserum and were subjected to 10% SDS-PAGE as described in "Materials and Methods". PEPC bands were observed only on precipitation with anti-PEPC antiserum. In controls (pre-immune serum) no PEPC bands was observed indicating that precipitation of the enzyme did not occur.

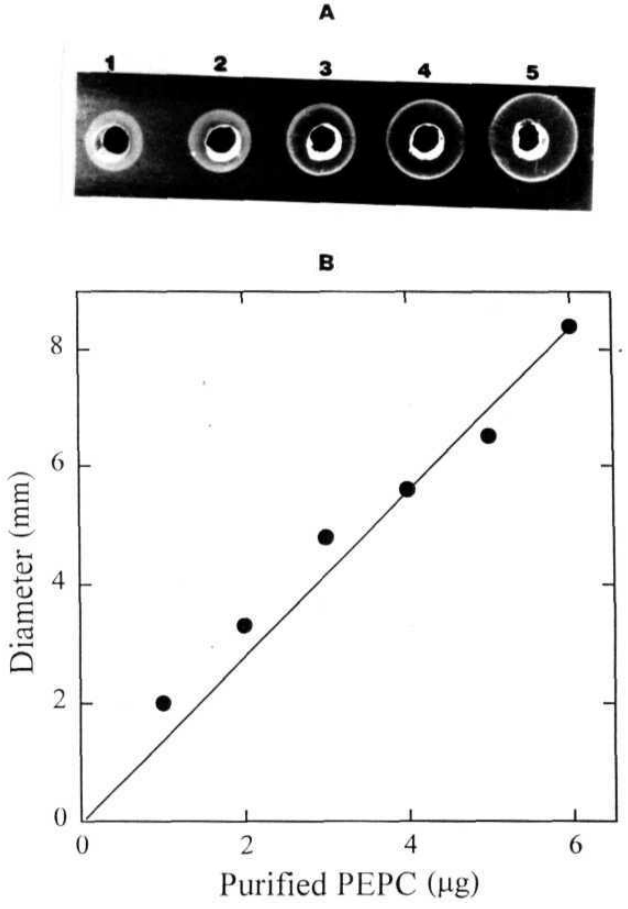

Figure 4.3. (A) Single radial immunodiffusion **of** PEPC. The wells contained 1 to 5 µg purified **PEPC-protein** (from *Amaranthus hypochondriacus*), in increasing order, from left to right. **The** diameter of the immunoprecipitin ring increased along **with** increase in PEPC concentration in the well. (B) The **quantitative** relation between the amount of purified PEPC in the agarose well **and** diameter of immunoprecipitate. Further details are described in "Materials and Methods".

measurements, we could estimate that the leaf extracts of *A. hypochondriacus* contained 1.2 µg of PEPC per mg of total protein (Plate 4.6).

Sugiyama et al. (1984) used single radial immunodiffusion to quantify the levels of PEPC protein in leaf extracts of maize, grown with different concentrations of nitrate. Huber and Sugiyama (1986) also have attempted to determine, by single radial immunodiffusion the amount of PEPC protein from illuminated or dark-adapted leaves of maize. They found only a marginal increase in PEPC-protein on illumination.

The cross reactivity of PEPC from *Amaranthus hypochondriacus*, a C_4 dicot, with PEPC in leaf extracts of C_3 or C_4 mono- and dicots was examined by Ouchterlony double-diffusion. When pre-immune serum was used and there was no immunoprccipitation with any of the plant species (Plate 4.7 A). The anti-PEPC antiserum, raised against PEPC of *Amaranthus hypochondriacus*, showed very strong reaction (as indicated by the precipitin-band) with *Amaranthus viridis* and *Alternanthera pungens* (C_4 dicots). On the other hand the same anti-PEPC antiserum, showed only a faint reaction with PEPC from *Zea mays*, a C_4 monocot (Plate 4.7 B). This suggests that PEPC from *Amaranthus hypochondriacus* has only limited identity with the tertiary structure of PEPC from either *Zea mays* (C_4-monocot) or C_3-dicot or C_3-C_4 intermediates. Iglesias et al. (1986) observed through the Ouchterlony technique, that the PEPC of *Zea mays* exhibited only limited cross reaction and thereby partial identity with anti-PEPC antiserum raised against PEPC purified from *A. viridis*. The distinctness of C_4 dicot-PEPC from that of C_4 monocot species was further confirmed by Western blot analysis.

Although, Ouchterlony double diffusion did not show much cross-reactivity of PEPC from *Amaranthus hypochondriacus*, a C_4 dicot with C_3 plants or C_3-C_4 intermediates (Plate 4.7 C & D), it could be also because of the limited sensitivity of Ouchterlony technique. Since the immune-reaction can be amplified with Western blot, we checked the cross-reactivity with C_3 and C_3-C_4

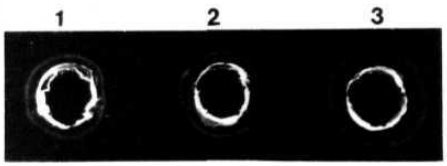

Plate 4.6. Single radial immunodiffusion to assess cross-reactivity of anti-PEPC antiserum with PEPC in leaf extracts of *Amaranthus hvpochondriacus.* The wells were loaded with different concentrations of protein (15, 30, 45 μg protein), from left to right.

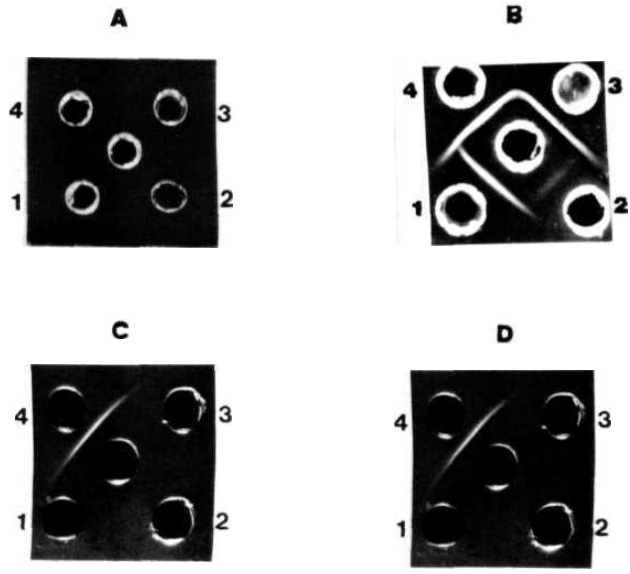

Plate 4.7. Ouchterlony double-diffusion, to assess the cross-reactivity of anti-PEPC antiserum (against PEPC of *Amaranthus hypochondriacus)* with PEPC in leaf extracts of C4 dicots or C4 monocots or C3 dicots or C3-C4 intermediates. The center wells contained either preimmune serum (A) or anti-PEPC antiserum of purified PEPC from *A. hypochondriacus* (B to D). The outer wells 1 to 3 contained in the given order. A: leaf extracts of C4 dicots - *A.* hypochondriacus, Amaranthus viridis, and Alternanthera pungens. B: Leaf extract of *A . pungens,* purified enzyme of *Zea mays* (C4 monocot), and leaf extract of *A. viridis.* C: Leaf extracts of C3 plants - *Pisum sativum*, *Alternanthera sessiles,* and *Commelina benghalensis.* D : Leaf extracts of C3-C4 intermediates - *Alternanthera tenella, Alternanthera ficiodes*, *Parthenium hysterophorus.* Outer well No 4 contained purified PEPC from *A. hypochondriacus* for an easy comparison.

species with that of C_4 dicot and C_4 monocot plants using anti-PEPC antiserum. The extent of homology of PEPC was examined (by Western blots), by using leaf extracts equal in terms of either protein or specific activity of PEPC. When probed with the anti-PEPC antiserum against PEPC of *A. hypochondriacus*, very good cross-reactivity was obtained (i.e.,intensely stained blots) with PEPC in leaf extracts of three C_4 dicot plants, *Amaranthus hypochondriacus, Amaranthus viridis* and *Alternanthera pungens* (Plate 4.8 A). In case of these C_4-dicots, the leaf extracts containing 40 µg were to be loaded on the gel for visual appearance of blot. The PEPC in the leaf extracts of three monocots *{Zea mays, Sorghum bicolor,* and *Pennisetum)* could also be visualized by Western blot using the anti-PEPC antiserum against C_4-dicot PEPC (Plate 4.8 B). However the protein to be loaded on the gel in case of C_4-monocots (60 µg of protein) was more than that (40 ug protein) needed in case of C_4 dicots (Plate 4.8 A & B). Such need for higher levels of C_4 monocot protein than that of C_4 dicot protein for similar intensity on Western blots, suggests that the PEPC of C_4-dicot is immunologically similar yet distinct from that of C_4 monocot.

In another set of experiments, leaf extracts of C_3 and C_3-C_4 were loaded in such way so as to have equal enzyme units, i.e.. specific activity. One blot was probed with anti-PEPC antiserum of PEPC from *A. hypochondriacus* and other blot was probed with anti-PEPC antiserum raised against commercially available PEPC from *Zea mays* (Sigma Chemical Co., USA). Since PEPC activity was more in C_4 dicot and monocot than with PEPC from C_3 and C3-C4 intermediates, the protein needed was about 60 ug for C_3 species (*Pisum sativwn, Commelina benghalensis, Alternanthera sessiles*) as seen in Plate 4.9 (A & B), C_3-C_4 intermediates *(Parthenium hysterophorus, Alternanthera ficoides, Alternanthera tenella)* (Plate 4.10 A & B) compared to only (3 (ig) equal to 0.01 U mg^{-1} protein needed for C_4 monocot *{Zea mays)* or C_4-dicots *{Amaranthus hypochondriacus, Alternanthera pungens, Amaranthus viridis)* (Plate 4.11 A & B). With these combinations, PEPC in C_3-species or C_3-C_4

Plate 4.8. Western blots of PEPC in leaf extracts of C4 dicots or C4-monocots. A: C4-dicots - Lane 1: purified PEPC from *Amaranthus hypochondriacus*, Lane 2: Leaf extract of *A. hypochondriacus*, Lane 3: *Amaranthus viridis*, and Lane 4: *Alternanthera pungens*. B: Monocots - Lane 1: Purified PEPC from *Zea mays*, Lane 2: *Sorghum bicolor*, Lane 3: *Pennisetum glaucum*. The amount of protein loaded on to the gel was either 10 µg (purified PEPC from *A. hypochondriacus* or 40 (ig (leaf extracts of C4-dicots) or 60 µg (leaf extracts of C4-monocots).

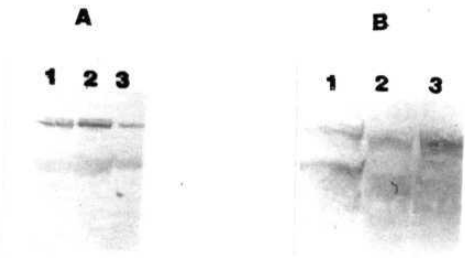

Plate 4.9. Western blots of PEPC in leaf extracts of a C3 dicot and two C3 monocots. The leaf extracts were assayed for PEPC activity and about 60 µg of protein (equal to 0.01 U of specific activity) were loaded in each lane for SDS-PAGE and for later developing the immunoblots. A: Immunoblot probed with anti-PEPC antiserum from *Amaranthus hypochondriacus*. Lane 1: *Pisum sativum*, Lane 2: *Commelina benghalensis*, Lane 3: *Alternanthera sessiles*. B: Immunoblot probed with anti-PEPC antiserum from *Zea mays*. Lane 1: *P. sativum*, Lane 2: *C. benghalensis*, Lane 3: *A. sessiles*.

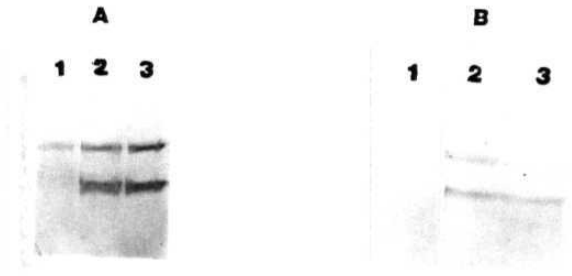

Plate 4.10. Western blots of PEPC in leaf extracts of three C3-C4 intermediate. The leaf extracts were assayed for PEPC activity and about 60 μg of protein (equal to 0.01 U of specific activity) were loaded in each lane for developing immunoblots. A: Immunoblot probed with anti-PEPC antiserum from *Amaranthus hypochondriacus*. Lane 1: *Parthenium hysterophorus*, Lane 2: *Alternanthera ficoides.* Lane 3: *Alternanthera tenella.* B: Immunoblot probed with anti-PEPC antiserum from *Zea mays.* Lane 1: *P. hysterophorus*, Lane 2: *A. ficoides*^ Lane 3: *A. tenella.*

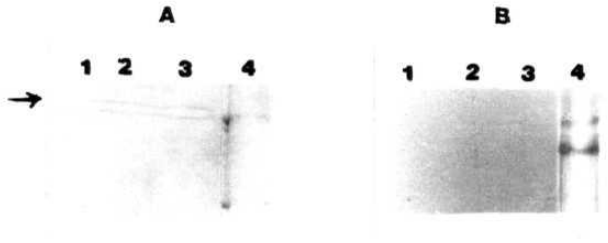

Plate **4.11.** Western blots of PEPC in leaf extracts of three C4 dicots and a C4 monocot. The leaf extracts were assayed for PEPC activity and about 3 μg of protein (equal to 0.01 U of specific activity) loaded in each lane for developing immunoblots. A: Immunoblot probed with anti-PEPC antiserum from *Amaranthus hypochondriacus*, Lane 1: *A. hypochondriacus*, Lane 2: *Amaranthus viridis*, Lane 3: *Alternanthera pungens*, Lane 4: *Zea mays*. B: Immunoblot probed with anti-PEPC antiserum from *Zea mays*. Lane 1: *A. hypochondriacus*, Lane 2: *A. viridis*, Lane 3: *A. pungens*, Lane 4: *Z. mays*.

intermediates could be visualized by anti-PEPC antiserum of *A. hypochondriacus*, but not of maize (Plates 4.9 & 4.10). The blot with leaf extracts of C_4 dicot species showed up when probed with only with antiserum against PEPC of *A. hypochondriacus*, but not of maize (Plate 4.11 A & B). The antiserum against maize PEPC could react and show up only the maize enzyme on the gels, but not of C_4 dicots.

These observations suggest that the antisera of *Amaranthus hypochondriacus*-PEPC recognized PEPC of different species in the following order: C_4 dicot > C_3-C_4 intermediate > C_3 dicot > C_4 monocot. On the other hand, antiserum of maize PEPC recognized C_4 dicot-PEPC is closer to C_3/CAM isoforms than to C_4 monocot as suggested by Lepiniec et al. (1993).

We noticed that at least two bands of PEPC appear on Western blots with leaf extracts of C_3 and C_3-C_4 intermediate species indicating **the** existence of different isoforms (94 kD to 100 kD) of PEPC. In C_4 species, only a single band was obtained on 10% SDS-PAGE gels or immunoblots, particularly when loaded with 30 or more ug of protein. The pattern of double-bands (with leaf extracts C_3 or C_3-C_4 intermediates) after immunoblotting remained unaltered even in presence of protease inhibitor like PMSF during extraction. We can therefore eliminate the possibility of proteolysis during extraction.

The appearance of two or three PEPC bands on Western blots indicating the presence of isoforms in C_3 have been noticed in extracts from C_3 plants (Hofner et al., 1989) and CAM plants (Weigend and Hincha. 1992; Slocombe et al., 1993). Weigend and Hincha (1992) have observed only one PEPC band with C_4 and CAM species while two bands appeared on immunoblots of extracts from C_3 plants using antiserum against maize PEPC. Schulz et al. (1992) has shown the presence of one PEPC band in mesophyll cell protoplasts, while two PEPC bands were detected in mesophyll tissue preparations, and three in guard cell protoplasts and epidermal tissue of *Vicia faba*. Western blot analysis of stomatal enzyme from *Vicia faba* showed two bands on cross

reacting with antibodies against *K. daigremontiana* (Denecke et al., 1993). Slocombe et al. (1993) has also shown two or three bands appearing in C_3 performing *M. crystallinum* using antisera directed against PEPC from CAM-performing *K. daigremontiana.* Sangwan et al. (1992) and Podesta and Plaxton (1994a) have also observed the presence of two PEPC bands in germinating cotyledons of *Ricinus communis* on probing with antiserum against maize leaf PEPC.

ELISA is useful to detect and quantitate the antigen-protein in given sample and can be employed in diagnostic kits. The standardization of ELISA technique with PEPC, has the potential of developing diagnostic kits for PEPC in leaf extracts of particularly C_4 species. The amounts of PEPC-protein in crude extracts of *Amaranthus hypochondriacus* and in *Pisum sativum*, a C_3 plant could be quantitated by ELISA. There was a linear **relationship** between the amount of color developed after ELISA and the amount of PEPC-protein used in the sample. However, the slopes of these linear curves, representing the avidity of the enzyme for the antibody, were different for the two forms of enzymes from pea or amaranth. The color development after ELISA was linear in the range of 0-100 ng ml" PEPC-protein in leaf extracts of *A. hypochondriacus* (Fig. 4.4), while the linearity was with a higher range of 0 to 35 $\mu g\ ml^{-1}$ PEPC-protein in case of leaf extracts from *Pisum sativum* (Fig. 4.5). If high concentrations of PEPC were used with leaf extracts of *A. hypochondriacus.* the color developed after ELISA was too strong to maintain linearity. In contrast, low levels (ng) of PEPC-protein from leaf extracts of *Pisum sativum* could not be detected as the color **was** faint and could not be read on the ELISA reader.

We conclude that ELISA could be used for quantitative detection of PEPC in leaf extracts, after proper standardization. The antiserum against PEPC of *A. hypochondriacus* could be used for both *A. hypochondriacus* and *P. sativum,* but the range of detection varied by markedly by an order of

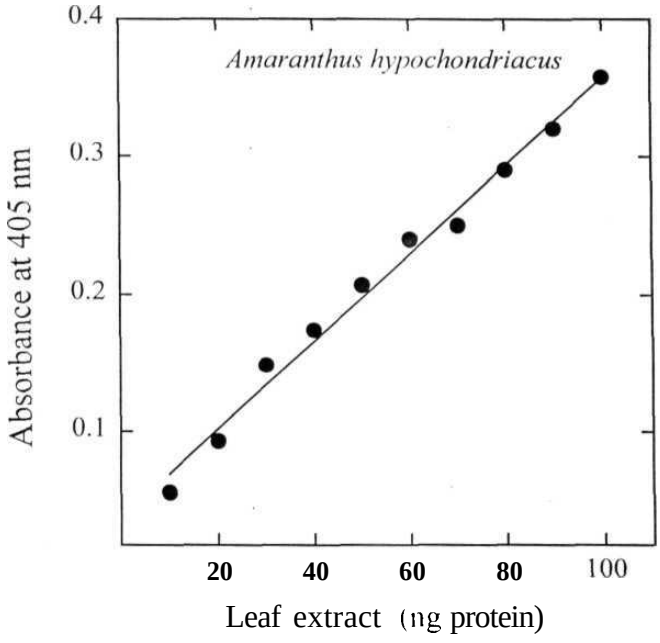

Figure 4.4. Application of ELISA **for detection** and determination **of PEPC** in leaf extracts of *Amaranthus hypochondriacus*. The specific activity **of PEPC** in leaf extracts of *A. hypochondriacus* was 0.19 U mg^{-1} protein. **The** wells of an HLISA plate were loaded with varying dilutions of leaf extracts, containing ng protein, as indicated. After HLISA, the color, based on assay with alkaline phosphatase was read at 405 nm. The absorbance at 405 nm was linear in the range of 20-100 µg protein in the wells. Further details are described in "Materials and Methods".

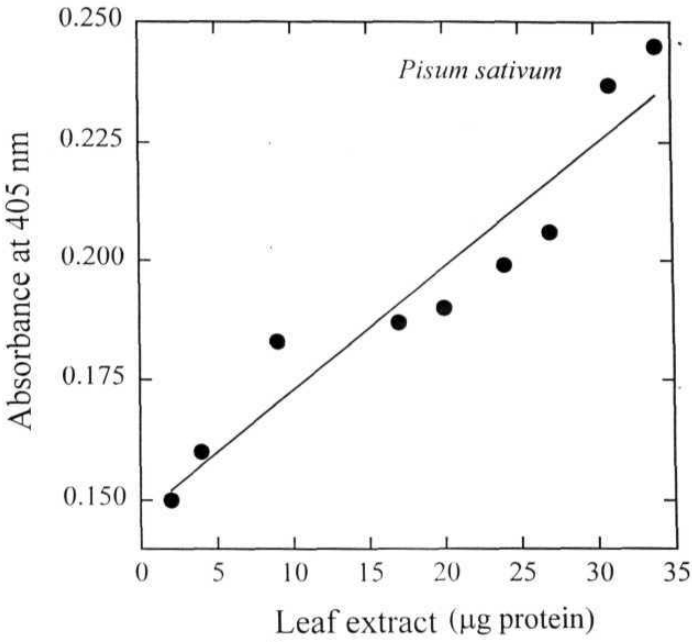

Figure 4.5. Use of ELISA to detect and determine **PEPC** in extracts of pea ***(Pisum sativum)*** leaves. The specific activity of **PEPC** in leaf extracts of *P. sativum* was 0.01 U mg^{-1} protein. The linearity **of** developed color was in the range of 5-35 µg of protein. The figure may be compared with Fig. 4.4. Further details are described in "Materials and Methods".

magnitude. ELISA can be used for detecting and determining PEPC at ng level in case of *Amaranthus hypochondriacus*, but could detect PEPC in extracts of *Pisum sativum* only at much higher levels of µg range. ELISA further confirms our data from Western blot analysis that there are both similarities and differences are present between the PEPC of C_3 and C_4 type of dicotyledonous plants, reflected immunological reaction of anti-PEPC antiserum.

There are very few reports of use of ELISA with PEPC or other enzymes of C_4 pathway. ELISA was used to evaluate the immunological difference between the NADP-malic enzymes extracted from C_3 and CAM type of plants (Saitou et al., 1994). ELISA has been used by Schnabl et al. (1993) to identify and purify the multiple isoforms of stomatal PEPC after gel clcctrophoresis. Our experiments (Figs. 4.4 and 4.5) demonstrate that ELISA can be used as a diagnostic kit to detect and determine PEPC-protein in C_4 or even C_3 plants.

Major conclusions from the results presented in this chapter are:

1. PEPC was purified from leaves of *A. hypochondriacus* with a very good specific activity of 54 U mg protein. This is one of the highest specific activities of PEPC reported in the literature.
2. Purified PEPC could be stored for at least 3 months in liquid nitrogen, along with 50% (v/v) glycerol, without any significant loss of specific activity.
3. Antibodies were raised in rabbits against purified PEPC from *A. hypochondriacus*. The anti-PEPC antiserum showed a titer value of 1: 100.
4. The monospecifity of PEPC-antiserum was confirmed by immunoprecipitation and the amount of PEPC protein in leaf extracts of *A. hypochondriacus* could be quantified by single radial immunodiffusion.

5. Ouchterlony double diffusion and Western blot analysis showed that the C_4 dicot-PEPC was similar to other C_4-dicots, but was distinct from C_4 monocots or C_3 species or C_3-C_4 intermediates, as indicated by immunological cross-reactivity.
6. An ELISA-technique was standardized to detect and determine PEPC in leaf extracts *of A. hypochondriacus* (a C_4-dicot) and *P. sativum* (a C_3-dicot).

Chapter 5

Interaction of PEPC with PEG and Other Compatible Solutes: Studies with Purified Enzyme and Crude Leaf Extracts

Chapter 5

Interaction of PEPC with PEG and Other Compatible Solutes: Studies with Purified Enzyme and Crude Leaf Extracts

The tetrameric form of PEPC is reported to be the most active form (Walker et al., 1986; Podesta et al., 1990; Wu et al., 1990), while dissociation of the enzyme results in a marked decrease in the catalytic activity of the enzyme (Walker et al., 1986; Wagner et al., 1987; Podesta et al., 1990; Willeford et al., 1990; Wu et al., 1990). The oligomeric status of the enzyme in solution depends on conditions such as pH (Walker et al., 1986), ionic strength (Wagner et al., 1987) or temperature (Wu and Wedding, 1987). *In vitro* experiments have indicated that dilution of the enzyme may be an important factor while inducing dissociation of PEPC into dimer and/or monomer (Angelopoulos and Gavalas, 1988; Wu and Wedding, 1987; Kleczkowski and Edwards, 1990). Thus, the dilution of the enzyme leads to a marked instability of PEPC.

Several authors have noted that the instability of PEPC activity in extraction or assay media could be overcome by addition of glyccrol (Manetas et al., 1986; Uedan and Sugiyama 1976; Manetas, 1982; Karabourniotis et al., 1983; Selinioti et al., 1987). The action of glycerol as a stabilizer of this protein structure and enzyme activity is known (Gekko and Timasheff, 1981). Besides glycerol, other organic solutes (PEG, proline, betaine) also promote self association of proteins and stabilize their structure (Timasheff et al., 1982). Besides PEPC, other cytosolic enzymes, known to be activated by PEG include pyruvate kinase from germinating castor seed endosperm (Podesta and Plaxton, 1993) and fructose-1,6-bisphosphatase from endosperm of germinating castor seeds (Hodgson and Plaxton, 1995). In agreement with above, we also have observed that PEPC is unstable when extracted, primarily due to dilution. Therefore detailed studies were made with purified enzyme and later with crude

extracts to use solutes, such as PEG or glycerol, to keep up the high activity and tetrameric state. Attempts were made to assess the oligomeric status, to see if any changes occur *in vivo.*

Experiments were designed to study in detail the interaction of PEPC, with four different solutes, namely PEG, glycerol, ethylene glycol and sorbitol. Initially, experiments were performed with purified enzyme and the enzyme was incubated with PEG or other solutes *in vitro.* In the next phase, the properties of PEPC were evaluated by including PEG and/or glycerol during both extraction of enzyme from leaves of *Amaranthus hypochondriacus* and subsequent assay of reaction. Inclusion of PEG or other solutes during the assay medium may reflect closely the physiological situation and is likely to be a way of mimicking the intracellular environment *in vivo.* Assays were run at pH 7.3, which seems to be close to microenvironment of PEPC *in vivo,* i.e.. cytosol. Similar approach was made while assaying PEPC at both optimal and sub-optimal pH in earlier reports (Huber and Edwards, 1975; Karabourniotis et al., **1985; Selinioti et** al., 1985).

The first set of experiments were conducted to know the effect of PEG-6000 or 8000 or 20,000 on the purified enzyme during the assay. All the three type of solutes activated the enzyme at both sub-saturating (0.5 mM PEP) and saturating (2.5 mM PEP) substrate concentrations (Fig. 5.1). Among the three types of PEGs, PEG -6000 exerted greater activation than PEG-8000 and 20,000 at both substrate concentrations. The stimulation by PEG-6000 of PEPC was nearly 3.5 fold at 0.5 mM PEP while being 2.5 fold at 2.5 mM PEP. The stimulation of PEPC was nearly 2 fold with PEG-8000 at either 0.5 or at 2.5 mM PEP. The stimulation by PEG-20,000 at 0.5 mM PEP was slightly less than that at 2.5 mM PEP.

The specific activities of PEPC on stimulation by PEG-6000 are shown in Fig. 5.2. The decrease in the effectiveness of PEG-8000 or PEG-20,000,

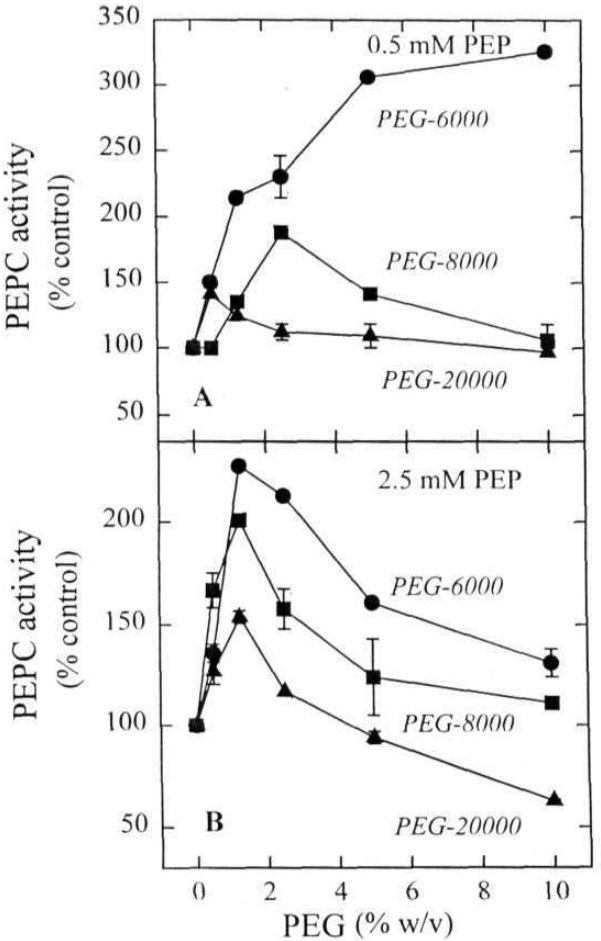

Figure 5.1. Effect of PEG on **the** activity of PEPC purified from leaves of *Amaranthus hypochondriacus.* PEPC was assayed at pH 7.3 with either 0.5 mM (sub-optimal) PHP (A) or 2.5 mM (optimal) PEP (B). The enzyme activity in absence of PEG (control) was 16 ± 0.5 and 46 ± 0.2 μmol min^{-1} mg^{-1} protein, when assayed **with** 0.5 mM and 2.5 mM PEP, respectively. The assay medium (1 ml) contained 2.0 **|ig** of purified PEPC. Data represents average ± SE of data from three independent experiments. Errors not seen are within the symbols. Further details are described in "Materials and Methods". **The** specific activities of PEPC with PEG-6000 arc directly plotted in **Fig.** 5.2.

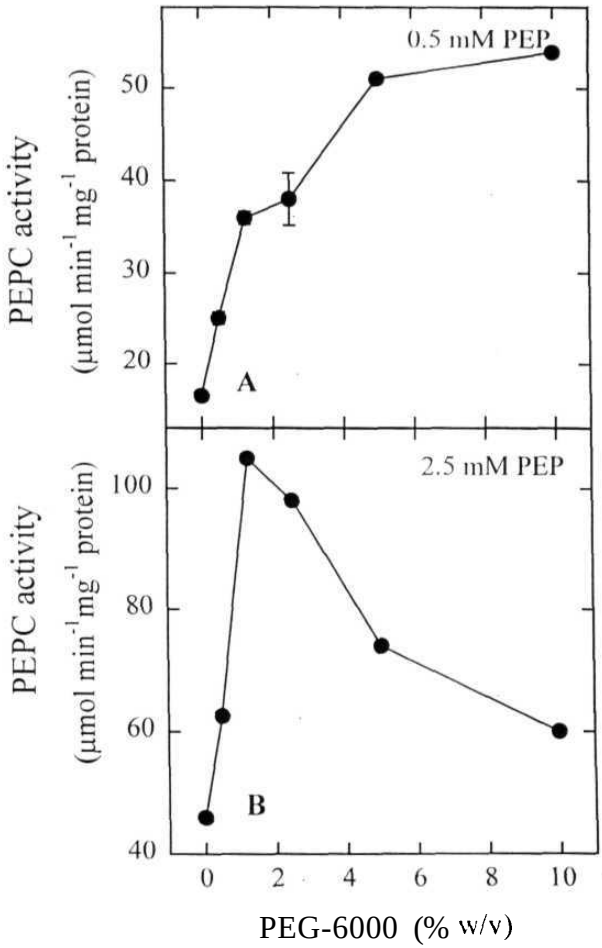

Figure 5.2. Stimulation **of purified PEPC by** PEG-6000, when the enzyme was assayed at 0.5 and 2.5 mM PEP. The activities are directly expressed for an easy comparison. Maximum stimulation was obtained with 1.25% (w/v) PEG at both substrate concentrations. Further details arc as in **Fig.** 5.1.

compared to that of PEG-6000, may be due to the increase in the viscosity of the assay medium with the enlargement of the polymer weight.

At 0.5 mM PEP, the activity of PEPC is stimulated by all three types of PEG even at high concentration as 10% (w/v). At 10% (w/v) PEG, the enzyme gets inhibited at 2.5 mM PEP. The high concentration of PEG-8000 may cause a precipitation of the protein which may result in the apparent decrease in the activity. Similar decrease in the extent of activation of cytosolic pyruvate kinase under high concentration (>10% w/v PEG) has been reported by Podesta and Plaxton (1993).

A concentration of 1.25% (w/v) PEG was optimal for stimulation of PEPC activity, irrespective of PEPC-protein concentration in the medium (Fig. 5.3). Similarly, a protein concentration of 2 ug ml^{-1}, PEPC was optimal for eliciting maximal stimulation by 1.25 % (w/v) PEG-6000 (Fig. 5.4). This indicates PEPC was stimulated at particularly low concentration of PEG and gets inhibited at higher concentrations of PEG. High concentrations of PEG up to 40% (w/v) have been used for precipitation of PEPC (Stamatakis et al., 1988; Angelopoulos and Gavalas, 1991).

The effect of solute on enzyme docs not seem to depend on the osmotic potential as the osmotic potentials of PEG at concentrations capable of PEPC stimulation are very low. Therefore, the activation of the enzyme by PEG can be best interpreted to be due to "exclusion-volume theory" which promotes self-association of enzyme-protein, and mimics the intracellular situation where the enzyme is usually much-concentrated. Stamatakis et al. (1988) have tested low concentrations of different PEGs: 200 to 20,000 (0.005M to 0.28 M), on PEPC from desalted extracts of *Cynodon dactylon* and have shown that stimulation of PEPC is not due to osmotic potential but by self-promotion of homologous protein molecules in solution.

Inclusion of ethylene glycol or glycerol, at a concentration of 1.25% (v/v), during the assay stimulated the activity of PEPC, similar to the

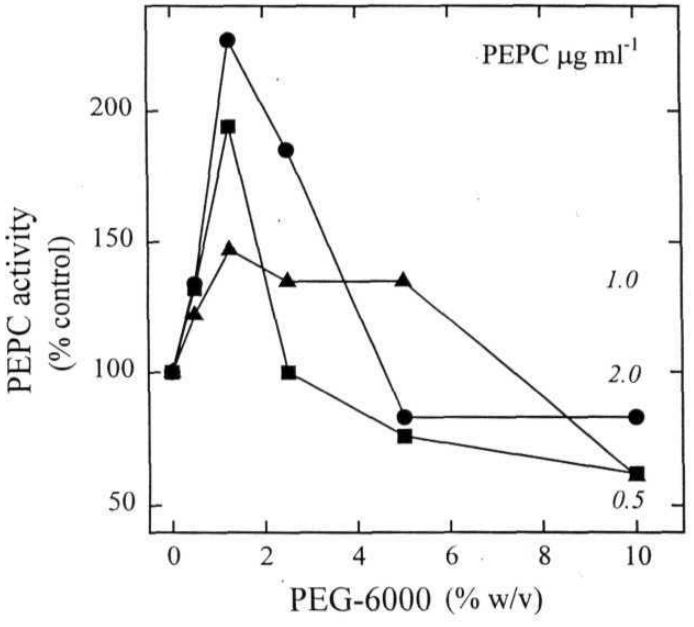

Figure 5.3. The effect of PEG at different PEPC concentration in the assay medium. Maximum stimulation of purified PEPC occurred at 1.25% PEG, when the enzyme was assayed at pH 7.3 with 2.5 mM PEP. The activity of PEPC in the control sets (without PEG) was 46 ± 0.2 μmol min^{-1} mg^{-1} protein.

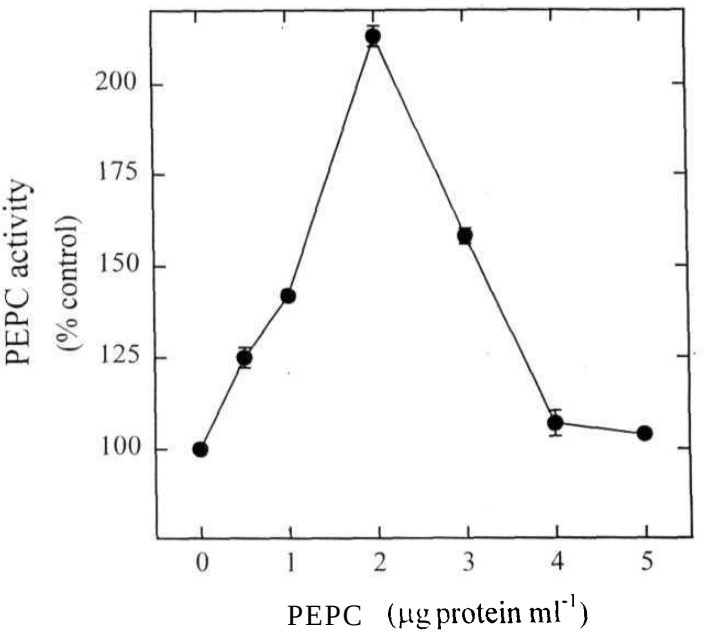

Figure 5.4. Aclivalioii of purified **PEPC** by **1.25%** PEG, as a (unction of PEPC **concentration in the** assay medium. Maximum **stimulation of PEPC** occurred at 2 μg ml"' of purified **PEPC.** PEPC activity in the absence of PEG (control) was 46 ± 0.2 μmol min^{-1} mg^{-1} protein. Further details are as in Fig. 5.1.

effect of like PEG-6000. There was a marked variation in the extent of response to glycerol at 0.5 mM or 2.5 mM PEP. Maximum stimulation of PEPC (2-fold) occurred at 1.25% (v/v) of glycerol when PEPC was assayed at sub-optimal (0.5 mM) PEP, while high concentration of glycerol (>10%, v/v) was needed to exert similar stimulation at optimal (2.5 mM) PEP (Fig. 5.5). A few earlier reports indicated that the addition of glycerol in assay medium increased the enzyme activity when assayed at low PEP levels of 1 mM (Gavalas et al., 1982; Stamatakis et al., 1988), or at suboptimal pH (Uedan and Sugiyama, 1976). Podesta and Andreo (1989) have shown that addition of glycerol (20%, v/v) to assay medium at pH 7.0 and at 1 mM PEP produced a marked increase in enzyme activity, up to 180% of the initial value. Podesta et al. (1995) have recently shown that dark-and light-form of purified PEPC from *A. viridis* shows stimulation by ethylene glycol up to 20% (w/v). However higher concentrations (>25% w/v) of ethylene glycol inhibit the enzyme.

Sorbitol had only a marginal effect of- about 14 fold stimulation - on PEPC activity at optimal (2.5 mM) PEP (Fig. 5.6). However, the effect of sorbitol on PEPC was less than that of other solutes namely PEG, glycerol and ethylene glycol. Manetas (1990) has reported that effect of sorbitol on PEPC is neutral and does not protect the enzyme against NaCl attack.

Our observations confirm that PEPC in solution can be stimulated in presence of even low concentrations of at least six different types of solutes. Drilias et al. (1994) have shown that the activation effect and protection of PEPC activity against temperature was not specific to only glycerol but was effected by other solutes like PEG. PEG and proline are effective in protecting enzyme-proteins at low concentrations (Lee and Lee, 1979). PEG also acts by promoting self association of the enzyme in solution and therefore underlies its use for fractional precipitation of proteins (Miekka and Ingham, 1978).

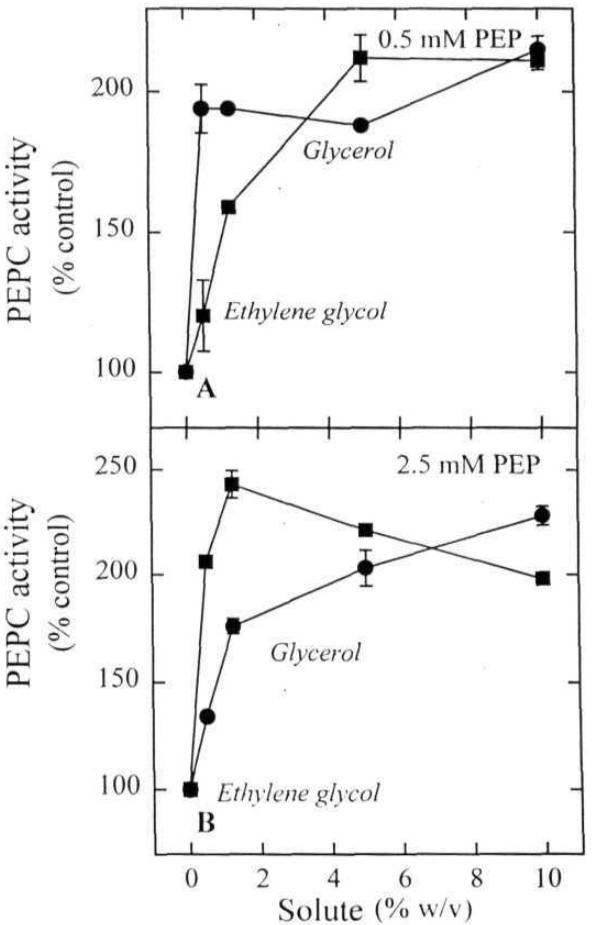

Figure 5.5. Stimulation of PEPC activity by the presence of solutes: glycerol (•) or ethylene glycol (•). The enzyme was assayed at pH 7.3 with either 0.5 mM PEP (A) or 2.5 mM PEP (B). The activities of PEPC in control sets, i.e., in the absence of glycerol or ethylene glycol were 18 ± 0.5 and 46 ± 0.2 μmol min^{-1} mg^{-1} protein, at 0.5 mM and 2.5 mM PEP respectively. Further details are as in Fig. 5.1.

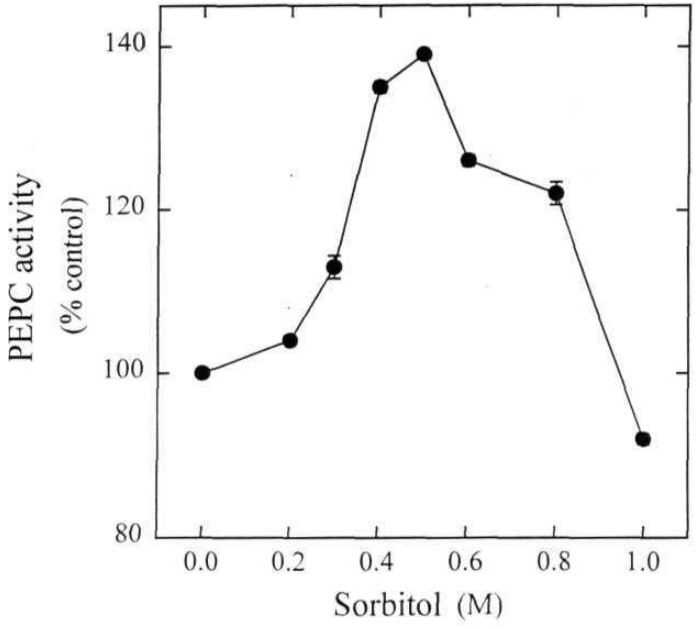

Figure 5.6. Effect of sorbitol. when included in the assay medium, on the activity of purified PEPC from leaves of *Amaranthus hypochondriacus.* PEPC activity was assayed at pH 7.3 with 2.5 mM PEP. Maximum stimulation of PEPC occurred at 0.5 M sorbitol. PEPC activity in absence of sorbitol (control) was 23 ± 0.7 μmol min^{-1} mg^{-1} protein.

As PEG-6000 exerted a much more marked stimulation of PEPC than that the other two forms of PEG (namely 8000 or 20,000), we have used PEG-6000 for further detailed studies with purified enzyme and crude extracts.

The kinetic characteristics of PEPC were evaluated in presence of PEG-6000. In presence of PEG, the affinity for PEP of PEPC decreased, while the sensitivity to malate or G-6-P decreased as indicated by the increase in the values of their K_i and K_A, respectively (Table 5.1). These observations are similar to a earlier reports in the literature. The decrease in the affinity for PEP, in presence of PEG was shown by Stamatakis et al. (1988). Huber and Sugiyama (1986) have shown that PEG relieves L-malate inhibition in both crude extracts and purified enzyme. Podesta and Andreo (1989) have also shown that presence of glycerol can help to **overcome** the inhibition of the enzyme by **L-malate.** We suggest that PEPC becomes less susceptible to L-malate inhibition in presence of PEG, possibly because of the shift of enzyme towards active tetrameric form.

It is a common experience that during the extraction and the assay of PEPC as per the normal procedures, a marked dilution of the enzyme occurs in leaf extracts. Such dilution can affect adversely the quaternary structure of PEPC due to the dissociation of the enzyme (Willeford and Wedding, 1992). These adverse effects can be counteracted either by increasing PEP concentration (Wagner et al., 1987; Willeford and Wedding, 1992) or by addition of solutes (Selinioti et al., 1987; Stamatakis et al., 1988; Podesta and Andreo, 1989). Hence, we extended our studies on interaction of PEPC with PEG and glycerol to crude leaf extracts. This serves two purposes: first to evaluate the effects of PEG on PEPC activity in crude leaf extracts and second, to identify the best way of extraction/assay of enzyme.

We have examined the effect of only PEG-6000 on PEPC in leaf crude extracts, as this compound was the most effective in stimulating the purified enzyme. The activity of PEPC was determined while including PEG-6000

Table 5.1. *Effect of inclusion of PEG, when included during the assay, on* ***the*** *characteristics of PEPC purified from leaves of* Amaranthus hypochondriacus

Parameter	Solute (w/v)	
	None	PEG-6000 1.25%
V_{max} (μmol min^{-} mg^{-} protein)	26 ±3.2	52 ±2.2
K_m PEP (mM)	0.27 ±0.01	0.19 ±0.06
K_i malate (mM)	0.4 ±0.03	0.9 ± 0.17
K_A G-6-P (mM)	0.5 ±0.05	0.7 ± 0.23

during either extraction alone, or only during assay or both during extraction and assay. Presence of PEG-6000 during both extraction and assay resulted in maximum catalytic activity of PEPC (Fig. 5.7). The effect is pronounced at 2.5 mM PEP (>4- fold stimulation compared to the activity in the absence of PEG) compared to that at 0.5 mM PEP (3.5 fold). Maximum stimulation occurred at 2% (w/v) PEG when PEPC was assayed with 0.5 mM PEP, although 5% (w/v) PEG was the most effective with 2.5 mM PEP. Thus, there was a slight variation in the optimal concentrations of PEG-6000 needed for maximal stimulation of PEPC in crude extracts or in the purified form. We have no immediate explanation for this phenomenon. However our observations demonstrate that inclusion of PEG during assay as well as extraction is necessary to maintain maximal **activity** of PEPC when assayed at either 0.5 or 2.5 mM PEP (Fig. 5.7).

The kinetic properties of PEPC changed in response to PEG even in leaf crude extracts. These changes are reflected in the sensitivity of PEPC to PEP or malate or G-6-P (Table 5.2). The K_m for PEP of PEPC decreased from 0.75 (control) to 0.44 (in presence of PEG), K_i for malate decreased from 1.04 (control) to 0.74 (+ PEG). On the other hand, the response to G-6-P, as indicated by K_A G-6-P, increased from 0.25 (control) to 1.17 mM (in presence of PEG).

Podesta and Plaxton (1993) have shown that in presence of PEG, the K_m for PEP and ATP of pyruvate kinase decreased. Hodgson and Plaxton (1995) have recently reported that PEG decreases the K_m for pyruvate kinase of fructose-1,6- bisphosphatase. K_m for PEP of PEPC in desalted extracts of *Cynodon dactylon* was lowered in presence of PEG or glycerol (Stamatakis et al., 1988; Manetas, 1990). We therefore conclude that PEG causes marked conformational changes in PEPC. In case of enzymic proteins, the aggregation state is often a significant factor determining the catalytic efficiency. The presence of solutes may therefore, modify dramatically the kinetic parameters

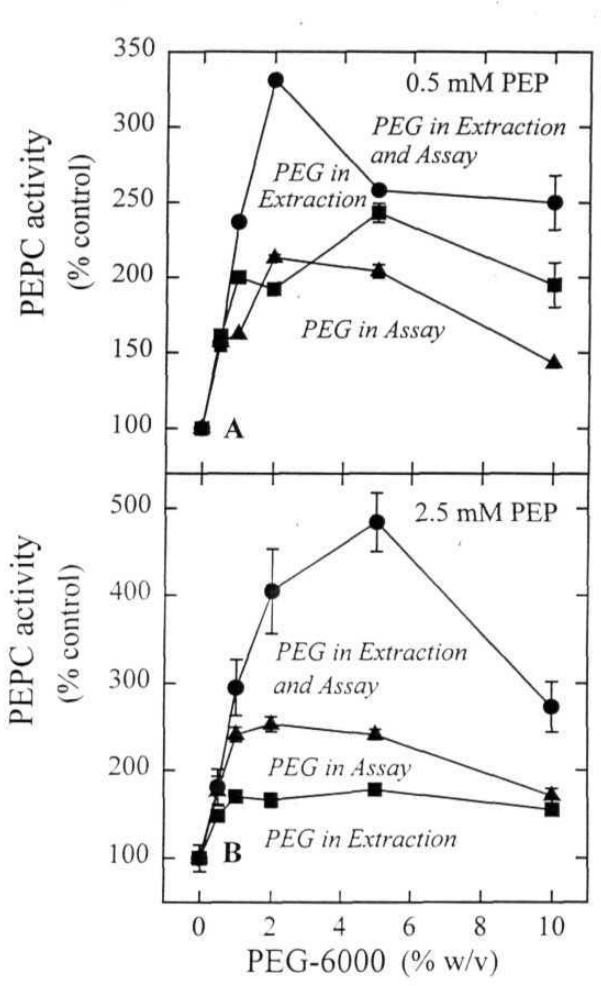

Figure 5.7. Activation by PEG of PEPC in extracts from leaves *of Amaranthus hypochondriacus*, when assayed **with** either 0.5 mM PEP (A) or 2.5 mM PEP (B). The extracts from dark-adapted leaf discs were assayed for PEPC activity at pH 7.3. The activities of PEPC in the absence of PEG (control) were 453 ± 54 and 606 ± 18 $\mu mol\ h^{-1}\ mg^{-1}$ chl when assayed at 0.5 mM PEP and 2.5 mM PEP, respectively.

Table 5.2. *Effect of 1%(w/v)* ***PEG*** *and/or 20% (v/v) glycerol during extraction and assay on the characteristics of PEPC in leaf extracts of* Amaranthus hypochondriacus

Parameter	Extraction medium		
	No PEG. no glycerol	+ Glycerol	Glycerol + PEG
V_{max} (μmol h^{-1} mg^{-1} chl)	581 ± 1.2	1316 ± 20	2323 ± 33
K_m PEP (mM)	0.75 ± 0.04	0.60 ± 0.26	0.44 ± 0.1
K_i malate (mM)	1.04 ± 0.21	0.20 ± 0.03	0.74 ± 0.14
K_A G-6-P (mM)	0.25 ± 0.07	0.17 ± 0.02	1.17 ± 0.31

of the enzyme reaction and/or the modulation of enzyme activity by metabolites (Stamatakis et al., 1988).

The light activation, and stability of PEPC in presence or absence of glycerol and/or PEG during extraction were studied. Even after 2 h of extraction, the enzyme was highly stable when glycerol and PEG were present in both extraction and assay media (Table 5.3). These results indicate that of all the combinations the presence of glycerol and PEG provides in a maximum protection of the enzyme against dilution, enhances the catalytic efficiency of PEPC and stabilize the protein. The effect of PEG appears to be primarily due to the rise in local protein concentration.

The influence of solute (PEG) on light- and dark-form of PEPC in crude leaf extracts was examined. When assayed at sub-optimal PEP and optimal-PEP concentrations the dark-form **of enzyme showed** much more marked stimulation by PEG than that of light-form (Fig. 5.8). The reports on the response of oligomerization/dissociation of PEPC-protcin in response to light or darkness are ambiguous. Stimulation of PEPC by PEG at 1.25 mM PEP was reported by Huber and Sugiyama (1986). The light-form of the enzyme is not stimulated than the dark-form of the enzyme in presence of PEG (Chadwick, 1994).

Glycerol is known to stabilize the activity of PEPC and is frequently included during extraction of enzyme from various plant tissues, including those of C_4 plants (Manetas, 1982; Karabourniotis et al., 1983; Manetas et al., 1986; Stamatakis et al., 1988; Jawali, 1990; Manetas, 1990; Drilias et al., 1994). Chidwick (1994) has observed that addition of 10% (w/v) PEG in extraction buffer, followed by rapid extraction, stabilizes PEPC in *Kalanchoë blossfeldiana.* However very few reports indicated the need for addition of glycerol while assaying PEPC.. Thus the use of compatible solutes during *in vitro* assay, appears to be highly relevant to *in vivo* situation, at least for oligomeric enzymes such as PEPC and PPDK (Salahas et al., 1990). Further

Table 5.3. *Effect of different combinations of 20 % (v/v) glycerol and 1 % (w/v) PEG during extraction/assay on the activity, light activation and stability of PEP C*

Extraction medium	Assay	Soon after extraction			After 2 h of extraction		
		Light	Dark	L/D	Light	Dark	L/D
		($\mu mol\ h^{-1} mg^{-1} chl$)		*Ratio*	($\mu mol\ h^{-1} mg^{-1} chl$)		*Ratio*
NoPEG.no **giycerol**	NoPEG.no glycerol	628 + 3.5	510 ± 2.8	1.2	716 ± 2.2	581 ± 4.2	**1.2**
	+ Glycerol	**968** + **5.6**	503 ± 2.0	1.9	1258 ±1.0	716 ±1.0	1.8
	Glycerol + PEG	1142 ±26	532 ±1.5	2.1	1142 ±15	610 ±6.0	1.9
+ Glycerol	+ Glycerol	943 ± 24	**561 ± 12**	1.7	1065 ±1.5	542 ±1.2	2.0
	+ Glycerol + PEG	1065 ± 20	639 ±16	1.7	1379 ±31	619 ±10	2.2
+ Glycerol + PEG	+ Glycerol + PEG	1258 ± 18	619 ± 22	2.0	1379 ±20	793 **±22**	1.7

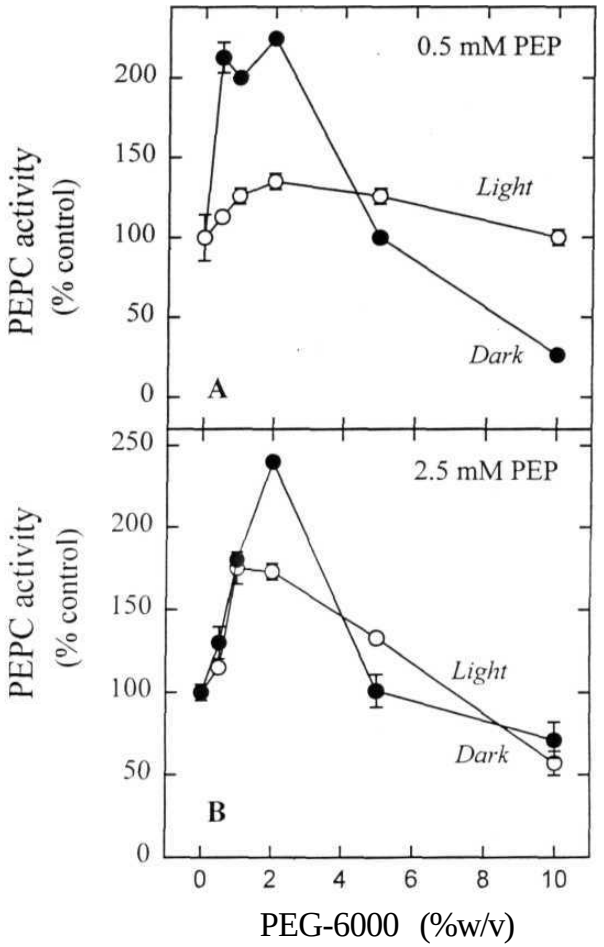

Figure 5.8. Stimulation by PEG of PEPC extracted from leaves of *Amaranthus hypochondriacus*, either adapted to darkness (•) or exposed to light (o). These leaves were extracted and PEPC was assayed at pH 7.3 **with** either 0.5 mM PEP or 2.5 mM PEP. PEG was included only during assay. The activities in the absence of PEG (control) in dark adapted leaves were 774 ± 12 and 1113 ± 14.5 μmol h^{-1} mg^{-1} chl when assayed at 0.5 mM and 2.5 mM PEP, respectively. The PEPC activity in control sets (no PEG) of illuminated leaves were 967 ± 3 (with 0.5 mM PEP) and **1597 ± 18** μmol h^{-1} mg^{-1} chl (with 2.5 mM PEP). **The** data are averages of four experiments ± SE.

studies are needed to elucidate the exact molecular mechanism involved in the regulation of PEPC by PEG and/or glycerol *invivo.*

Our results demonstrate that the presence of PEG and/or glycerol during extraction and assay not only enhances the catalytic activity of PEPC but also improves the stability of enzyme. The main reason for this effect can be assumed to be the prevention of the PEPC dilution during the extraction. We therefore recommend that PEG and/or glycerol could be valuable additives to the medium to determine maximum catalytic efficiency of the enzyme. However it should also be kept in mind that PEG and/or glycerol change the kinetic properties of PEPC.

Oligomerization state of PEPC in crude leaf extracts

To gain further insight into the possible interaction of PEPC with compatible solutes, we have examined the quaternary structure of PEPC by gel filtration in crude leaf extracts of PEPC on Sephadex G-200 column.

The elution profile of the enzyme in crude extracts was compared with the calibration of the column with standard proteins of known molecular weight (Fig. 5.9). The calibration curve obtained by elution pattern of four protein standards of different molecular weight was used to assess the oligomeric status of PEPC and to determine the form of PEPC: tctramer, dimer, or oligomer. The number of the fraction at which a peak of elution can be taken as the indication of its apparent molecular weight. Thus the protein peaks eluting at the fraction Nos. 10, 18 and 25 correspond to molecular weights of about 450 kD, 225 kD and 100 kD. respectively. These molecular weights correspond to tetramer, dimer and monomeric forms of PEPC in leaf extracts.

In one set of experiments, glycerol and/or PEG are included during both extraction of the enzyme from leaves as well as elution from Sephadex column. In another set of experiments 5% (w/v) PEG and/or 20% (v/v) glycerol were

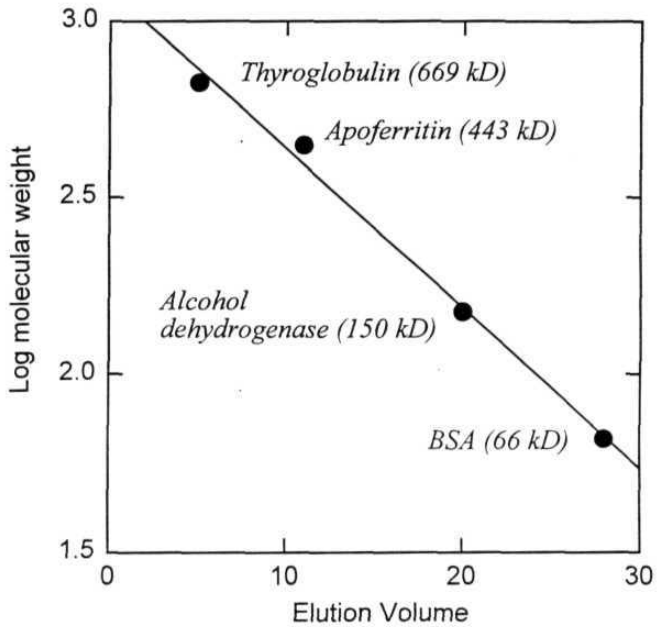

Figure 5.9. Elution profile of molecular standards from the column of Sephadex G-200 during calibration The buffer used for elution, at a flow rate of 10 ml h^{-1}, contained 50 mM Hepes-KOH (pH 7.3). 500 μg of protein in 500 μl was loaded.

present only during extraction but were excluded while eluting from the column.

When the leaves of *A. hypochondriacus* were extracted with a medium containing neither glycerol nor PEG, PEPC eluted at two peaks numbering 18 and 25 (Fig. 5.10). These peaks correspond to dimer and monomers. If the extracts were prepared with glycerol and/or PEG in the buffered medium, PEPC eluted mainly at fraction No. 12, i.e. as a tetramer of about 450 kD (Fig. 5.11 & 5.12). On the other hand, if PEG or glycerol were omitted while eluting the column with only buffer, PEPC appeared as a mixture of tetramer and dimer, as indicated by peaks at No.10 and 18 (Fig. 5.13 & 5.14). These results provide a good demonstration of variability in oligomeric status of PEPC in leaf extracts, and its modulation by glycerol and PEG.

The osmotic potential of the cell sap (> 330 mM) is much above that of the normal buffer strength (up to 50 to 100 mM) used during extraction or assays. During organelle isolation, 0.3 to 0.4 M sorbitol or mannitol is employed to mimic the osmotic microenvironment of cell sap. We have therefore examined the effect of 0.3 M sorbitol on the size of PEPC in leaf extracts. The presence of 0.3 M sorbitol in extraction and elution buffer a minor tetramer peak appears along with dimer and monomer peaks (Fig. 5.15). This is in well correlation with the marginal stimulation observed with our spectrophotometric assays. Sorbitol also can shift the enzyme towards active tetramer form.

Gel filtration technique has frequently been employed to assess the molecular size of PEPC from a few C_3-, C_4- and CAM species. While many of these attempts were with purified enzyme, some of the reports used crude extracts. Jones et al. (1978) reported a dissociation, on dilution, of purified PEPC from a CAM plant, *Bryophyllum fedtschenkoi*, using a Sepharose CL-6B column. McNaughton et al. (1989) detected changes in the oligomeric status of the PEPC, extracted from illuminated and dark-adapted leaves of

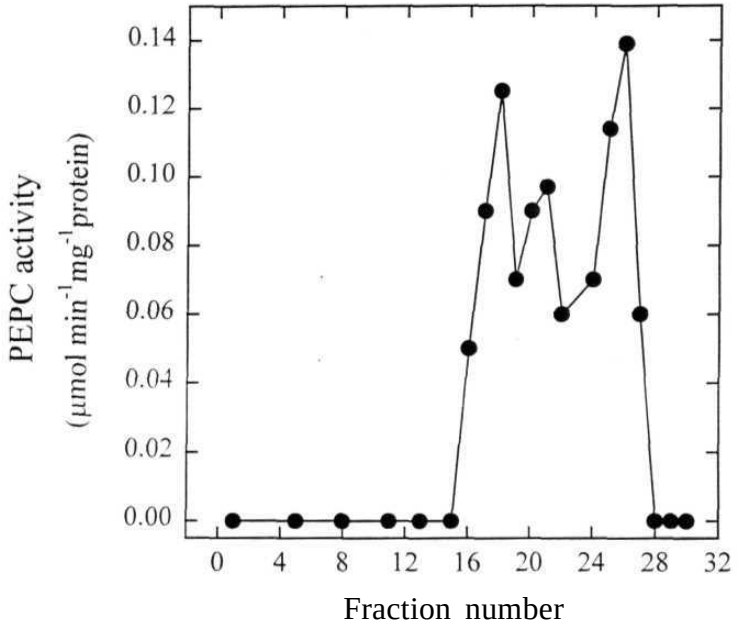

Figure 5.10. Elution profile from a Sephadex G-200 column, of PEPC in leaf extracts of *Amaranthus hypochondriacus*. The dark-adapted leaves were extracted with the medium, containing neither glycerol nor PEG. Leaf extract equal to about 500 μg of protein was loaded on to the column and eluted with a buffered medium containing 50 mM Hepes-KOH pH 7.3. One ml fractions were collected and assayed at pH 7.3 with 2.5 mM PEP. Further details **are** described in •'Materials and Methods".

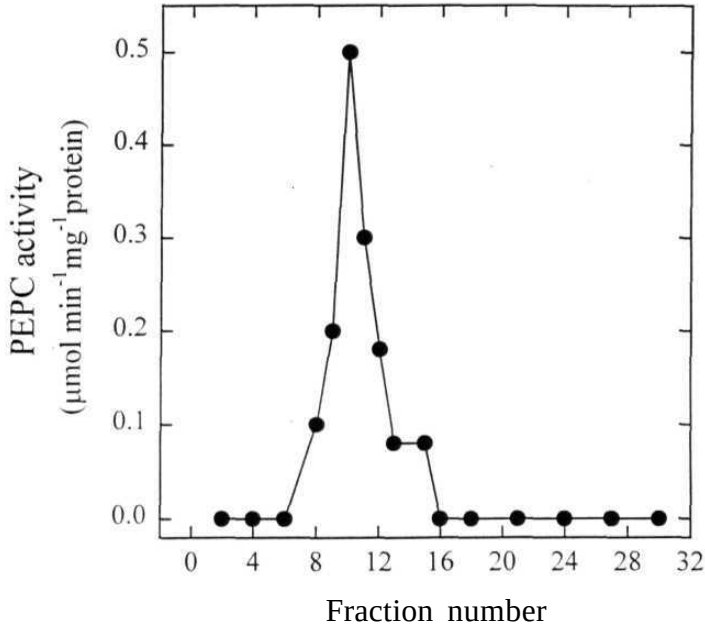

Figure 5.11. The pattern of **elution** of PEPC (in leaf extracts of *Amaranthus hypochondriacus*) from Sephadex **G-200** column, when 20% (v/v) glycerol was included in both the extraction medium and the elution buffer. Further details are as in Fig. 5.10.

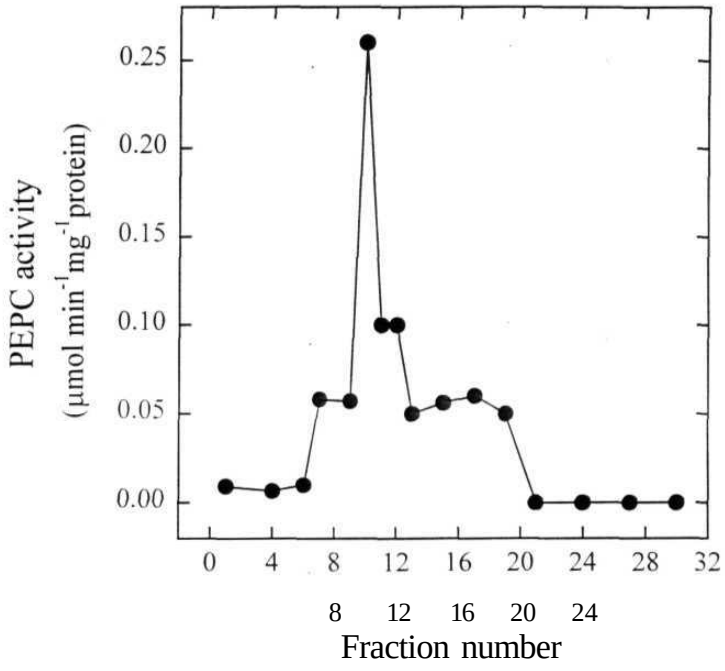

Figure 5.12. The elution profile of PEPC (in leaf extracts of *Amaranthus hypochondriacus*) from Sephadex G-200 column, in presence of 5% (w/v) PEG and 20% (v/v) glycerol were included in both the extraction medium and the elution buffer. Further details are as in Fig. 5.10.

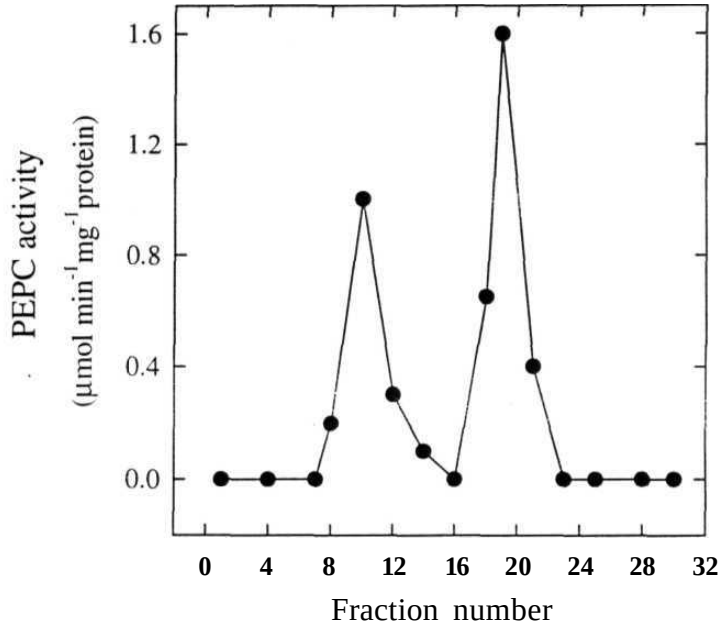

Figure 5.13. Effect of glycerol **on** the oligomeric status of PEPC in leaf extracts of *Amarantlms hypochondriaciis.* as indicated by its elution from a Sephadex G-200 column. 20% (v/v) glycerol was included in the extraction medium, but was excluded from elution buffer. Further details are as in Fig 5.10.

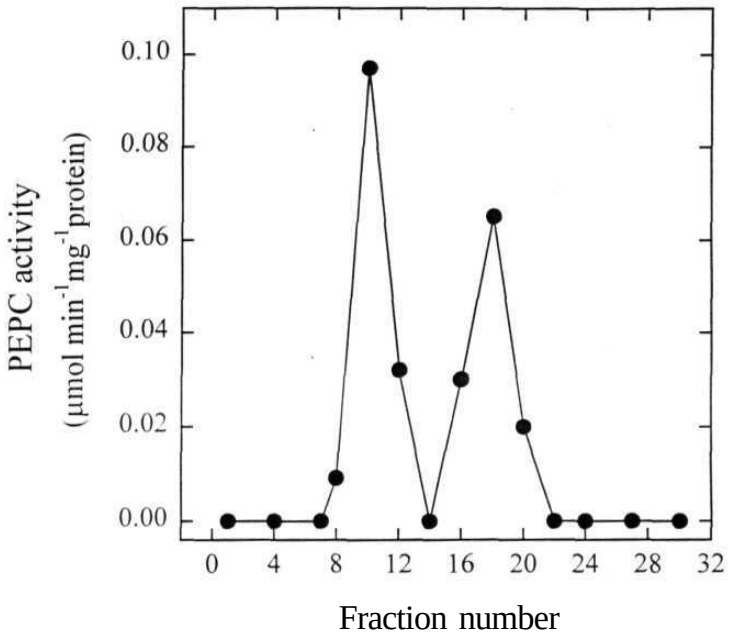

Figure 5.14. Effect of PEG on the oligomeric status of PEPC in leaf extracts of *Amaranthus hypochondriacus*, as indicated by its elution from a Sephadex G-200 column. 5% (w/v) PEG and 20% (v/v) of glycerol were **included** in the extraction medium, but was excluded from elution buffer. Further details are as **in Fig** 5.10.

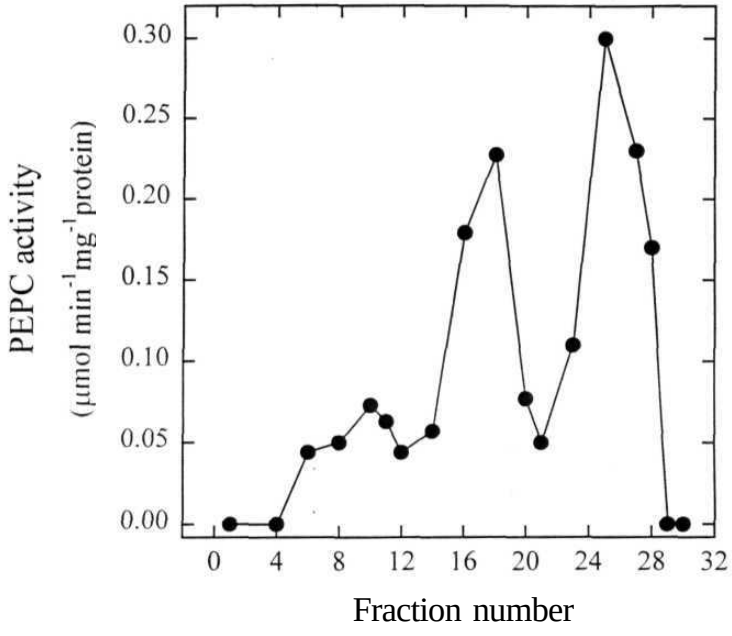

Figure 5.15. The pattern of elution of PEPC (in leaf extracts of *Amaranthus hypochondriacus*) from Sephadex G-200 column, in presence of 0.3 M sorbitol in both the extraction medium and the elution buffer. Further details are as in Fig. 5.10.

maize when passed through a Superose 6 column. Further, the oligomeric status of PEPC was found to change with dilution of enzyme suspension. The enzyme eluted as a single peak, with molecular weight corresponding to tetramer state, when the column was loaded with 500 μg of protein. However, on loading with a low amount of protein (0.5 μg), the enzyme appeared as a dimer. Jawali (1990) purified PEPC from maize which appeared as a dimer on Superose 12 column. Willeford et al. (1990) have also reported that PEPC predominantly exists as tetramer in presence of saturating substrate concentrations and as dimer under sub-saturating (<0.4 mM PEP) substrate level.

Podesta and Andreo (1989) have examined the effects of glycerol on oligomeric status of purified PEPC from maize. They **observed** that PEPC eluted as a tetramer on eluting **with** buffer containing glycerol. However in the absence of glycerol in elution buffer the enzyme appeared as both tetramer and dimer at pH 7.0. NaCl induces dissociation even in presence of glycerol and shifts the enzyme to dimeric state (McNaughton et al., 1989; Podesta and Andreo, 1989; Willeford et al., 1990).

Although the existence of different oligomers (mono, di- and tetramers) of PEPC is well documented, the information on the activity of monomer or dimer of PEPC is ambiguous and controversial. Jones et al. (1978) have shown that the PEPC-dimer, from *Bryophyllum fedtschenkoi*, possess about 50% of the specific activity of that of tetramer in PEPC. Huber et al. (1986) have shown that PEPC eluted as a tetramer from both illuminated and darkened maize leaves *in vivo*. Under such high concentrations of PEPC the addition of 2 mM malate in elution buffer did not alter the elution pattern. There are reports showing that under high protein concentration, PEPC exists as tetramer *in vivo* (O'Leary, 1982; Andreo et al., 1987; Wagner et al., 1987; Wu and Wedding 1987; Stiborová, 1988). Willeford et al. (1990) have shown that maize PEPC eluted as a dimer on a size-exclusion HPLC at pH 7.0, in the presence of

20% (v/v) glycerol and 100 mM NaCl. They found that the dimer had considerably enzyme activity under standard assay conditions or getting converted to the tetramer form in the assay. On the other hand, Meyer et al. (1991) reported that only a tetramer, but not a dimer, is active on native gels. On loading a native gel, with 20 µg of protein, PEPC appears as homotetramer, while diluting the enzyme to 10 to 5 µg shows a dimer which is not active on staining.

Apart from PEPC, several oligomeric enzymes are markedly influenced by the presence of PEG or glycerol. Examples for such enzymes are: Rubisco activase and PPi-dependent phosphofructokinase (Salvucci, 1992; Podesta and Plaxton, 1993; Moorehead et al., 1994), cytosolic pyruvate kinase (Podesta and Plaxton. 1993) and cytosolic FBPase (Hodgson and Plaxton. 1995). The addition of PEG or glycerol shifted the enzyme to active tctramcric form, in case of pyruvate kinase (Podesta and Plaxton, 1993) or FBPase (Hodgson and Plaxton, 1995). In the absence of PEG or glycerol, the enzymes appeared mostly in an inactive form.

Stimulation of PEPC activity was possibly due to microconcentration of enzyme (or) removal of water/hydrophobic interaction. Such possibility is further confirmed by the effect of sorbitol with enzyme from *Amaranthus hypochondriacus* (present studies, see Fig. 5.6). Podesta et al. (1995) have shown stimulation with other solvents, such as methanol or ethylene glycol on PEPC purified from *Amaranthus viridis*. We therefore suggest that an increase in homologous protein-protein interaction, probably promoting subunit aggregation. is responsible for the PEG-mediated activation.

Most of the earlier reports on the effects of PEG or glycerol on PEPC focussed on the properties, regulation and oligomeric status of enzyme only *in vitro*. The present work is the first attempt to assess in detail the oligomeric status of PEPC, on extraction from leaves in presence or absence of solutes in the medium. Our results demonstrate that PEG or glycerol or even sorbitol help

in keeping PEPC in its native active tetrameric form even in crude leaf extracts. Besides glycerol or PEG-6000 or sorbitol can also be used to modulate the oligomeric status of PEPC. These studies are quite relevant to the situation *in vivo*, and may be extended further to assess changes in oligomerization of PEPC, for e.g. on illumination.

Most of the observations on changes in oligomeric status of PEPC were made *in vitro* (Wu and Wedding, 1985; Wagner et al., 1987; Podesta and Andreo, 1989; Willeford et al., 1990). Attempts to record such changes *in vivo* of oligomerization or dissociation of PEPC have so far not been successful (Wagner et al., 1987; Podesta and Andreo, 1989; Weigend and Hincha, 1992). As a result, the physiological significance of changes in oligomeric status of PEPC has been questioned (McNaughton et al.. 1989; Wu et al., 1990).

High concentrations of salt, such as 200-400 mM NaCl induce dissociation of PEPC (Wagner et al., 1987), while presence of PEP or G-6-P counteract the salt effect, favoring equilibrium toward tetramer formation (Walker et al., 1986; Wagner et al.. 1987). McNaughton et al. (1989) have shown that purified maize PEPC dissociates into dimcric form with progressive dilution. Using light scattering as the probe, Willeford et al. (1990) have shown that PEPC exists *in vitro* in an equilibrium of aggregates. The enzyme shifted to a predominantly tetramer state on addition of Mg-PEP. while malate (an inhibitor of PEPC) shifted the equilibrium to dimer. Wu et al. (1990) have shown that at neutral pH, the enzyme exists as tetramer. At low or high pH, the tetramer form is dissociated. resulting in a largely inactive form. Meyer et al. (1991) also have indicated that dilution induced dissociation of the active tetramer to a less active dimer. Incubation in presence of PEP or G-6-P stabilizes the tetramer while presence of malate induced dimer formation in *Crassula* species. Wedding et al. (1992) have shown that dissociation of tetrameric form of PEPC into their subunits in presence of 1.5 M urea. The dissociation by urea was protected by PEP and G-6-P. Thus, the presence of

substrate (PEP) or an activator (G-6-P) promote aggregation of PEPC, while the inhibitor promotes enzyme dissociation.

Our findings confirm that the structural, kinetic and regulatory properties of PEPC are influenced by the microenvironment. For example, the concentration of PEPC while being assayed *in vitro,* is far below its expected concentration ***in*** *vivo.* Precautions must therefore be taken while studying the properties and regulation of PEPC. A similar situation may exist in case of also other plant enzymes, particularly cytosolic ones, such as pyruvate kinase (Podesta and Plaxton, 1993).

Major conclusions from the results presented in this chapter are:

1. The inclusion in assay medium of solutes such as PEG. glycerol or even sorbitol, stimulated the activity of purified PEPC. Of all the solutes, PEG-6000 was the most effective in stimulating PEPC.
2. In the presence of PEG, the affinity for PEP of purified PEPC or PEPC in leaf extracts decreased, the K_A of G-6-P increased, while the K_i of malate decreased suggesting that the regulation of PEPC is dampened.
3. Addition of both PEG and glycerol during both extraction and assay helps to stabilize the enzyme and to maintain the highest activities, possibly preventing dilution and dissociation of enzyme.
4. The stimulation by PEG of the dark-form of PEPC was more than that of the light-form under sub-optimal or optimal PEP concentrations.
5. Gel filtration of PEPC in leaf extracts on Sephadex G-200, showed up the existence of three different oligomeric forms: tetramer, dimer and monomer.

6. The absence of **PEG,** glyccrol or sorbitol during extraction and subsequent assay, resulted in a marked shift of **the** enzyme into dimer and/or monomer, with a very small proportion of **tetramer.**
7. Presence of PEG and/or glycerol both during extraction and/or elution results in the enzyme maintaining predominantly a tetrameric shape.

Chapter 6

Effect of Inorganic Ions on the Activity of PEPC from Amaranthus hypochondriacus in vitro

Chapter 6

Effect of Inorganic Ions on the Activity of PEPC from *Amaranthus hypochondriac us In Vitro*

Inorganic ions like nitrate or ammonium are not only constituents of plant components but also regulate plant metabolism by modulating the biosynthesis and activity of enzymes. The activities of at least three key enzymes namely: cytosolic sucrose phosphate synthase (SPS), nitrate reductase (NR) and PEPC, respond to L/D transitions and appear to be modulated by regulatory protein phosphorylation in response to light, N, or both (Duff and Chollet, 1995). An attractive hypothesis is that nitrate or a downstream product of its assimilation would activate PEPC and inactivate SPS by modulating the respective protein kinase/phosphatase activity ratios to favor increased phosphorylation (Van Quy et al., 1991 a, b; Champigny and Foyer, 1992).

Thus, nitrate functions as a signal metabolite activating the cytosolic protein kinase, modulating the activities of the two key enzymes and redirecting the flow of carbon away from sucrose synthesis towards amino acid synthesis (Champigny and Foyer, 1992). Biosynthesis of PEPC in leaves is effected by the extent and form of nitrogen available to the plant. For e.g. the levels of PEPC increased when the leaves of maize were fed with nitrate or ammonium. Sugiharto and Sugiyama (1992) have reported that ammonium salt is a much more effective inducer of PEPC biosynthesis than nitrate. However these effects ***in*** *vivo* of nitrate or ammonium are long-term ones on biosynthesis of PEPC protein.

Ammonia is a toxic metabolite and inhibits several enzymes of plant and animal metabolism (WHO, 1968). Nevertheless, a few enzymes such as pyruvate kinase are stimulated by ammonium (Hiller, 1970; Kanazawa et al., 1972; Peterson and Evans, 1978). Ammonium ions enhance assimilation of carbon into C_4 acids in higher plants, algal cells and cyanobacteria (Ohmori et

al., 1986; Miiller et al., 1990; Vanlerberghe et al., 1990). Further, ammonia assimilation rates *in vivo* are well correlated with PEPC activity in green alga, *Selanastrum minutum* (Vanlerberghe et al., 1990). When leaf discs and mesophyll cells were fed with ammonia, PEPC and pyruvate kinase activity were stimulated (Hammel et al., 1979). Although an increase in the activity of **PEPC** is assumed to be the reason for elevated rates of dark carbon fixation, the direct effects of ammonium salts on PEPC activity are not assessed.

The present chapter is an attempt to examine the effect *in vitro* of ammonium and several other inorganic ions on PEPC from L/D adapted leaves of *Amaranthus hypochondriacus*, a NAD-ME type of C_4 plant.

The activity of PEPC was stimulated markedly by ammonium chloride, particularly at low concentrations. The enzyme activity was enhanced by 80% (over control) at 50 μM NH_4Cl. However, the extent of stimulation declined as NH_4Cl concentration was raised above 100 μM. The effect of NH_4Cl, at 3-4 mM, on PEPC was only marginal and the enzyme activity decreased at 5 mM NH_4Cl (Fig. 6.1).

When a range of different salts were tested, PEPC was stimulated only by ammonium salts and to some extent by potassium ions. Besides chloride, acetate and sulphate salts of ammonium also stimulated the activity of PEPC. On the oilier hand, monovalent ions like lithium, sodium and rubidium had only a marginal effect on PEPC (fable 6.1). The presence of calcium chloride decreased the activity of PEPC. **The** inhibition of PEPC by calcium has been reported earlier (Gavalas and Manetas, 1980).

The stimulation by potassium ions was **not** as marked as that by ammonium ions. At 0.5 mM KC1, the activity **of** PEPC was stimulated by 35% over control. However, the activity of PEPC was slightly decreased at higher concentrations of potassium ions (about 20-25% inhibition at 2-5 mM KC1) (Fig. 6.2). Potassium ions, which promote stomatal opening (Raschke, 1979) and swelling of guard cell protoplasts (Hampp and Schnabl, 1984) are

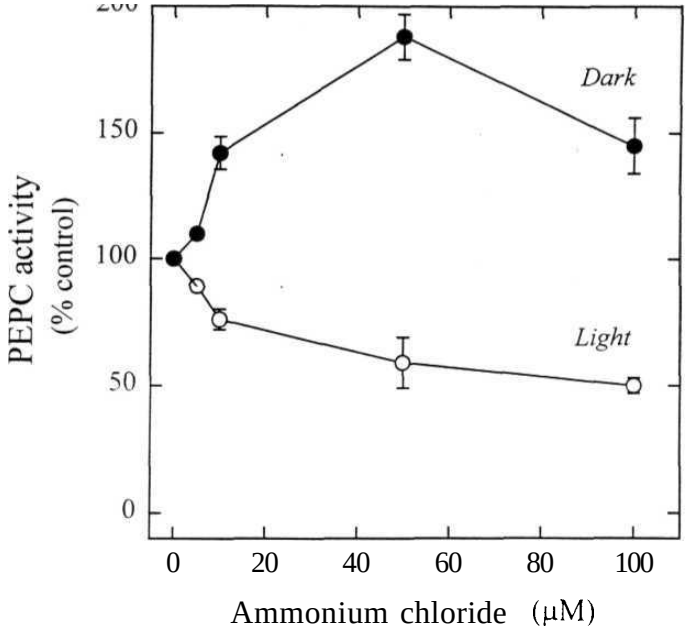

Figure 6.1. Stimulation by NH_4Cl *in vitro* of PEPC extracted from dark-adapted leaves of *Amaranthus hypochondriacus*. The enzyme was assayed at pH 7.3 and the reaction was started by 2.5 mM PEP. The inset shows the response of the enzyme to low concentrations of ammonium chloride. Maximum stimulation of about 80% (over control) occurred at 50 uM ammonium chloride. Enzyme activity in the absence of ammonium chloride (control) was 544 ± 10 umol h^{-1} mg^{-1} chl. Data are averages ± SE of at least three separate experiments. If not seen, the errors are within the symbols.

Table 6.1. *Effect of different salts* in vitro *on the activity of PEPC extracted from leaves of* Amaranthus hypochondriacus

Salt	Optimal concentration	Enzyme activity	Activity compared to control
		($\mu mol\ h^{-1}\ mg^{-1} chl$)	(%)
None (control)	0	627 ± 23	100
Ammonium chloride	50	1130 ± 10	180
Ammonium sulphate	50	1150 ± 5	183
Ammonium acetate	50	1121 ± 9	179
Potassium chloride	500	821 ± 1	131
Sodium chloride	500	730 ± 1	116
Lithium chloride	500	697 ± 1	111
Rubidium chloride	500	697 ± 1	111
Calcium chloride	500	372 ± 15	41

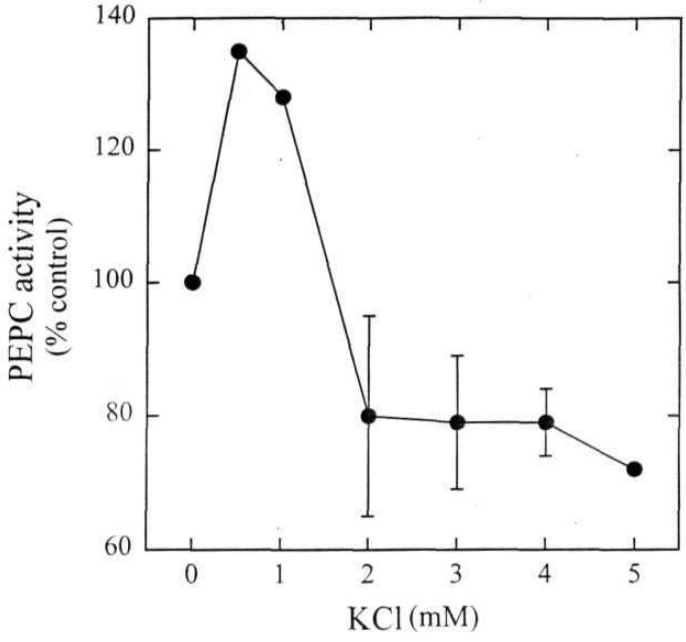

Figure 6.2. Stimulation *in vitro* of PEPC from dark by KC1. Maximum stimulation was at 0.5 mM KC1, while PEPC activity decreased at higher concentrations of KCl. The activity of PEPC in the absence of KCl (control) was 627 ± 23 μmol h^{-1} mg^{-1} chl. Further details were as in Fig. 6.1.

reported to stimulate PEPC activity in guard cell protoplasts (Michalke and Schnabl, 1990) and epidermal strips (Willmer et al., 1990).

In the presence of NH_4Cl, the affinity to PEPC was not much altered, although V_{max} increased by 85%. The double reciprocal plots revealed similar K_m for PEP in the presence or absence of NH_4Cl (Table 6.2). Also, there was no significant change in the sensitivity of the enzyme to malate in the presence of NH_4Cl (Table 6.2).

G-6-P is an allosteric activator of PEPC from C_4 plants (O'Leary, 1982). However, the response to G-6-P was quite different in the presence of NH_4Cl. G-6-P activated PEPC markedly in the absence but not in the presence of NH_4Cl. The enzyme was stimulated by more than two fold by G-6-P. But there was only a marginal stimulation (<20%) by G-6-P in the presence of NH_4Cl. Further, there was a decrease in PEPC activity at higher concentrations of G-6-P (>3 mM) in the presence of NH_4Cl (Fig. 6.3). The K_A of PEPC for G-6-P was 0.17 mM in the absence (control) and 0.94 mM in the presence of NH_4Cl (Table 6.2). A major reason for ineffectiveness of G-6-P appears to be that the enzyme is already activated by NH_4Cl. Similarly, the decrease in the enzyme activity at higher concentrations of G-6-P may be due to the marked alteration of regulatory site by ammonium ions. Therefore, NH_4Cl appears to modulate PEPC *in vitro* by changing the sensitivity of the allosteric regulatory site.

On illumination, the activity of PEPC is stimulated two to three fold in leaves of C_4 plants (Rajagopalan et al., 1994; Doncaster and Leegood, 1987). We have therefore tested the response to the ammonium of the enzyme extracted from illuminated leaves. However, unlike the dark-adapted enzyme, the light-activated form of PEPC was not stimulated, but was inhibited (Fig. 6.4). The light-activated form of PEPC differs from the dark-adapted form in several characteristics: malate sensitivity, phosphorylation status and K_m for PEP (Rajagopalan et al., 1994). When leaves are illuminated, conformational

Table 6.2. *Kinetic characteristics of PEPC from leaves of* Amaranthus hypochondriacus *in the presence or absence of 50 μM ammonium chloride*

Parameter	Control	NH_4Cl
V_{max} (μmol h mg chl)	1316 + 20	2436 ± 17
K_m PEP (mM)	0.60 ± 0.03	0.70 ±0.12
K_i malate (raM)	0.20 ± 0.05	0.25 ±0.01
K_A G-6-P (raM)	0.17 ±0.01	0.94 + 0.22 **

** Apparent, due to non-typical kinetics.

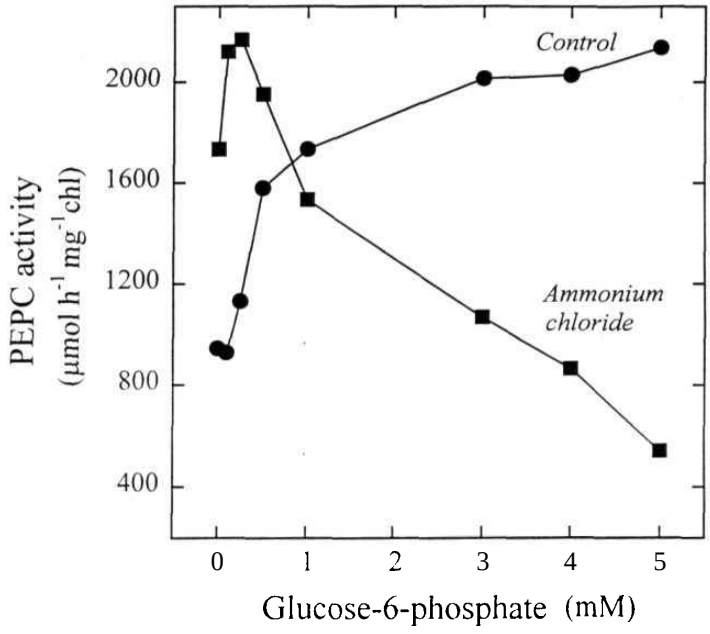

Figure 6.3. Response to G-6-P of PEPC from dark-adapted leaves of *Amaranthus hypochondriacus*. The enzyme was **assayed** at pH 7.3 in the presence or absence of 50 μM ammonium chloride and different concentrations of G-6-P. PEPC was activated markedly (>two fold) by G-6-P only in the absence of ammonium chloride. The stimulation by G-6-P was marginal (<20%) in the presence of ammonium chloride. Further details were as in Fig. 6.1.

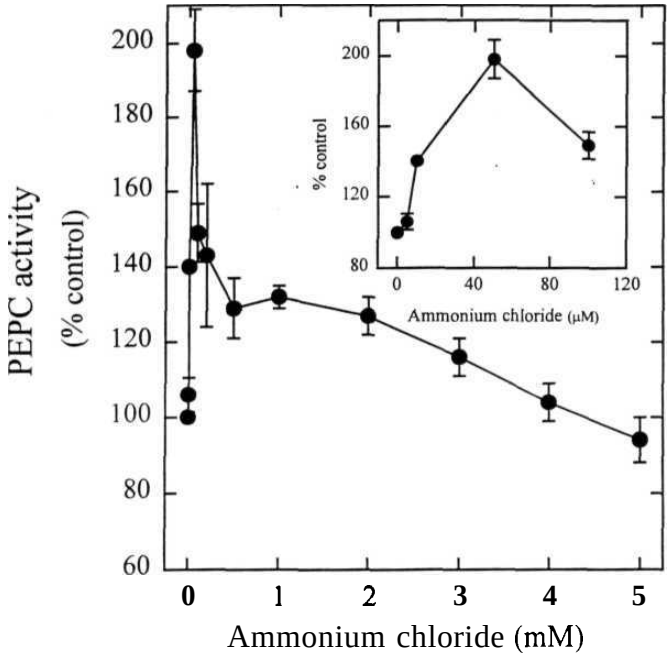

Figure 6.4. Response to ammonium chloride of PEPC extracted from dark-adapted or illuminated leaves of *Amaranthus hypochondriacus.* Leaves which were kept in darkness for 2 h, were either retained in darkness or illuminated at 1200 μE m^{-2} s^{-1} for 20 min. PEPC was assayed at pH 7.3 with 2.5 mM PEP. The activities of PEPC in the control sets (i.e. without NH_4Cl) were 836 ± 4 μmol h^{-1} mg^{-1} chl (dark-adapted leaves) and 2090 ± 10 μmol h^{-1} mg^{-1} chl (illuminated leaves). Further details were as in Fig. 6.1.

changes are likely to occur in PEPC. The absence of stimulation by NH_4Cl or PEPC from illuminated leaves indicates that the dark-form of the enzyme alone has the conformational status capable of responding to ammonium ions.

Light dependent phosphorylation of PEPC in mesophyll protoplasts of sorghum was promoted by weak bases such as ammonium chloride and methylamine (Pierre et al., 1992). These ions were expected to increase the cytosolic pH, raise the levels of calcium and activate either PEPC or PEPC-PK or both. Our present observations demonstrate that ammonium ion is an allosteric activator of PEPC from C_4 plants.

In C_4 plants, the reactions of carbon and nitrogen metabolism are spatially separated between the mesophyll and bundle sheath cells (Hatch, 1987). PEPC is located in cytosol of mesophyll cells in leaves of C_4 plants. The reduction of nitrate to ammonium also occurs in C_4 mesophyll cells (Hatch, 1987). Allosteric activation by ammonium of PEPC fits well with intercellular enzymic distribution. An increase in the availability of ammonium ions can stimulate PEPC and thus promote carbon and amino acid metabolism. Our observations can therefore form an additional basis of a better nitrogen use efficiency in C_4 plants than that in C_3 species (Hatch, 1987).

This is the first report on direct activation by ammonium ions ***in*** *vitro* of PEPC, a key enzyme of C_4 pathway. The effects of calcium on PEPC are studied further in the next chapter.

Major conclusions from the results presented in this chapter are:

1. Among the different salts tested acetate, chloride and sulphate salts of ammonium were the most effective in enhancing the activity of PEPC, during assay i.e., ***in*** *vitro.* PEPC was stimulated to a limited extent also by potassium ions.

2. Ammonium chloride stimulated PEPC activity at a low concentration of 50 μM in dark-adapted leaves from *Amaranthus hypochondriacus.*

3. The extent of stimulation of PEPC by ammonium chloride in dark-adapted leaves was more than the light-adapted leaves.
4. The V_{max} and malate sensitivity was not much altered in presence of ammonium chloride. However, G-6-P activation decreased in the presence of ammonium chloride.
5. Ammonium chloride modulates PEPC activity *in vitro* by changing the sensitivity of the allosteric regulatory site.

Chapter 7

Effect of Calcium on PEPC and PEPC-PK in Leaves of Amaranthus hypochondriacus

Chapter 7

Effect of Calcium on PEPC and PEPC-Protein Kinase in Leaves of *Amaranthus hypochondriacus*

Reversible protein phosphorylation of enzymes is an important mechanism of metabolic regulation in plants (Budde and Chollet, 1988). During this cascade, the target enzyme (protein) is phosphorylated by a protein kinase, while the dephosphorylation is catalyzed by a protein phosphatase. Both of these steps form a reversible cycle. In most cases, both the phosphorylation and dephosphorylation reactions are active at the same time and the relative activities would determine the steady state level of phosphorylation status of enzyme (Stadtman and Chock. 1977).

PEPC is one of the best examples of plant **enzymes** regulated by phosphorylation-dephosphorylation cascade. PEPC is phosphorylated by **a** PEPC-PK and dephosphorylatcd by a type-2A protein phosphatase (Budde and Chollet, 1988; Huber et al., 1994; Rajagopalan et al., 1994). The coordination of both PEPC-PK and PEPC-phosphatase(s) determines the phosphorylation status of PEPC and its sensitivity to malate during L/D transitions. The phosphorylated form of the enzyme is more active than the dephosphorylated form. The physiological significance of such modification is that the phosphorylated form of PEPC is less sensitive to malate and G-6-P than the dephosphorylated form.

The post-translational modification of PEPC involves phosphorylation at the serine residues. The phosphory lation of PEPC occurs on scr in tobacco, ser in sorghum, ser " in maize and ser in *Mesembryanthemum* (Lepiniec et al., 1994; Rajagopalan et al.. 1994).

Despite the extensive literature available on PEPC-phosphorylation, the regulation by metabolites or the pattern of messengers involved in the transduction of light signal is not completely characterized. Phosphorylation of

PEPC was not affected by a number of putative light-modulated cytoplasmic effectors including calcium/calmodulin, fructose-2,6-bisphosphate, PPi, and thioredoxin h (Jiao and Chollet, 1989; Chollet et al., 1990; McNaughton et al., 1991).

Calcium acts as a secondary messenger in a variety of physiological responses. The marked changes on illumination in the levels of cytosolic calcium suggested that calcium could be a part of light-transduction mechanism in plants (Pooviah and Reddy, 1993; Bush, 1995). Gavalas and Manetas (1980) have shown that C_4 plants are calciphobes, i.e., they keep soluble calcium at low levels in their leaf tissues, and this property may be a prerequisite for normal functioning of the C_4-pathway. However, the reports on involvement of calcium in the function of PEPC phosphorylation or PEPC-PK is not clear. Some of the reports described the occurrence of a calcium-independent PEPC-PK in C_4 plants (Carter et al., 1991; Jiao and Chollet, 1991; Bakrim et al., 1992; Li and Chollet, 1994; Wang and Chollet, 1993). On the contrary, PEPC phosphorylation was reported to be mediated by a calcium-dependent PEPC-PK by some authors (Echevarria et al., 1988; Bakrim et al., 1992; Ogawa and Izui, 1992; Ogawa et al., 1992; Pierre et al., 1992).

The present Chapter describes our experiments designed to reexamine the role and regulation by calcium of PEPC in extracts from leaves of *Amaranthus hypochondriacus*. Attempts were made to assess the activity of PEPC in leaf extracts in the presence or absence of calcium, when assayed under sub-optimal (pH 7.3) or optimal pH (pH 7.8). Initially, the experiments were performed on PEPC activity. Later, the experiments were extended to study the pattern of PEPC-phosphorylation in the presence or absence of calcium.

The presence of calcium inhibited the PEPC activity at both pH 7.3 and pH 7.8. However, the extent of inhibition by calcium chloride, particularly at 0.5 or 1.0 mM was more at pH 7.8 (> 50%), than that at pH 7.3 (<20%)

(Fig. 7.1). At low concentrations of calcium chloride (0-100 μM), there was a marginal stimulation of PEPC activity when assayed at pH 7.3.

It is possible that the response of PEPC to calcium changes when the leaves are illuminated, since light stimulates PEPC activity in leaves of particularly C_4 plants. We have therefore checked the effect of calcium chloride on PEPC activity in leaf extracts prepared from illuminated or dark-adapted leaves.

In light treated samples, the inhibition by 0.5 mM calcium chloride was less than 30% at pH 7.8 while there was no inhibition at pH 7.3 (Fig. 7.2). There was, in fact , a slight stimulation by low concentrations of (<100 μM) calcium chloride of PEPC at both pH (7.3 and 7.8). We conclude from the data of Figs. 7.1 and 7.2 that low concentration of calcium is beneficial for PEPC activity of particularly the light-form while high concentrations are inhibitory for both dark- and light-forms (Fig. 7.2).

These results are similar to our earlier observations (in previous Chapter 6) that calcium chloride inhibits PEPC enzyme (Gayathri and Raghavendra, 1994). Mukerji (1977) reported that calcium inhibited the residual activity of PEPC obtained without magnesium, but detailed investigation of this effect has not been made. Gavalas and Manetas (1980) have shown that calcium inhibits PEPC activity at a concentration of 1 mM in *Atriplex tatarica* at pH 7.7. Calcium acted not only as an inhibitor of the enzyme but also as a stabilizer at low concentrations of PEP (< 0.6 mM) and in absence of dithiothreitol.

It is possible that calcium affects the phosphorylation of PEPC by modulating PEPC-PK. The phosphorylation of PEPC can be studied either directly or indirectly. When leaf extracts containing PEPC (and presumably PEPC-PK) are incubated with ATP and $MgCl_2$, the activity of PEPC is stimulated while the inhibition by malate of PEPC is decreased. Since the decrease in malate sensitivity is a reflection of the PEPC-phosphorylation, the stimulation of PEPC activity by ATP-incubation is taken as a measure of

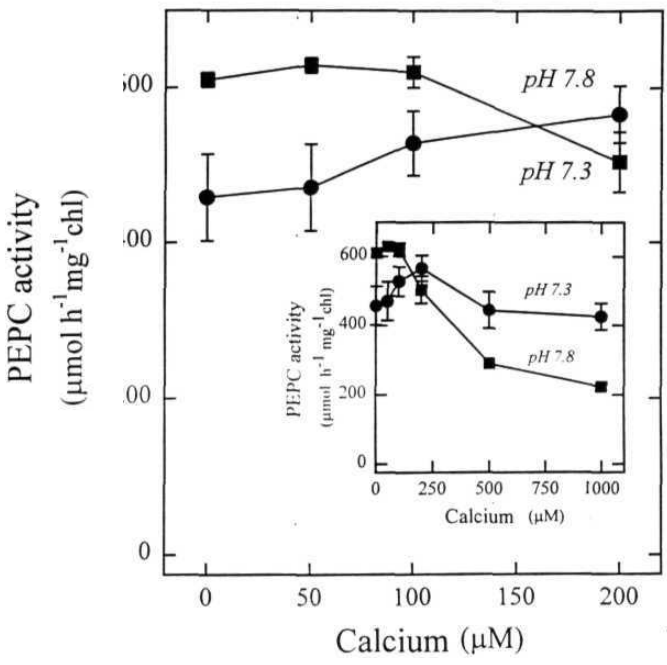

Figure 7.1. Effect of calcium chloride on PEPC activity in leaf extracts from dark-adapted leaves of *Amaranthus hypochondriacus*, when assayed with 2.5 mM PEP either at sub-optimal pH (7.3) or optimal pH (7.8). Various concentrations of calcium chloride were included in the assay medium while measuring the PEPC activity. The inset shows the response of enzyme to calcium up to a high concentration of 1000 µM. Data represent the averages ± SE of at least three independent experiments. Further details are described in "Materials and Methods".

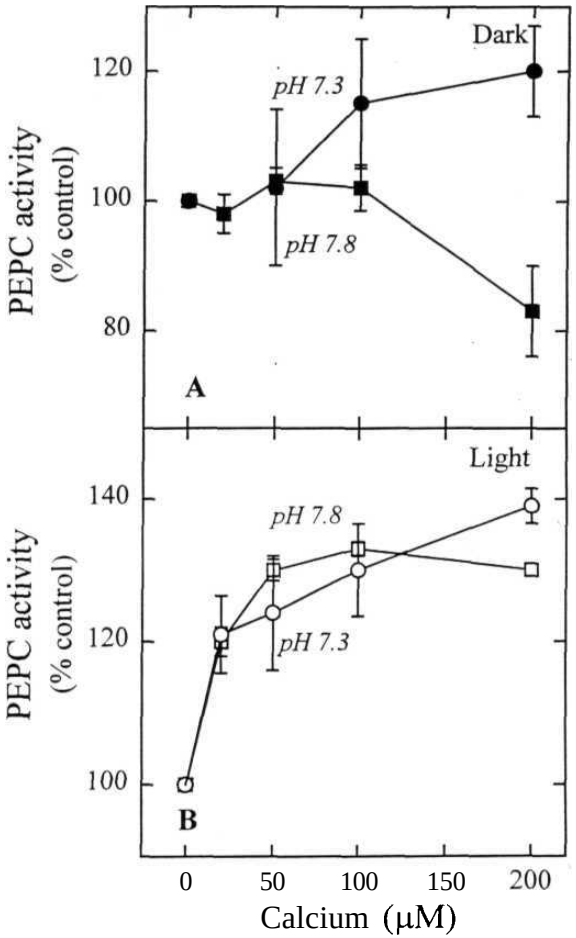

Figure 7.2. The response to calcium chloride of PEPC activity in leaf extracts prepared from dark-adapted (A) or illuminated (B). The enzyme was assayed at either sub-optimal (pH 7.3) or optimal (pH 7.8). The activities of PEPC in dark-adapted leaves when assayed without calcium (control) were 458 ± 56 and 610 ± 10 $\mu mol\ h^{-1}\ mg^{-1}$ chl, at pH 7.3 and pH 7.8, respectively. The activities of PEPC in light-adapted leaves when assayed without calcium (control) were 852 ± 63 and 1752 ± 29 $\mu mol\ h^{-1}\ mg^{-1}$ chl, at pH 7.3 and pH 7.8, respectively. Further details were as in Fig 7.1.

PEPC-phosphorylation and/or PEPC-PK activity. We have therefore measured the PEPC activity, before and after incubating the crude leaf extracts with 1 mM ATP and 5 mM $MgCl_2$.

When extracts prepared from leaves were preincubated with ATP and $MgCl_2$ for an hour, the activity of PEPC was stimulated. Stimulation by ATP was more when PEPC was assayed at pH 7.8 than that when assayed at pH 7.3 (Table 7.1). Such ATP dependent stimulation of PEPC activity occurred in extracts from illuminated leaves but not of dark-adapted leaves, irrespective of PEPC assay: at pH 7.3 or pH 7.8. As a result of ATP-stimulation of PEPC activity, the L/D ratio increased from 1.9 to 3.2 at pH 7.3 and 1.6 to 3.1 at pH 7.8 (Table 7.1). The presence of calcium chloride increased the extent of stimulation of PEPC activity by ATP. when assayed at either optimal or sub-optimal pH. However, the extent of stimulation by ATP of PEPC activity was nearly 80% at pH 7.8, while being 50% at pH 7.3 (Fig. 7.3).

Our observations confirm that PEPC is phosphorylated in leaf extracts by an endogenous protein kinase and this process can further be stimulated by the addition of ATP to the medium. We also feel that the PEPC-PK activity in leaf extracts is obviously limited by ATP and/or Ca^{2+} levels in leaf cytosol. These results emphasize that calcium is a positive effector of PEPC-PK activity, the enzyme responsible for PEPC-phosphorylation.

As per the literature, there were very few attempts to incubate the tissue extracts with ATP to modulate PEPC activity. Jiao and Chollet (1988) have shown that on incubation with ATP with purified PEPC in presence of partially purified PEPC-PK the stimulation in PEPC activity occurs within 60 min. Schuller and Werner (1993) have shown stimulation of PEPC activity on incubating with Mg-ATP for 60 min in desalted extracts of soybean root nodules.

Since there will be some calcium within the leaf, we have attempted to chelate the internal calcium so that it would be possible to assess the effect of

Table 7.1. *Stimulation by ATP of PEP C activity in extracts prepared from leaves exposed to either light or darkness*

Assay pH	Exposure of leaves	Incubation		Stimulation (+) or inhibition (-) by ATP
		No ATP (Control)	+ ATP	
		($\mu mol\ h^{-1}\ mg^{-1} chl$)		(%)
7.3	Darkness	458 ± 56	394 ± 64	<-) 13
	Light	852 ± 63	1277 ± 21	(+)50
	Light/Dark (Ratio)	1.9	3.2	
7.8	Darkness	610 ± 10	566 ± 51	(-)7
	Light	992 + 44	1752 + 30	(+)77
	Light/Dark (Ratio)	1.6	3.1	

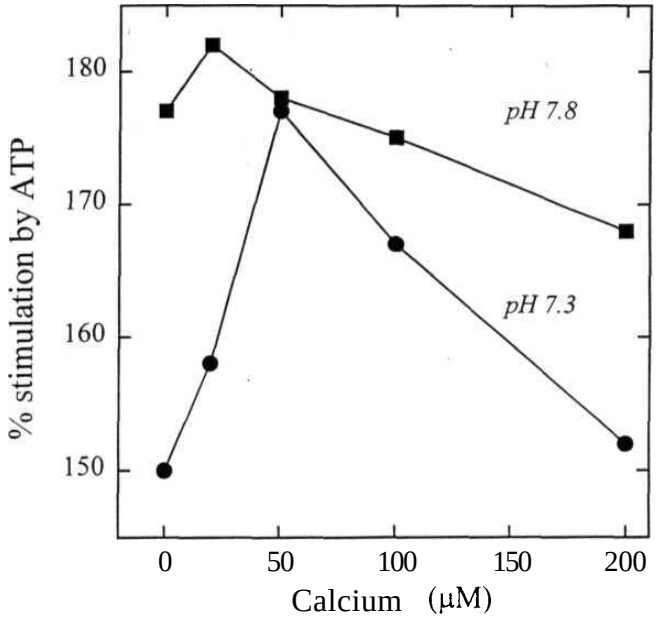

Figure 7.3. ATP-dependent activation of PEPC activity in leaf extracts from illuminated leaves, when assayed at either sub-optimal pH of 7.3, or optimal pH of 7.8. The extracts from illuminated leaves were incubated with 1 mM ATP and 5 mM $MgCl_2$ for 1 h and assayed for PEPC activity in presence or absence of calcium chloride. The stimulation by ATP is a measure of PEPC-PK activity. Further details were as in Fig. 7.2.

externally added calcium on both PEPC and PEPC-PK activity. EGTA is an effective chelator of calcium. We, therefore, fed the leaf with EGTA and studied the effect of calcium on PEPC activity in leaf extracts, before and after incubation with ATP. PEPC activity was assayed at pH 7.3.

On feeding with EGTA, there was not much effect on the activity of PEPC in dark-adapted leaves, indicating that calcium was not inhibitory for basal PEPC activity. However, there was a marked decrease in PEPC activity in extracts from illuminated leaves, when preincubated with EGTA and a conspicuous amplification in the response to external calcium (Table 7.2). On incubation with ATP, of extracts prepared from leaves fed with EGTA, there was stimulation in PEPC activity and such response of apparent PEPC-PK activity increased markedly on addition of calcium chloride. The response to different concentrations of calcium chloride was particularly remarkable in leaves preincubated with EGTA and on incubation of these extracts with ATP (Fig. 7.4). These data indicate that the pretreatment with EGTA sharpens the response to calcium in presence or absence of ATP at pH 7.3, and that calcium is an important factor while regulating PEPC-PK activity.

We could not locate in the literature any attempts of pretreatment of leaves with EGTA while assessing PEPC activity in C_4 plants. However, EGTA was included by some authors during phosphorylation assays *in vitro* and recovery of PEPC activity was noticed in presence of calcium chloride (Echevarria et al., 1988; Ogawa and Izui, 1992; Izui et al., 1992).

After using ATP-stimulation of PEPC activity as a measure of PEPC phosphorylation, we have attempted to examine directly the phosphorylation of PEPC and its response to calcium. PEPC phosphorylation can be studied directly in two ways: *in vivo* and *in vitro*. For *in vivo* studies, leaves were fed with ^{32}Pi, left in light for at least 2 to 4 h, so as to label PEPC and later PEPC in leaf extracts was immunoprecipitated. In our *in vitro* experiments, leaf extracts were incubated with γ-$AT^{32}P$ and the incorporation of radioactivity into

Table 7.2. *Effect of calcium chloride on PEPC* ***activity*** *before and after incubation with ATP in leaves treated with or without EGTA.*

Pretrealment	Activity		Stimulation by ATP
	Before incubation with **ATP**	After **incubation** with **ATP**	
	(μmol h^{-1} mg^{-1} chl)		*(%)*
No EGTA			
No calcium	852 + 63	**1277** ± 21	**50**
+ 20 μM calcium chloride	968 ± 12	1597 ± 22	**65**
EGTA			
No calcium	589 ± 16	629 ± 49	**s**
+ 20 μM calcium chloride	920 ± 49	1210 + 49	32

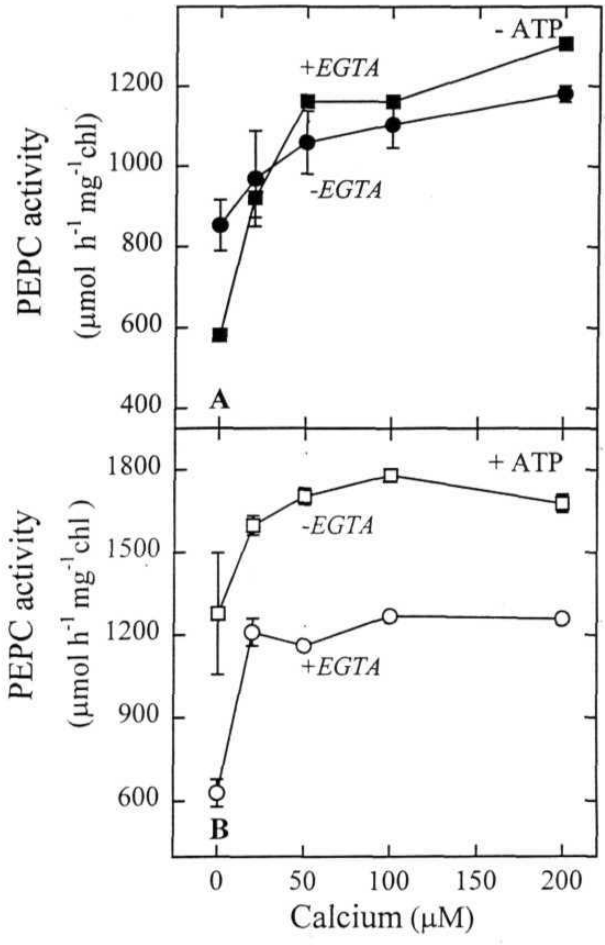

Figure 7.4. Effect of calcium chloride on PEPC activity in leaves without or with pretreatment of 2 mM EGTA. The extracts were incubated with or without 1 mM ATP and 5 mM $MgCl_2$ and PEPC activity was assayed at pH 7.3. The stimulation of PEPC activity by ATP-incubation is considered to be a measure of PEPC-PK activity. The response of PEPC to calcium chloride on PEPC was amplified on treatment with EGTA irrespective of incubation of leaf extracts without (A) or with ATP (B). Further details were as in Fig. 7.1 and 7.3.

PEPC-protein was assessed at pH 7.3. Most of the recent work on C_4-PEPC has been performed at pH of around 7.3, since this pH is physiologically relevant (Echevarria et al., 1994).

When leaves were fed with "Pi and equilibrated, the extent of phosphorylation of PEPC was greater in illuminated tissues than that in dark-adapted leaves (Plate 7.1). Phosphorylation of PEPC has always been observed to be more in illuminated leaf tissue than that in dark-adapted leaves of sorghum or maize (Nimmo et al., 1987; Vidal et al., 1990; Jiao and Chollet, 1991). Phosphorylated enzyme is more active and less sensitive to malate inhibition. These reports and our observations (Plate 7.1) show that PEPC-PK was activated considerably by light and its activity was almost not detectable in dark-adapted tissue. Most of the experiments on PEPC phosphorylation. both *in vivo* and *in vitro* were therefore performed with illuminated leaves.

While PEPC is phosphorylated by PEPC-PK, the enzyme (protein) is also dephosphorylated by a protein phosphatase. In our system, the inclusion of NaF was necessary to visualize phosphorylation of PEPC-protein. Hence we included NaF a non-specific protein phosphatase inhibitor in all our preliminary phosphorylation studies. Inclusion of NaF during the preparation and incubation of leaf extracts was essential and enhanced the phosphorylation of PEPC, presumably inhibiting the endogenous phosphatase(s). In the absence of NaF, we were unable to detect any phosphorylation and the level of phosphorylation declined, presumably due to hydrolysis (Plate 7.2). An incubation period of 45 min was better than 30 min for observing PEPC-phosphorylation. Presence of calcium and magnesium. besides NaF, was also necessary.

Incubation of crude leaf extracts with NaF and ATP-regenerating system and adenylate kinase inhibitor was necessary to overcome the problem of endogenous protein phosphatase(s) (Budde and Chollet, 1986). Echevarría et al. (1988) and Ogawa et al. (1992) have shown that addition of KF was necessary

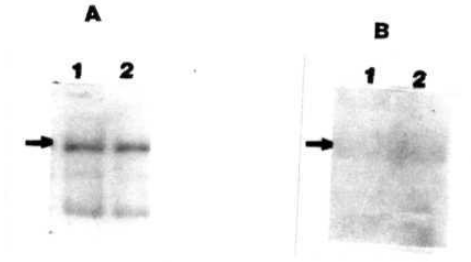

Plate 7.1. Phosphorylation of PEPC in *vivo*. Leaves were fed through petiole 60 μCi $KH_2{}^{32}PO_4$. After a period of illumination or incubation in darkness, the leaves were extracted and PEPC was immunoprecipitated with anti-PEPC antiserum. The precipitated proteins were separated by 10% SDS-PAGE. The gel was stained with Coomassie brilliant blue R-250, destained, dried under vacuum and autoradiographed at -80 °C for 20 to 25 days. The top part (A) represents Coomassie blue stained gel, while bottom set (B) represents the corresponding autoradiogram. Lane1: Extracts from dark-adapted leaves, Lane 2: Extracts from illuminated leaves. Further details are described in "Materials and Methods".

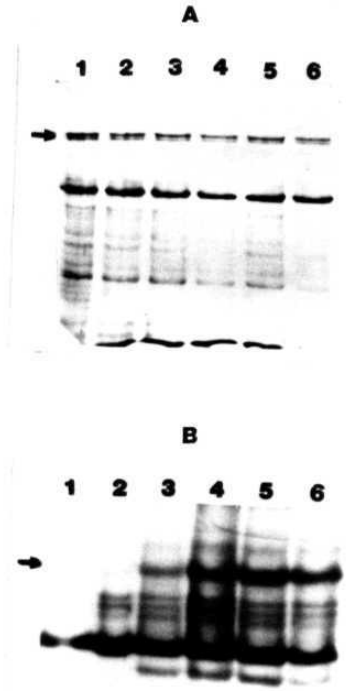

Plate 7.2. Phosphorylation of PEPC from total leaf extracts *in vitro*. Illuminated leaves were extracted and incubated with phosphorylation mixture containing γ–(AT^{32}P) and with or without NaF. Later the proteins were separated on 10% SDS-PAGE. The gel was stained with Coomassie brilliant blue R-250, destained, dried under vacuum and autoradiographed at -80 °C for 4 to 5 days. The top part (A) represents Coomassie blue stained gel. while bottom set (B) represents the corresponding autoradiogram. Lanes 1 and 2: contained extracts without NaF, lanes 3 to 6 contained 10 mM NaF during incubation with AT^{32}P, Lanes 5 and 6: 20 uM $CaCl_2$ was present during incubation. Lane 1, 3 and 5: Extracts incubated for 30 min. Lanes 2, 4, 6: Incubation for 45 min. Further details are described in "Materials and Methods".

to stabilize the phosphorylation of PEPC. Leaves of C_4 plants, like *Amaranthus hypochondriacus*, contain adenylate kinase which will rapidly randomize the labelled phosphate between ADP and ATP. Thus, the adenylate kinase inhibitor AP_5A was included in all our *in vitro* phosphorylation studies. We have also added an ADP-scavenging system composed of phosphocreatine and creatine phosphokinase. Budde and Chollet (1986) have also included AP_5A, creatine and creatine phosphokinase for phosphorylation by ATP of PEPC *in vitro*.

To reconfirm if PEPC-PK activity was dependent on calcium, the leaves were pretreated with EGTA and the effect of calcium was investigated. On pretreatment with EGTA, there was decrease in the incorporation of ^{32}Pi into PEPC in light treated leaves than in the dark. The extent of PEPC phosphorylation recovered considerably by feeding with 20 μM calcium in the pretreated EGTA leaves. This effect was enhanced in the presence of both calcium and magnesium (Plate 7.3).

Incubation with only calcium alone, or magnesium alone, did not show any enhanced PEPC phosphorylation in extracts from leaves exposed to light. But inclusion of both calcium and magnesium promoted PEPC phosphorylation and this effect was sharpened further in EGTA-fed leaves (Plate 7.4 A & B). These observations suggest that low concentration calcium is needed for PEPC-PK activity, though calcium was inhibitory at higher concentrations for PEPC activity. EGTA amplified the response to calcium. Both calcium and magnesium had a synergistic effect on PEPC phosphorylation.

Similar effect of EGTA on light-adapted leaves was observed under *in vitro* conditions. When EGTA was fed to the leaves under light, the phosphorylation of PEPC was almost suppressed at 2 mM EGTA and recovered on addition of different concentrations of calcium chloride (Plate 7.5 A & B). Echevarria et al. (1988) have reported that calcium effect was abolished by EGTA treatment and recovered back on adding calcium. Ogawa et al. (1992) suggested that the protein kinase that phosphorylated and activated

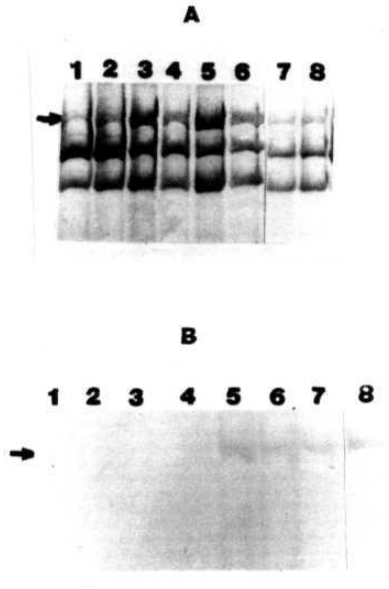

Plate 7.3. The effect of EGTA, calcium and magnesium on phosphorylation of PEPC *in vivo.* Leaves were fed with 2 mM EGTA followed by either water or $CaCl_2$ and/or $MgCl_2$. The top part (A) represents Coomassie blue stained gel, while bottom set (B) represents the corresponding autoradiogram. Lanes 1 to 4: Extracts from dark-adapted leaves and lanes 5 to 8: Extracts from illuminated leaves. Lanes 1 and 5 are control samples (leaves fed with water). Lanes 2 to 4 and 6 to 8 are of samples from leaves pretreated with EGTA and fed with either water or contain both 20 μM $CaCl_2$ and 5 mM $MgCl_2$ (lanes 4 and 8:). Lanes 2 and 5, i.e., EGTA , 20 μM $CaCl_2$ (lanes 3 and 7:), Further details were as in Plate 7.1.

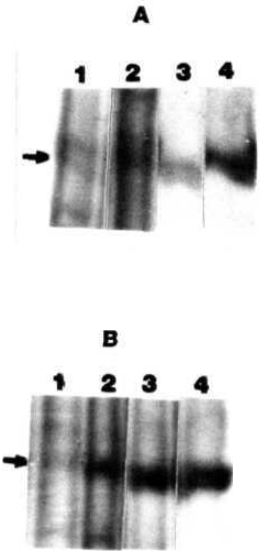

Plate 7.4. The effect of EGTA, calcium or magnesium on phosphorylation of PEPC *in vitro*. The leaves were pretreated with or without 2 mM EGTA and then exposed to light for 20 min. The extracts from illuminated leaves were incubated with $AT^{32}P$ for 45 min in presence or absence of 20 uM $CaCl_2$ and/or 5 mM $MgCl_2$. At the end of incubation, the reaction was stopped with sample buffer and proteins were separated by 10% SDS-PAGE. The gels were subjected to autoradiography. The top set (A) represents leaves which were not treated with EGTA (control), while the bottom set (B) is of leaves which were pretreated with 2 mM EGTA. Lane 1: Control (no calcium and no magnesium during phosphorylation), Lane 2: Only 5 mM $MgCl_2$, Lane 3: only 20 uM CaCl2, Lane 4: Both $CaCl_2$ and $MgCl_2$. Further details were as in Plate 7.2.

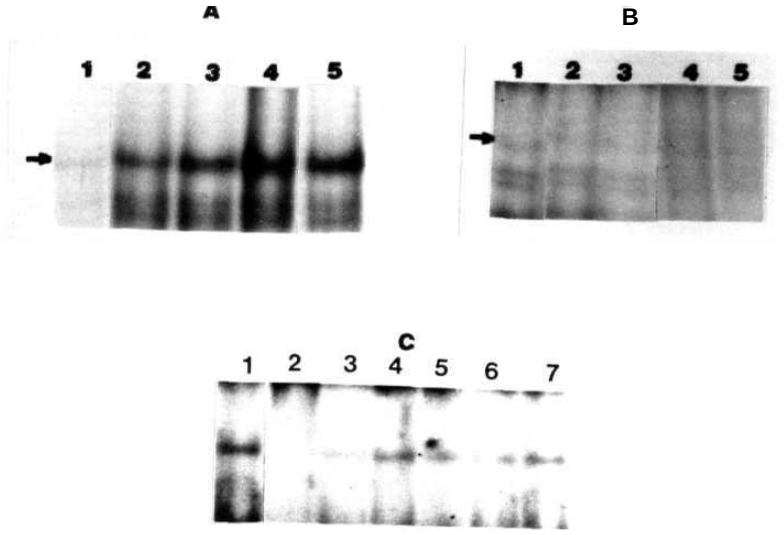

Plate 7.5. Effect of $CaCl_2$ on phosphorylation of PEPC *in vitro* in extracts prepared from leaves treated with or without EGTA. Leaves were extracted after illumination and phosphorylation of PEPC was allowed *in vitro* in presence of different concentrations of $CaCl_2$ in the reaction medium. Set A: Lane 1: control (no $CaCl_2$), Lanes 2 to 5: Increased concentrations of $CaCl_2$, in the order of 10, 20, 50, and 100 μM. Set B: Leaves were fed with different concentrations of EGTA and then illuminated before preparing the extracts for phosphorylation assays. Lane 1: control (no $CaCl_2$) Lanes 2 to 5: Increasing concentrations of EGTA, in the order of 0.5, 1, 2 and 3 mM. **Set** C: Illuminated leaves were fed with 2 mM EGTA expect in case of Lane 1: (water-control) Lanes 2 to 7: are extracts from leaves pretreated with EGTA. Lane 2: No $CaCl_2$, Lanes 3 to 7: increasing concentrations of $CaCl_2$, in the order of 20, 50, 100, 200, 500 μM during phosphorylation assays. Further details as described in Plate 7.2.

maize PEPC was calcium-dependent. In their experiments, the activity of partially purified PEPC kinase preparations could be inhibited completely by micromolar levels of EGTA, and the inhibition was reversed by 20 uM calcium but not magnesium.

Bakrim et al. (1992) purified partially two protein kinases from sorghum leaves. One of the protein kinases was calcium-dependent, while the other was calcium-independent. Ogawa et al. (1992) have reported that partial proteolysis of PEPC-PK can cause desensitization to calcium. The phosphorylation of PEPC activity was inhibited by the additions of EGTA in standard phosphorylation assays (Ogawa and Izui, 1992). The kinase responsible for PEPC-phosphorylation can therefore be attributed to the calcium-dependent protein kinases rather than calcium-independent kinases. The group of Ogawa suggests that at least four protein kinases or its catalytic subunit in the PEPC-PK preparation, may be present and two of them are calcium-dependent. Immunopurified C_4 PEPC from sorghum and maize could be phosphorylated *in vitro* by various kinases, including calcium- or magnesium-dependent protein kinase, both in crude extract and in a reconstituted system (Bakrim et al., 1992) or catalytic-subunit of cAMP-dependent protein kinase from bovine heart (Tcrada et al., 1990; Bakrim et al., 1992).

On the other hand, PEPC-PK preparations partially purified through Blue-dextran agarose either from maize or sorghum were reported by several groups to be active even under calcium depleted conditions (Chollet et al., 1990; Echevarria et al., 1990; McNaughton et al., 1991). Vidal et al. (1990) isolated a calcium-dependent protein kinase(s) from sorghum leaves that could phosphorylate dark-form PEPC *in vitro,* but phosphorylation did not change the properties of the target enzyme, i.e., PEPC. Thus, the reports on regulation of C_4 PEPC-PK by calcium have been controversial.

Our experiments endorse the opinion that PEPC-phosphorylation (and PEPC-PK activity) is promoted by calcium in crude extracts. The effect may be direct but could also involve other cytosolic components, such as calmodulin. Further experiments are needed to purify PEPC-PK from C_4 leaves and elucidate its exact nature by performing reconstitution assays *in vitro.* As a preliminary attempt, we examined the effect of calcium-calmodulin antagonists and calcium-channel blockers on PEPC-phosphorylation *in vitro.*

Leaves were fed with either TFP (calmodulin antagonist), or diltiazem, verapamil and lanthanum (calcium channel blockers). All of them inhibited PEPC phosphorylation in light (Plate 7.6 A & B), suggesting that apart from calcium, calmodulin is also involved in PEPC phosphorylation. These data suggest that PEPC from *Amaranthus hypochondriacus* is phosphorylatcd in a calcium-CaM dependent manner.

TFP (calmodulin antagonist) inhibited calmodulin activity in protoplasts of carrot (Gilory et al., 1987) and guard cells of *Vicia faba* (Shimazaki et al., 1992). Lanthanum and verapamil (calcium channel blockers) caused a reduction of internal calcium in suspension cultures of *Zinnia* (Roberts and Haigler, 1990). Diltiazem also inhibited ATP dependent calcium uptake in maize root microsomes (Vaughan et al., 1984).

Calcium-dependent protein kinase from wheat embryo was inhibited by calmodulin antagonists such as TFP or chlorpromazine and also by lanthanides (Polya and Micucci. 1985). TFP inhibited the protein kinase of soluble starch synthase in spinach leaves indicating that the protein kinase is calmodulin-dependent (Dreier et al., 1992). Verapamil, diltiazem and lanthanum are known to restrict Ca^{2+} influx and inhibit the related physiological responses such as spore germination, organogenesis, and setting of circadian rhythms (Bush, 1995).

Our results indicate that calcium promotes PEPC-PK activity and PEPC phosphorylation. But low concentration of calcium is necessary for optimizing

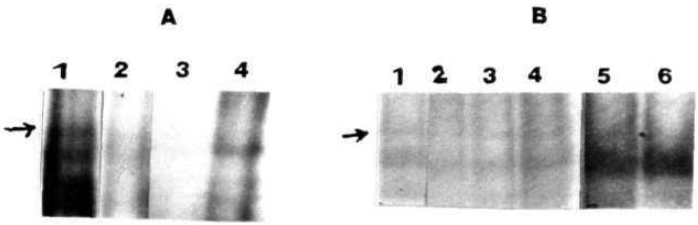

Plate 7.6. Effects of calcium- or calmodulin antagonists on PEPC-phosphorylation *in vitro*. Leaves were fed with either $CaCl_2$ or these metabolic inhibitors, and were illuminated before preparing the extracts and examining phosphorylation of PEPC *in vitro*. **Set** A: Lane 1: Leaves fed with 20 μM $CaCl_2$ (control), Lane 2: 2 mM EGTA, Lane 3: 100 μM TFP, Lane 4: 2 mM EGTA + 20 μM $CaCl_2$. **Set** B: Lane 1: 20 μM $CaCl_2$ (control), Lane 2: 100 μM verapamil, Lane 3: 200 μM verapamil, Lane 4: 1 mM lanthanum, Lane 5: 0.5 mM diltiazem, Lane 6: 1 mM diltiazem. Further details were as described in "Materials and Methods" and in Plate 7.2.

the PEPC activity, since high concentrations inhibit PEPC in leaf extracts of *A. hypochondriacus*. The effect of calcium appears to be dependent on calmodulin and calcium channel activities. Ca-calmodulin-dependent PEPC-protein kinases occur in plant tissues (Echevarría et al., 1988; Ogawa and Izui, 1992). The activity of calcium channels can regulate cytosolic free calcium in plant cells (Bush, 1995). We suggest that a fine tuning of calcium levels is essential for maximal PEPC activity particularly at alkaline pH prevalent in mesophyll cytosol of illuminated leaves of C_4 plants.

Major conclusions from the results presented in this chapter are:

1. Calcium inhibits PEPC activity at both pH 7.3 and pH 7.8 in extracts from dark- adapted leaves of *Amaranthus hypochondriacus.*
2. Low concentrations of calcium (20-50 uM) stimulated PEPC activity in extracts from light-adapted leaves, when the enzyme was assayed at either pH 7.3 or 7.8.
3. Incubation of leaf extracts (particularly from illuminated leaves) with ATP and $MgCl_2$ stimulated PEPC activity, suggesting that PEPC was phosphorylated, presumably by PEPC-PK in leaf extracts. The extent of ATP stimulation, indicating the PEPC-phosphorylation, was more at pH 7.8 than that at pH 7.3.
4. 20-50 μM $CaCl_2$ enhanced markedly (by about 80%) the stimulation by ATP of PEPC activity, indicating the stimulation of PEPC phosphorylation/PEPC-PK activity by calcium.
5. Thus, calcium affected PEPC and PEPC-PK in different ways.
6. Pretreatment of leaves with EGTA amplified the response of PEPC and PEPC-kinase activity to calcium.

7. Presence of NaF was essential to stabilize the phosphorylation of PEPC presumably by minimizing the dephosphorylation by protein phosphatase. Besides Mg^{+}, calcium was necessary to activate the process of phosphorylation. Both calcium and magnesium have synergistic effect in stimulating PEPC phosphorylation.
8. The phosphorylation of PEPC in extracts from illuminated leaves was inhibited by pretreatment of leaves with TFP (a calmodulin antagonist), or diltiazem, or verapamil or lanthanum (calcium channel blockers). These obsen'ations indicate that phosphorylation of PEPC is dependent not only on free-calcium but also calmodulin and calcium-channel activity.

Chapter 8

Summary and Conclusions

Chapter 8

Summary and Conclusions

PEPC is an important enzyme, mediating the primary carbon fixation in C_4 and CAM plants. In C_3 plants and algae, the enzyme plays an auxiliary role (O'Leary, 1982; Latzko and Kelly, 1983; Andreo et al., 1987). Due to its strategic importance, the properties, regulation, molecular biology and evolution of C_4 PEPC have been studied extensively (Lepiniec et al., 1994; Rajagopalan et al., 1994; Toh et al., 1994). The main objective of present work is to study the properties and regulation of C_4-PEPC using the leaves of *Amaranthus hypochondriacus*, a NAD-ME type C_4 plant, as the source of the enzyme.

The first set of experiments were designed to purify PEPC from leaves of *A. hypochondriacus* and to examine the stability of PEPC under different conditions of storage. As per literature, PEPC has been purified from several C_4 plants (Andreo et al., 1987; Rajagopalan et al., 1994). Our purification method involved 40-60% ammonium sulphate fractionation, followed by DEAE-Sepharose, HAP chromatography and finally through a Seralose 6-B column. The protocol was efficient and resulted in a high yield of nearly 50% and with PEPC having one of the highest specific activities reported for C_4 plants (54 Units mg^{-} protein). The purified PEPC was homogenous and appeared as a single band on 10% SDS-PAGE. Purified PEPC appeared as two bands on native gels and stained positively when examined for PEPC activity. Two-dimensional electrophoresis of these two bands resulted in a single band on SDS-PAGE indicating that PEPC is composed of only one-type of subunits, with a molecular mass of about 100-kD. We suggest that these two bands may be isozymes of a homotetramer, since dimer or monomer are not expected to stain for PEPC activity.

Purified PEPC is highly unstable and loses its N-terminal. The loss of the N-terminal is reflected in the loss of its malate sensitivity (McNaughton et al., 1989). Proteolysis of PEPC during purification is observed, particularly in the absence of protease inhibitors like PMSF or chymostatin (McNaughton et al., 1989). We have used PMSF as the protease inhibitor, so as to avoid proteolysis and to maintain the stability of the enzyme during extraction and purification.

In literature, there are several reports that the instability of PEPC could be prevented by inclusion of glycerol under *in vitro* conditions (Uedan and Sugiyama, 1976; Manetas et al., 1987; Selinioti et al., 1987; Podesta and Andreo, 1989). Hence, we studied the properties of the purified enzyme after storing at different temperatures (i.e.. room temperature. 4 °C, -20 "C, liquid nitrogen) in the presence or absence of glycerol. High activity of PEPC was maintained for up to four months, in liquid nitrogen when stored along with 50% (v/v) glycerol. A study of the purified PEPC, after storage at different temperatures, revealed that the enzyme stored in liquid nitrogen with 50% (v/v) glycerol maintained all the three key characteristics, even after 4 months: high V_{max}, stimulation by G-6-P, and inhibition by malate.

Although extensive literature is available on purification, properties and regulation of PEPC, studies on immunological properties of PEPC are quite limited. An attempt has therefore been made to study the immunological properties of PEPC and assess its cross-reactivity with the enzyme in leaf extracts of a few C_3- or C_4-type dicots, or C_3-C_4 intermediates or C_4-monocots.

Antibodies were raised in rabbits against the purified PEPC from *A. hypochondriacus*. The antibody exhibited a titer value of 1/100 against the PEPC of *A. hypochondriacus* (in leaf extracts and purified enzyme) and *Alternanthera pungens*. The antibody showed limited cross-reactivity with the PEPC from C_4 monocot (*Zea mays),* as indicated by the intensity of precipitation. This was confirmed further by Western blot analysis.

Immunoprecipitation experiments were conducted to assess the specificity of the antiserum. A fixed volume of leaf extract was mixed with different volumes of antibody solution. In the control samples, a serum of non-immunised rabbit was used. When the concentration of the antibody was increased, the amount of PEPC precipitated from the supernatants increased. In contrast, there was no PEPC precipitation with non-immunised serum. There was a proportionate decrease in PEPC activity in the supematants with increase in PEPC precipitation. The immunoprecipitates were later analysed by 10% SDS-PAGE to confirm the identity and quantity of PEPC protein.

Single radial immunodiffusion was performed to quantitate the amount of PEPC protein in crude leaf extracts using the anti-PEPC antiserum of *Amaranthus hypochondriacus*. There was a proportionate increase in the diameter of the ring with the increase in the antigen concentration.

The cross-reactivity between PEPC of C_3, C_3-C_4 and C_4 plants was examined by Ouchterlony double diffusion. The anti-PEPC antiserum raised against PEPC from *Amaranthus hypochondriacus* showed very strong immunoreaction with PEPC in leaf extracts of *Amaranthus viridis*, *Alternanthera pungens* (C_4 dicots) and faint reaction with *Zea mays*, a C_4 monocot. However, we could not detect much cross-reactivity with PEPC in leaf extracts of C_3 plants or C_3-C_4 intermediates. Hence we evaluated the immunological identity with also Western blots.

Anti-PEPC antiserum raised against *A. hypochondriacus* enzyme showed high cross-reactivity with purified PEPC from *A. hypochondriacus*, or the enzyme from leaf extracts of *Amaranthus viridis* or *Alternanthera pungens* (all three C_4 dicots), but limited reactivity with that of C_4 monocots: maize, sorghum or pearl millet. PEPC in leaf extracts of C_3 plants (*Pisum sativum, Commelina benghalensis, Alternanthera sessiles)* and C_3-C_4 intermediates (*Parthenium hysterophorus, Alternanthera tenella, Alternanthera ficoides*), exhibited stronger cross-reactivity with anti-PEPC antiserum of

A. hypochondriacus than that with the anti-PEPC antiserum against maize-PEPC. All these results indicated that C_4 dicot PEPC was closer to C_3 species, or C3-C4 intermediates than with C_4 monocot PEPC.

Using ELISA, it was possible to quantitate very low levels (ng) of PEPC protein in crude leaf extracts of *A. hypochondriacus*, as indicated by the linear relationship between immuno-precipitate-linked absorbance and PEPC protein in leaf crude extracts. In contrast, µg amounts of PEPC protein was required for detecting PEPC in pea leaf extracts indicating that the cross-reactivity of C_3 dicot PEPC with C_4-PEPC was less than that of C_4 dicot PEPC.

The tetrameric form of PEPC is reported to be the most active form (Walker et al., 1986; Podesta et al., 1990 Wu et al., 1990). Organic solutes such as PEG, proline, betaine play an important role in maintaining the integrity of the enzyme, even in adverse conditions (Drilias et al., 1994). The presence of solute helps in maintenance of the homologous interaction of the protein and thus increase enzyme stability. Normally, the enzyme in crude leaf extracts tends to be in diluted state. Several authors have reported that the instability of PEPC in extraction or assay media could be overcome by addition of glycerol (Manetas, 1982; Selinioti et al., 1987). Besides glycerol, other organic solutes (PEG, proline, betaine) also promote self-association and stabilize the structure of proteins (Timasheff et al., 1982). We have therefore studied in detail the interaction of PEG with PEPC from *A. hypochondriacus* purified form as well as in crude leaf extracts.

The effect of three different PEGs (PEG-6000, 8000 and 20,000) were studied on purified enzyme at either saturating (2.5 mM) or sub-saturating (0.5 mM) concentration of PEP. The activity of PEPC was markedly enhanced by PEG and such stimulation by PEG-6000 was more than that by PEG-8000 or PEG-20,000, irrespective of substrate concentration. The extent of activation by (1.25% w/v) PEG-6000 was more at 0.5 mM PEP, than at 2.5 mM PEP. There was a decrease in PEPC activity as the PEG concentration

increased above 10% (w/v). This may be due to the possible precipitation of the enzyme at higher levels of PEG. The activity of PEPC increased also in presence of ethylene glycol, or glycerol but the extent of activation, when compared to that with PEGs, was low. Sorbitol had only a marginal effect on PEPC.

In presence of PEG, the affinity for PEP of PEPC decreased and the enzyme was less malate sensitive. This indicates that PEG relieved malate inhibition of PEPC while activating the enzyme. The K_A for G-6-P increased in the presence of PEG. G-6-P and PEG were reported to shift PEPC from maize to an active state (Huber and Sugiyama, 1986).

Further experiments were taken up to assess the effect of PEG on PEPC during enzyme extraction from leaves. Different concentrations of PEG were used during either extraction, or assay, or both. Maximal activation was obtained when PEG was present during both extraction and assay.

In the next set of experiments, the effect of PEG was studied on the light or dark-form of PEPC. Light activation of PEPC was achieved by illuminating leaf discs at 1500 $\mu E\ m^{-2}$ for 20 min (after pre-darkening for 2 h). The light-form of PEPC was 2 to 3 times more active and less sensitive to malate than the dark-form. The activation by PEG of the light-form was less than that of the dark-form, particularly at sub-optimal PEP.

Glycerol is usually included while extracting leaves for studies on PEPC. Efforts were therefore made to study the effect of a combination of glycerol and PEG during extraction and/or assay on the activity, stability and light activation of PEPC. The activity of PEPC remained high even after 2 h of extraction if both PEG and glycerol were present. Further, the extent of light activation also improved in presence of PEG and glycerol. Of all the combinations, inclusion of glycerol plus PEG during extraction as well as in assay medium was the best for maintaining high stability and maximum light activation of PEPC.

From the above experiments we have observed that activity of PEPC was enhanced in presence of organic solutes. However the changes in the oligomeric structure of PEPC in presence of organic solutes will allow us to gain further insight into the possible interaction of PEPC with compatible solutes. Hence we examined the quaternary structure of PEPC in crude leaf extracts by gel filtration on Sephadex G-200 column.

In the absence of either PEG or glycerol, PEPC eluted as a mixture of dimer and monomers. If the extracts were prepared with glycerol and/or PEG in buffered medium, PEPC appeared predominantly as a tetramer. On the other hand, if PEG or glycerol were omitted while eluting the column with only buffer, PEPC eluted as a mixture of tetramer and dimer. The presence of 0.3 M sorbitol in both extraction and elution buffer resulted in the appearence of enzyme as a mixture of tetramer. dimer and monomers. These results provide a good demonstration of variability in oligomeric status of PEPC in leaf extracts and its modulation by glycerol and PEG.

PEPC is located in cytosol of mesophyll cells in leaves of C_4 plants. The reduction of nitrate to ammonium also occurs in C_4 mesophyll cells (Hatch, 1987). Biosynthesis of PEPC in C_4 leaves is modulated by the extent and form of nitrogen available to the plant. For e.g. the levels of PEPC increased when leaves of maize were fed with nitrate or ammonium (Sugiharto and Sugiyama, 1992). The effectiveness of ammonium salt as an inducer of PEPC biosynthesis was two fold greater than that of nitrate.

When a range of different salts were tested, PEPC in leaf extracts of *A. hypochondriacus* was stimulated by ammonium salts and to some extent by potassium ions. Acetate and sulphate salts of ammonium, besides chloride, stimulated the activity of PEPC. On the other hand, monovalent ions like lithium, sodium and rubidium had only a marginal effect on PEPC. The stimulation by potassium was significant, but not as marked as that by ammonium. The presence of calcium chloride decreased the activity of PEPC.

The inhibition of PEPC by calcium has been reported earlier (Gavalas and Manetas, 1980).

Although ammonium ions could stimulate *in vitro* the activity of PEPC (dark-form of the enzyme), the light-activated form of PEPC was not stimulated, but was slightly inhibited. The light-activated form of PEPC is known to differ from the dark-form in several characteristics: malate sensitivity, phosphorylation status and K_m for PEP (Rajagopalan et al., 1994). The absence of stimulation by ammonium chloride of PEPC from illuminated leaves indicates that only the dark-form of the enzyme has the conformational status capable of responding to ammonium ions.

In the presence of ammonium chloride, the V_{max} increased but the affinity to PEP was not altered. Double reciprocal plots revealed that the K_m for PEP, in the presence or absence of ammonium chloride, was similar. Also, there was no significant change in the sensitivity of the enzyme to malate in the presence of ammonium chloride. However, the response to G-6-P was quite different in the presence of ammonium chloride. There was only a marginal stimulation of G-6-P in the presence of ammonium chloride. Further, there was a decrease in PEPC activity at high concentrations of G-6-P in presence of ammonium chloride.

Our observations demonstrate that ammonium ion is an allosteric activator of PEPC from C_4 plants *in vitro*. Allosteric activation by ammonium of PEPC fits well with intercellular enzymic distribution. An increase in the availability of ammonium ions can stimulate PEPC and promote carbon amino acid metabolism. These results form an additional basis of a better nitrogen use efficiency in C_4 plants than that in C_3 species (Hatch, 1987).

PEPC is subjected to phosphorylation-dephosphorylation cascade during light/dark transitions *in vivo* (Jiao and Chollet, 1991; Nimmo, 1993; Rajagopalan et al., 1994). A protein-serine kinase phosphorylates PEPC, while a type 2A protein phosphatase dephosphorylates the enzyme. Experiments with

mesophyll protoplasts of maize (Devi and Raghavendra, 1992) and sorghum (Pierre et al., 1992) have shown that pH and calcium are important factors during light activation and phosphorylation of PEPC. Yet, the mechanism of action of calcium and its interaction with pH during PEPC phosphorylation are not clear. Experiments were therefore designed to investigate if calcium modulates PEPC or PEPC-PK or both.

The effect of calcium on PEPC was examined at either optimal (pH 7.8) or sub-optimal (pH 7.3) levels of pH. The presence of calcium inhibited PEPC, the inhibitory effect being more at pH 7.8 than at pH 7.3. The effect of calcium on PEPC was dependent on the light- or dark-form of the enzyme. The extent of inhibition by calcium (at pH 7.8) was only marginal in light-form of PEPC compared to dark-form. At pH 7.3, the degree of inhibition was marginal in case of both light and dark-forms. Low concentration of calcium was necessary for optimal activity of PEPC at both pH 7.3 and 7.8 in extracts from illuminated leaves.

The activity of PEPC-PK or PEPC-phosphorylation were studied either directly or indirectly. An indirect measure of PEPC-PK is the stimulation of PEPC activity on incubation of leaf extracts with ATP. Incubation with ATP stimulated PEPC activity and decreased the malate sensitivity particularly in extracts from illuminated leaves. As a result of marked stimulation by ATP of PEPC activity in extracts from illuminated leaves, there was an increase in L/D ratio. The effect of ATP was dependent on pH. The extent of stimulation by ATP was more at pH 7.8 than at pH 7.3. Calcium (even at a low concentration of calcium 20-50 μM) stimulated the PEPC activity during ATP incubation. Such stimulation by calcium was more at pH 7.3 than at pH 7.8. Pretreatment of leaves with EGTA *in vivo* decreased the extent of stimulation by ATP of PEPC activity (a measure of PEPC-PK) particularly in leaves exposed to light. Pretreatment with EGTA sharpened further the response to calcium.

A major conclusion from these experiments is that calcium affects PEPC and PEPC-kinase in different ways. Presence of calcium inhibits PEPC, particularly at pH 7.8. On the other hand, calcium is essential for PEPC-PK and promotes phosphorylation of PEPC.

Direct evidence of PEPC-phosphorylation was obtained by either incubation of leaf extracts with y-AT P *(in vitro)* or feeding intact leaves with Pi *(in vivo). In vivo* labelling of leaves with ^{32}Pi showed that the phosphorylation of PEPC was more in light than that in dark treated leaves. Most of the experiments were therefore conducted with illuminated leaves. Pretreatment of leaves with EGTA *in vivo* decreased the extent of PEPC phosphorylation. With 2 mM EGTA, phosphorylation of PEPC was reduced to almost nil, but could be restored by addition of calcium during incubation with γ-AT˜P. The stimulatory effect of calcium (20 μM being optimal) was amplified due to the pretreatment of leaves with EGTA. Besides calcium, magnesium also was necessary for PEPC phosphorylation and both had a synergistic effect.

A few experiments were undertaken to study if calcium-related secondary messengers, such as calmodulin, play any role in PEPC-phosphorylation. When leaves were fed through petiole, with metabolic inhibitors such as TFP (a calmodulin antagonist), phosphorylation of PEPC in light decreased. Phosphorylation of PEPC was also inhibited by verapamil, lanthanum and diltiazem (calcium channel blockers). These results suggest that besides calcium, calmodulin and calcium channel-activity play an important role phosphorylation of PEPC ***in*** *vitro.*

To summarize, major conclusions from the present study are:

1. PEPC purified from *Amaranthus hypochondriacus*, had one of the highest activities reported in C_4 plants. The enzyme was stable for more than 4 months, if stored in liquid nitrogen along with 50% (v/v) glycerol.

2. The antibody of PEPC from *A. hypochondriacus* (a C_4-dicot) reacted with PEPC from both C_3 and C_4 plants, but with a large variation in the extent of cross-reaction. The antibody of PEPC from *A. hypochondriacus*, a C_4 dicot showed much stronger cross-reaction with the enzyme of *A. viridis* and *A. pungens* (C_4 dicots), than with that of C_3 species or C_3--C_4 intermediates or Z. *mays* (C_4 monocot). Similarly, anti-PEPC antiserum from Z. *mays* showed faint cross-reactivity with the enzyme in leaf extracts of C_4 dicots, or C_3 species or C_3-C_4 intermediates.

3. The presence of PEG, a compatible solute, enhanced markedly the activity of enzyme, when checked with either purified enzyme or crude leaf extracts. Maximum activation of PEPC was obtained, when PEG was present during both extraction from leaves and assay of the enzyme. The affinity for PEP decreased, the response to G-6-P was enhanced and the enzyme became less malate sensitive in presence of PEG.

4. The oligomeric status of PEPC (tetramer, dimer and monomer) was examined by gel filtration in presence or absence of compatible solutes during enzyme extraction from leaves.

5. Presence of both PEG and/or glycerol both during extraction and/or elution helped in maintaining PEPC predominantly in tetrameric form.

6. In the absence of PEG or glycerol and sorbitol during extraction and elution. PEPC was present as a dimer and/or monomer, with a small proportion of tetramer.

7. Ammonium ions stimulated the PEPC activity *in vitro* from C_4 plants. The activation by ammonium of PEPC was at the allosteric site of enzyme.

8. Calcium affected PEPC and PEPC-PK in different ways and the effect was dependent on pH. The presence of calcium inhibited PEPC activity, particularly at pH 7.8, but had very little effect at pH 7.3. Calcium promoted PEPC phosphorylation, both *in vitro* and *in vivo.*

9. Marked stimulation by ATP in the presence of calcium either at pH 7.3 or pH 7.8 indicated that calcium promoted phosphorylation of PEPC. Both calcium and magnesium were necessary for PEPC-phosphorylation and have a synergistic effect on PEPC-PK.

10. Pretreatment of leaves with EGTA *in vivo* decreased the activity and phosphorylation of PEPC in leaves exposed to light (but not in darkness). Pretreatment with EGTA sharpened the response to calcium.

11. Labelling of leaves with ^{32}Pi *in vivo* revealed that the phosphorylation of PEPC was more in light than that in dark-adapted leaves.

12. TFP (a calmodulin antagonist) as well as verpamil, lanthanum, diltiazem (calcium channel blockers) decreased phosphorylation of PEPC in light. These results indicate that both calcium and calmodulin are involved during PEPC phosphorylation enhanced under illumination.

Chapter 9

Literature Cited

Chapter 9

Literature cited

Adams CA, Leung F, Sun SSM (1986) Molecular properties of phospho*enol*-pyruvate carboxylase from C_3, C_3-C_4 intermediates, and C_4 *Flaveria* species. Planta **167:** 216-225

Andreo CS, Gonzalez DH, Iglesias AA (1987) Higher plant phospho*enol*pyruvate carboxylase. Structure and regulation. FEBS Lett **213:** 1-8

Angelopoulos K, Gavalas NA (1988) Reversible cold inactivation of C_4-phospho*enol*pyruvate carboxylase: Factors affecting reactivation and stability. J Plant Physiol **132:** 714-719

Angelopoulos K, Gavalas NA (1991) Effects of anions in ion exchange chromatography on the specific activity of C_4 phospho*enol*pyruvate carboxylase. J Plant Physiol **137:** 363-367

Angelopoulos K, Zervondakis G, Vonquiouklakis S, Gavalas NA (1990) Differences among C_4 species in cold inactivation of phospho*enol*pyruvate carboxylase and the effect of pH on it. Physiol Plant **79:** suppl., 2 (Abstr) 58

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris.* Plant Physiol **24:** 1-15

Arrio-Dupont M, Bakrim N, Echevarria C, Gadal P, Le Marechal P, Vidal J (1992) Compared properties of phospho*enol*pyruvate carboxylase from dark- and light-adapted sorghum leaves. Use of a rapid purification technique by immunochromatography. Plant Sci **81:** 37-46

Ausenhus SL, O'Leary MH (1992) Hydrolysis of phospho*enol*pyruvate catalyzed by phospho*enol*pyruvate carboxylase from *Zea mays.* Biochemistry **31:** 6427-6431

Bakrim N, Echevarria C, Cretin C, Arrio-Dupont M, Pierre JN, Vidal J, Chollet R, Gadal P (1992) Regulatory phosphorylation of sorghum leaf phospho*enol*pyruvate carboxylase. Identification of the protein-serine kinase and some elements of the signal-transduction cascade. Eur **J** Biochem **204:** 821-830

Bandarian V, Poehner WJ, Grover SD (1992) Metabolite activation of crassulacean acid metabolism and C_4 phospho*enol*pyruvate carboxylase. Plant Physiol **100:** 1411-1416

Bandurski RS, Greiner CM (1953) The enzymatic synthesis of oxaloacetate from phosphoryl-*enol*pyruvate and carbon dioxide. J Biol Chem **204:** 781-786

Baur B, Dietz K-J, Winter K (1992) Regulatory protein phosphorylation of phospho*enol*pyruvate carboxylase in the facultative crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. Eur J Biochem 209:95-101

Betz M, Dietz K-J (1991) Immunological characterization of two dominant tonoplast polypeptides. Plant Physiol 97: 1294-1301

Blum H, Beier H, Grass HJ (1987) Improved silver stain of plant proteins. RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93-97

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Brulfert J, Arrabaca MC, Guerrier D, Queiroz O (1979) Changes in the isozymic pattern of phosph*enol*pyruvate. An early step in periodic control of crassulacean acid metabolism. Planta **146:** 129-133

Buchanan BB (1991) Regulation of CO_2 assimilation in oxygenic photosynthesis: The ferrodoxin/thioredoxin system. Perspective on **its** discovery, present status and future development. Arch Biochem Biophys **288:** 1-9

Buchanan BB (1992) Carbon dioxide assimilation in oxygenic and anoxygenic photosynthesis. Photosynth Res **33:** 147-162

Budde RJA, Chollet R (1986) *In vitro* phosphorylation of maize leaf phospho*enol*pyruvate carboxylase. Plant Physiol **82:** 1107-1114

Budde RJA, Chollet R (1988) Regulation of enzyme activity in plants by reversible phosphorylation. Physiol Plant 72: 435-439

Bush DS (1995) Calcium regulation in plant cells and its role in signalling. Annu Rev Plant Physiol Plant Mol Biol. **46:** 95-122

Carter PJ, Nimmo HG, Fewson CA, Wilkins MB (1991) Circadian rhythms in the activity of a plant protein kinase. EMBO J **10:** 2063-2068

Champigny M-L and Foyer CH (1992) Nitrate activation of cytosolic protein kinases diverts photosynthesis carbon from sucrose to amino acid biosynthesis. Plant Physiol **100:** 7-12

Chen JH, Jones RF (1970) Multiple forms of phospho*enol*pyruvate carboxylase from *Chlamydomonas reinhardtii.* Biochim Biophys Acta **214:318-325**

Chidwick K (1994) Development of an assay for phospho*enol*pyruvate carboxylase from crassulacean acid metabolism plants. Biochem Soc Trans **22:** 204S

Chollet R, Budde RJA, Jiao J-A and Roeske CA (1990) Light/dark regulation of C_4-photosynthesis enzymes by reversible phosphorylation. *In* M Baltchefsky, ed, Current Research in Photosynthesis, Vol. IV. Kluwer Academic Publishers. Dordrecht, Pp 135 -142.

Coombs J, Baldry CW, Bucke C (1973) The C-4 pathway in *Pennisetum purpureum*. The allosteric nature of PEP carboxylase. Planta **110:** 95-107

Coombs J, Maw SL, Baldry CW (1974) Metabolic regulation in C_4 photosynthesis: PEP carboxylase and energy charge. Planta **117:** 279-292

Coombs J, Maw SL, Baldry CW **(1975)** Metabolic regulation in C_4 photosynthesis: The inorganic carbon substrate for PEP carboxylase. Plant Sci Lett **4: 97-102**

Cretin C, Keryer E, Tagu D, Lepiniec L, Vidal J, Gadal P (1990) Complete cDNA sequence of sorghum phospho*enol*pyruvate carboxylase involved in C_4 photosynthesis. Nucl Acid Res **18:** 658

Cretin C, Santi S, Keryer E, Lepiniec L, Tagu D, Vidal J, Gadal P (1991) The phospho*enol*pyruvate carboxylase gene family of sorghum: Promoter structures, amino acid sequences and expression of genes. Gene **99:** 87-94

Cretin C, Vidal J, Suzuki A, Gadal P (1984) Isolation of plant phospho*enol*-pyruvate carboxylase by high performance size-exclusion chromatography. J Chromatogr **315:** 430-434

Dai Z, Ku MSB, Edwards GE (1994) Effects of growth regulators on the induction of crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* L. Planta **192:** 287-294

Davis BJ (1964) Disc electrophoresis I. Background and theory. Ann N Y Acad Sci **121:** 321-349

Davis DD (1973) Control of and by pH. Symp Soc Expt Biol **27:** 513-530

Davis DD (1979) The central role of phospho*enol*pyruvate in plant metabolism. Annu Rev Plant Physiol **30:** 131-158

Denecke M, Schulz M, Fischer C, Schnabl H (1993) Partial purification and characterisation of stomatal phospho*enol*pyruvate carboxylase from *Vicia faba*. Physiol Plant **87:** 96-102

Devi MT, Raghavendra AS (1992) Light activation of phospho*enol*pyruvate carboxylase in maize mesophyll protoplasts. J Plant Physiol **138: 434-439**

Doncaster HD, Leegood RC (1987) Regulation of phospho*enol*pyruvate carboxylase activity in maize leaves. Plant Physiol **84:** 82-87

Dreier W, Preusser E, Griindel M (1992) The regulation of the activity of soluble starch synthase in spinach leaves by a calcium-calmodulin dependent protein kinase. Biochem Physiol Pflanzen **188:** 81-96

Drilias P, Gousias H, Manetas Y, Gavalas NA (1994) Temperature dependence of phosopho*enol*pyruvate carboxylase activity in the presence of cosolutes. Photosynthetica **30:** 225-232

Duff SMG, Andreo CS, Pacquit V, Lepiniec L, Sarath G, Condon SA, Vidal J, Gadal P, Chollet R (1995) Kinetic analysis of the non-phosphorylated, in vitro phosphorylated, and phosphorylation-site-mutant (Asp8) forms of intact recombinant C_4 phospho*enol*pyruvate carboxylase from sorghum. Eur J Biochem **228:** 92-95

Duff SMG, Chollet R (1995) ***In*** *vivo* regulation of wheat-leaf phospho*enol*pyruvate carboxylase by reversible phosphorylation. Plant Physiol **107:** 775-782

Echevarría C, Pacquit V, Bakrim N, Osuna L, Delgado B, Arrio-Dupont M, Vidal J (1994) The effect of pH on the covalent and metabolic control of C_4 phospho*enol*pyruvate carboxylase from sorghum leaf. Arch Biochem Biophys **315:** 425-430

Echevarría C, Vidal J, Le Maréchal P, Brulfert J, Ranjeva R, Gadal P (1988) The phosphorylation of sorghum leaf phospho*enol*pyruvate carboxylase is a Ca^{2+}-calmodulin dependent process. Biochem Biophys Res Commun **155:** 835-840

Echevarria C, Vidal J, Jiao J-A, Chollet R (1990) Reversible light activation of phospho*enol*pyruvate carboxylase protein-serine kinase in maize leaves. FEBS Lett 275: 25-28

Edwards GE, Huber SC (1981) The C_4 pathway. *In* MD Hatch, NR Boardman, eds, The Biochemistry of Plants, A Comprehensive Treatise, **Vol** 8. Photosynthesis. Academic press, New York, pp 237-280.

Edwards GE, Ku MSB, Monson RK (1985) C_4 photosynthesis. *In* Barber J, Baker NR, eds, Topics in Photosynthesis, Vol VI. Photosynthetic Mechanisms and the Environment. Elsevier, New York, pp 287-327.

Edwards GE, Walker DA (1983) C_3, C_4: Mechanisms and Cellular and Environmental Regulation of Photosynthesis. Blackwell Scientific, Oxford, UK.

Fujita N, Miwa T Ishijima S (1984) The primary structure of phospho*enol*-pyruvate carboxylase of *Escherichia coli*. Nucleotide sequence of the ppc gene and deduced amino acid sequence. J Biochem **95:** 909-916

Furbank RT, Foyer CH (1988) C_4 plants as valuable model experimental systems for the study of photosynthesis. New Phytol **109:** 265-277

Furbank RT, Hatch MD (1987) Mechanism of C_4 photosynthesis: The size and composition of the inorganic carbon pool in bundle sheath cells. Plant Physiol **85:** 958-964

Gadal P (1983) Phospho*enol*pyruvate carboxylase and nitrogen fixation. Physiol Veg **21:** 1069-1074

Gavalas NA, Manetas Y (1980) Calcium inhibition of phospho*enol*pyruvate carboxylase: Possible physiological consequences for C_4 photosynthesis. Z Pflanzenphysiol **100:** 179-184

Gavalas NA, Caravatas S, Manetas Y (1981) Sigmoid rate curves of photosynthetic (C_4) PEP carboxylase as an artifact of the assay: Factors affecting inactivation-reactivation of the enzyme. *In* G Akoyunoglou, ed, Photosynthesis, Vol 4. Balaban International Science Services, Philadelphia, pp 91-98.

Gavalas NA, Caravatas S, Manetas Y (1982) Factors affecting **a** fast and reversible inactivation of photosynthetic phospho*enol*pyruvate carboxylase. Photosynthetica 16: 49-58

Gayathri J, Raghavendra AS (1994) Ammonium ions stimulate *in vitro* the activity of phospho*enol*pyruvate carboxylase from leaves of *Amaranthus*

hypochondriacus, a C_4 plant: Evidence for allosteric activation. Biochem Mol Biol Int **33:** 337-344

Gekko K, Timasheff SN (1981) Mechanism of protein stabilization by glycerol: Preferential hydration in glycerol-water mixtures. Biochemistry 20: 4667-4676

Gillina HJ, Greally JF (1993) Enzyme-linked immunosorbent assay for measurement of IgG in serum of rabbit. Clin Chim Acta **216:** 23-28

Gilory S, Hughes WA, Trewavas AJ (1987) Calmodulin antagonists increase free cytosolic calcium levels in plant protoplasts *in vivo*. FEBS Lett **212:** 133-137

Goatly MB, Smith H (1974) Differential properties of phospho*enol*pyruvate carboxylase from etiolated and green sugar cane. Planta **117:** 67-73

González DH, Andreo CS **(1989)** The use of substrate analogues to study the active-site structure and mechanism of PEP carboxylase. Trends Biochem Sci **14:** 24-27

Gonzalez DH, Iglesias AA, Andreo CS (1984) On the regulation of phospho*enol*pyruvate carboxylase activity from maize leaves by L-malate. Effect of pH. J Plant Physiol **116:** 425-434

Gonzalez DH, Iglesias AA, Andreo CS (1987) Interaction of acetylphosphate and carbamyl phosphate with plant phospho*enol*pyruvate carboxylase. Biochem J **241:** 543-548

Gupta SK, Ku MSB, Lin JH, Zhang D, Edwards GE (1994) Light/dark modulation of phospho*enol*pyruvate carboxylase in C_3 and C_4 species. Photosynth Res **42:** 133-143

Guern J, Mathieu Y, Kurkdjian A (1983) Phospho*enol*pyruvate carboxylase activity and the regulation of intracellular pH in plant cells. Physiol Veg **21:** 855-866

Hague DR, Sims TL (1980) Evidence for light-stimulated synthesis of phospho*enol*pyruvate carboxylase in leaves of maize. Plant Physiol 66: 505-509

Hammel KE, Cornwell KL, Bassham JA (1979) Stimulation of dark CO_2 fixation by ammonia in isolated mesophyll cells of *Papaver somniferum* L. Plant Cell Physiol **20:** 1523-1529

Hampp R and Schnabl H (1984) Adenine and pyridine nucleotide status of isolated *Vicia* guard cell protoplasts during K^+-induced swelling. Plant Cell Physiol **25:** 1233-1239

Hatch MD (1987) C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochim Biophys Acta 895: 81-106

Hatch MD (1992) C_4 photosynthesis: An unlikely process full of surprises. Plant Cell Physiol 33: 333-342

Hatch MB, Heldt HW (1985) Synthesis, storage and stability of (4-^{14}C) oxaloacetic acid. Anal Biochem **145:** 393-397

Hatch MD, Osmond CB, Troughton JH, Bjorkman O (1972) Physiological and biochemical characteristics of C_3 and C_4 *Atriplex* species and hybrids in relation to the evolution of the C_4 pathway. Carnegie Inst Wash Year book **71:** 135-141

Hayakawa S, Matsunaga K, Sugiyama T (1981) Light induction of phospho*enol*pyruvate carboxylase in etiolated maize leaf tissue. Plant Physiol 67: 133-138

Hermans J, Westhoff P (1990) Analysis of expression and evolutionary relationships of phospho*enol*pyruvate carboxylase genes in *Flaveria trinervia* (C_4) and *F. pringlei* (C_3). Mol Gen Genet **224:** 459-468

Miller RG (1970) Transients in the photosynthetic carbon reduction cycle produced by iodoacetic acid and ammonium ions. J Exp Bot **21:** 628-638

Hodgson RJ, Plaxton WC (1995) Effect of polyethylene glycol on the activity, intrinsic fluorescence and oligomeric structure of castor seed cytosolic fructose-1,6-bisphosphatase. FEBS Lett **368:** 559-562

Hofner R, Vazquez-Moreno L, Abou-Mandour AA, Bohnert HJ, Schmitt JM (1989) Two isoforms of phospho*enol*pyruvate carboxylase in the facultative CAM plant *Mesembryanthemum crystallinum*. Plant Physiol Biochem 27: 803-810

Huber SC, Edwards GE (1975) Inhibition of phospho*enol*pyruvate carboxylase from C_4 plants by malate and aspartate. Can J Bot **53:** 1925-1933

Huber SC, Huber JL, McMichael RW **Jr (1994)** Control of plant enzyme activity by reversible protein phosphorylation. Int Rev Cytol **149:** 47-98

Huber SC, Sugiyama T (1986) Changes in sensitivity to effectors of maize leaf phospho*enol*pyruvate carboxylase during light/dark transitions. Plant Physiol 81: 674-677

Huber SC, Sugiyama T, Akazawa **T (1986)** Light modulation of maize leaf phospho*enol*pyruvate carboxylase. Plant Physiol 82: 550-554

Hudspeth RL, Glackin CA, Bonner J, Grula JW (1986) Genomic and cDNA clones for maize phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase: Expression of different gene-family members in leaves and roots. Proc Natl Acad Sci USA **83:** 2884 - 2888

Hudspeth RL, Grula JW (1989) Structure and expression of the maize gene encoding the phospho*enol*pyruvate carboxylase isozyme involved in C_4 photosynthesis. Plant Mol Biol 12: 579-589

Iglesias AA, Andreo CS (1984) On the molecular mechanism of maize phospho*enol*pyruvate carboxylase activation by thiol compounds. Plant Physiol 75: 983-987

Iglesias AA, Andreo CS (1989) Purification of NADP-malic enzyme and phospho*enol*pyruvate carboxylase from sugarcane leaves. Plant Cell Physiol **30:** 399-405

Iglesias AA, Gonzalez DH, Andreo CS (1986) Purification and molecular and kinetic properties of phospho*enol*pyruvate carboxylase from *Amaranthus viridis* L. leaves. Planta 168:239-244

Ishijima S, Katagiri F, Kadaki T, Izui K, Katsuki H, Nishikawa K, Nakashima H, Ooi (1985) Comparison of amino acid sequences between phospho*enol*pyruvate carboxylases from *Escherichia coli* (allosteric) and *Anacystis nidulans* (non-allosteric): identification of conserved and variable regions. Biochem Biophys Res Commun **133:** 436-441

Izui K, Ishijima S, Yamaguchi Y, Katagiri F, Murata T, Shigesada K, Sugiyama T, Katsuki H (1986) Cloning and sequence analysis of cDNA encoding active phospho*enol*pyruvate carboxylase of the C_4 pathway from maize. Nucl Acid Res 14: 1615-1628

Izui K, Kawamura T, Okumura S, Toh H (1992) Molecular evolution of phospho*enol*pyruvate carboxylase for C_4 photosynthesis in maize. *In* N Murata, ed, Research in Photosynthesis, Vol III. Kluwer Academic Publishers, Dordrecht, pp 827-830

Janc JW, O'Leary MH, Cleland WW (1992a) A kinetic investigation of PEPC from *Zea mays.* Biochemistry **31:** 6421-6426

Janc JW, Urbauer JL, O'Leary MH, Cleland WW (1992b) Mechanistic studies of phospho*enol*pyruvate carboxylase from Zea mays with (Z)"- and (E)-3-fluorophospho*enol*pyruvate as substrates. Biochemistry **31:** 6432-6440

Jawali N (1990) The dimeric form of phospho*enol*pyruvate carboxylase isolated from maize: Physical and kinetic properties. Arch Biochem Biophys **277:** 61-68

Jiao J-A, Chollet R (1988) Light/dark regulation of maize leaf phospho*enol*-pyruvate carboxylase by ***in*** *vivo* phosphorylation. Arch Biochem Biophys **261:** 409-417

Jiao J-A, Chollet R (1989) Regulatory seryl-phosphorylation of C_4 phospho*enol*pyruvate carboxylase by **a** soluble protein kinase from maize leaves. Arch Biochem Biophys **269:** 526-535

Jiao J-A, Chollet R (1990) Regulatory phosphorylation of serine-15 in maize phospho*enol*pryuvate carboxylase by a C_4-leaf protein-serine kinase. Arch Biochem Biophys **283:** 300-305

Jiao J-A, Chollet R (1991) Posttranslational regulation of phospho*enol*pyruvate carboxylase in C_4 and Crassulacean acid metabolism plants. Plant Physiol 95: 981-985

Jiao J-A, Chollet R (1992) Light activation of maize phospho*enol*pyruvate carboxylase protein-serine kinase activity is inhibited by mesophyll and bundle sheath-directed photosynthesis inhibitors. Plant Physiol 98: 152-156

Jiao J-A, Vidal J, Echevarria C, Chollet R (1991) *In vivo* regulatory phosphorylation site in C_4-leaf phospho*enol*pyruvate carboxylase from maize and sorghum. Plant Physiol 96: 297-301

Jones R, Wilkins MB, Coggins JR, Fewson CA, Malcolm ADB (1978) Phospho*enol*pyruvate carboxylase from the crassulacean plant *Bryophyllum fedtschenkoi* Hamet et Perrier. Purification, molecular and kinetic properties. Biochem J **175:** 391-406

Kanazawa T, Kanazawa K, Krik M, Bassham JA (1972) Regulatory effects of ammonia on carbon metabolism in *Chlorella pyrenoidosa* during photosynthesis and respiration. Biochim Biophys Acta 256: 656-669

Karabourniotis G, Manetas Y, Gavalas NA (1983) Photoregulation of phospho*enol*pyruvate carboxylase in *Salsola soda* L. and other C_4 plants. **Plant** Physiol **73:** 735-739

Karabourniotis G, Manetas Y, Gavalas NA (1985) Detecting photoactivation of phospho*enol*pyruvate carboxylase in C_4 plants. An effect of pH. Plant Physiol **77:** 300-302

Kawaura T, Shigesada K, Toh H, Okumura S, Yanagisawa S, Izui K (1992) Molecular evolution of phospho*enol*pyruvate carboxylase for C_4 photosynthesis in maize: Comparison of its cDNA sequence with a newly isolated cDNA encoding an isozyme involved in the anaplerotic function. J Biochem **112:** 147-154

Kleczkowski LA, Edwards GE (1990) Hysteresis and reversible cold inactivation of maize PEPC. Z Naturforsch 45: 42-46

Kluge M (1983) The role of phospho*enol*pyruvate carboxylase in C_4-photosynthesis and crassulacean acid metabolism. Physiol Veg **21:** 817-825

Kluge M, Maier P, Brulfert J, Faist **K, Wollny, B (1988)** Regulation of phospho*enol*pyruvate carboxylase in crassulacean acid metabolism: ***in*** *vitro* phosphorylation of the enzyme. J Plant Physiol **133:** 252-256

Koizumi N, Sato F, Terano Y, Yamada Y (1991) Sequence analysis of cDNA encoding phospho*enol*pyruvate carboxylase from cultured tobacco cells. Plant Mol Biol **17:** 535-539

Krall JP, Edwards GE (1993) PEP carboxylase from two C_4 species of *Panicum* with markedly different, susceptibilities to cold inactivation. Plant cell Physiol **34:** 1-11

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature **227:** 680-685

Latzko E, Kelly GJ (1983) The many-faceted function of phospho*enol*pyruvate carboxylase in C_3 plants. Physiol Veg **21:** 805-815

Leblova S, Strakosova A, Vojtechova M (1991) Regulation of activity of phospho*enol*pyruvate carboxylase isolated from germinating maize *(lea mays* L.) seeds by some metabolites. Biol Plant (Praha) **33:** 66-74

Lee JC, Lee LY (1979) Interaction of calf brain tubulin with poly(ethylene glycols). Biochemistry 18: 5518-5526

Leegood RC (1993) Carbon dioxide-concentrating mechanisms. *In* Lea PJ, Leegood RC, eds, Plant Biochemistry and Molecular Biology. John Wiley & Sons Ltd, pp **48-72**

Lepiniec L, Keryer E, Philippe H, Gadal P, Cretin C (1993) Sorghum phospho*enol*pyruvate carboxylase gene family: structure, function and molecular evolution. Plant Mol Biol 21: 487-502

Lepiniec L, Vidal J, Chollet R, Gadal P, Cretin C (1994) Phospho*enol*pyruvate carboxylase: Structure, regulation and evolution. Plant Sci 99: 111-124

Li B and Chollet R (1994) Salt induction and the partial purification/characterization of phosph*enol*pyruvate carboxylase protein-serine kinase from an inducible crassulacean-acid-meatbolism (CAM) plant, *Mesembryanthemum crystallinum* L. Arch Biochem Biophys **314:** 247-254

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin *phenol* reagent. J Biol Chem 193:265-275

Maheswari V, Bharadwaj R (1991) Photoactivation and regulation of maize leaf phospho*enol*pyruvate carboxylase. Indian J Exp Biol 29: 1058-1061

Manetas Y (1982) Changes in properties of phospho*enol*pyruvate carboxylase from the CAM plant *Sedum praealtum* DC. upon dark/light transition and their stabilization by glycerol. Photosynth Res 3: 321-333

Manetas Y (1990) A re-examination of NaCl effects on phospho*enol*pyruvate carboxylase at high (physiological) enzyme concentrations. Physiol Plant **78:** 225-229

Manetas Y, Gavalas NA (1982) Evidence for essential sulphydryl groups in photosynthetic phospho*enol*pyruvate carboxylase: protection by

substrate, metal-substrate and glucose-6-phosphate against *p*-chloro mercurbenzoate inhibition. Photosynthetica **16:** 59-66

Manetas Y, Petropoulou Y, Karabourniotis G (1986) Compatible solutes and their effects on phospho*enol*pyruvate carboxylase of C_4-halophytes. Plant Cell Environ 9: 145-151

Mares J, Leblova S (1980) Phospho*enol*pyruvate carboxylase from leaves of maize, sorghum and millet. Photosynthetica 14: 25-31

Maruyama H, Lane MD (1962) Purification and properties of phospho-*enol*pyruvate carboxylase from the germinating peanut cotyledon. Biochim Biophys Acta 65: 207-218

McElwain EF, Bohnert HJ, Thomas JC (1992) Light moderates the induction of phospho*enol*pyruvate carboxylase by NaCl and abscisic acid in *Mesembryanthemum crystallinum*. Plant Physiol 99: 1261-1264

McNaughton GAL, Fewson CA, Wilkins MB, Nimmo HG (1989) Purification, oligomerization state and malate sensitivity of maize leaf phospho*enol*pyruvate carboxylase. Biochcm J **261:** 349-355

McNaughton GAL, Macintosh C, Fewson CA, Wilkinson MB, Nimmo HG (1991) Illumination increases the phosphorylation state of maize leaf phospho*enol*pyruvate carboxylase by causing an increase in the activity ofaprotein kinase. Biochim Biophys Acta **1093:** 189-195

Medina A, Aragon **JJ,** Sols A (1985) Effects of polyethylene glycol on the kinetic behaviour of pyruvate kinase and other potentially regulatory liver enzymes. FEBS Lett **180:** 77-80

Mehta M, Saharan MR, Singh R (1995) Purification and characterization of phospho*enol*pyruvate carboxylase from developing seeds of *Brassica*. J Plant Biochem Biotech 4: 11-16

Melzer E, O'Leary **MH** (1987) Anapleurotic CO_2 fixation by phospho*enol*pyruvate carboxylase in C_3 plants. Plant Physiol 84: 58-60

Meyer CR, Willeford KO, Wedding RT (1991) Regulation of phospho*enol*pyruvate carboxylase from *Crassula argentea*: Effect of incubation with ligands and dilution on oligomeric state, activity, and allosteric properties. Arch Biochem Biophys **288:** 343-349

Michalke B, Schnabl H (1990) Modulation of the activity of phospho*enol*pyruvate carboxylase during potassium induced swelling of guard cell protoplasts of *vicia faba* L. after light and dark treatments. Planta **180:** 188-193

Miekka SI, Ingham KS (1978) Influence of self-association of proteins on their precipitation by poly(ethylene glycol). Arch Biochem Biophys **191:** 525-536

Miyachi S, **Miyachi** S (1985) Ammonia induces starch degradation in *Chlorella* cells. Plant Cell **Physio] 26:** 245-252

Miziorko HM, Nowak T, Mildvan AS (1974) Spinach leaf phospho*enol*pyruvate carboxylase: Purification, properties, and kinetic studies. Arch Biochem Biophys **163:** 378-389

Moorehead GBG, Hodgson RJ, Plaxton WC (1994) Copurification of cytosolic fructose-1,6-bisphosphatase and cytosolic aldolase from endosperm of germinating castor oil seeds. Arch Biochem Biophys **512:** 326-335

Miiller R, Steigner W, Gimmler H, Kaiser WM (1990) Effect of ammonium on dark-CO_2 fixation and on cytosolic and vacuolar pH values in *Eremosphaera viridis de bary* (Chlorococcales). J Expt Bot **41:** 441-448

Mukerji SK (1977) Corn leaf phospho*enol*pyruvate carboxylase. Purification and properties of two isoenzymes. Arch Biochem Biophys **182:** 343-351

Mukerji SK, Ting IP (1971) Phospho*enol*pyruvate carboxylase isoenzymes. Separation and properties of three forms from cotton leaf tissue. Arch Biochem Biophys **143:** 297-317

Nakamoto H, Ku MSB, Edwards GE (1983) Photosynthetic characteristics of C_3-C_4 intermediate *Flaveria* species. II. Kinetic properties of phospho*enol*pyruvate carboxylase from C_3, C_4 and C_3-C_4 intermediate species. Plant Cell Physiol **24:** 1387-1393

Nash D, Paleg LG, Wiskich JT (1982) Effect of proline, betaine and some other solutes on the heat stability of mitochondrial enzymes. Aust J Plant Physiol 9: 47-57

Nimmo HG (1993) The regulation of phospho*enol*pyruvate carboxylase by reversible phosphorylation. *In* NH Battey, HG Dickinson, AM Hetherington, eds, Society for Experimental Biology Seminar Series, Vol. 53. Post-translational Modifications in Plants. Cambridge University Press. Cambridge. pp 161-170.

Nimmo GA, McNaughton GAL, Fewson CA, Wilkins MB, Nimmo HG (1987) Changes in the kinetic properties and phosphorylation state of phospho*enol*pyruvate carboxylase in Zea mays leaves in response **to** light and dark. FEBS Lett **213:** 18-22

Nimmo HG, Nimmo GA (1982) A general method for the localization of enzymes that produce phosphate, pyrophosphate or CO_2 after polyacrylamide gel electrophoresis. Anal Biochem **121:** 17-22

Nimmo GA, Nimmo HG, Fewson CA, Wilkins MB (1984) Diurnal changes in the properties of phospho*enol*pyruvate carboxylase in *Bryophyllum* leaves: a possible covalent modification. FEBS Lett **178:** 199-203

Nimmo GA, Nimmo HG, Hamliton ID, Fewson CA, Wilkins MB (1986) Purification of the phosphorylated night form and dephosphorylated day form of phospho*enol*pyruvate carboxylase from *Bryophyllum fedtschenkoi*. Biochem J **239:** 213-220

Nishikido T, Takanashi H (1973) Glycine activation of PEP carboxylase from monocotyledonous C_4 plants. Biochem Biophys Res Commun 45: 716-722

Nott DL, Osmond CB (1982) Purification and properties of phospho*enol*pyruvate carboxylase from plants with crassulacean acid metabolism. Aust J Plant Physiol **9:** 409-422

Ogawa N, Okumara J, Izui K (1992) A Ca^{2+}-dependent protein kinase phosphorylates phospho*enol*pyruvate carboxylase in maize. FEBS Lett **302:** 86-88

Ogawa N, Izui K (1992) Regulatory phosphorylation of C_4-phospho*enol*pyruvate carboxylase by a Ca^{2+}-dependent protein kinase from maize leaves. *In* N Murata, ed, Research in Photosynthesis, Vol. III. Kluwer Academic Publishers, Dordrecht, pp 831-834.

Ohmori M, Oh-hama T, Furihata K, Miyachi S (1986) Effects of ammonia on cellular pH of *Anabena cylindrica* determined with ^{31}P NMR spectroscopy. Plant Cell Physiol 27: 563-566

Oishi M (1971) The separation of T-even bactetiophage DNA from host DNA by hydroxyapatite chromatography. *In* L Grossman, K Moldave, eds, Methods of Enzymology, Vol 21. Academic press, New York, **pp** 140-147.

O'Leary MH (1982) Phospho*enol*pyruvate carboxylase: An enzymologist`s view. Annu Rev Plant Physiol **33:** 297-315

O'Leary MH (1983) Mechanism of action of phospho*enol*pyruvate carboxylase. Physiol Veg **21:** 883-888

O'Leary MH Rife JE, Slater JD (1981) Kinetic and isotope effects studies of maize phospho*enol*pyruvate carboxylase. Biochemistry **20:** 7308-7314

Outlaw WH Jr (1990) Kinetic properties of guard-cell phospho*enol*pyruvate carboxylase. Biochem Physiol Pflanzen **186:** 317-325

Paleg LG, Douglas TJ., Van Daal, A Keech DB (1981) Proline, betaine and other organic solutes protect enzymes against heat inactivation. Aust J Plant Physiol 8: 107-114

Pathirana SM, Vance CP, Müller SS, Gantt JS (1992) Alfalfa root nodule phospho*enol*pyruvate carboxylase: characterization of the cDNA and expression in effective and plant-controlled ineffective nodules. Plant Mol Biol **20:** 437-450

Pays AGG, Jones R, Wilkins MB, Fewson CA, Malcolm ADB (1980) Kinetic analysis of effectors of phospho*enol*pyruvate carboxylase from *Bryophyllum fedtschenkoi.* Arch Biochem Biophys **614:** 151-162

Perrot-Rechenmann, Vidal J, Brulfert J, Brulet A, **Gadal P (1982)** A comparative immunocytochemical localization study of phospho*enol*pyruvate carboxylase in leaves of higher plants. Planta **155:** 24-30

Peterson **JB,** Evans HJ (1978) Properties of pyruvate kinase from soybean nodule cytosol. Plant Physiol **61:** 909-914

Peterson JB, Evans HJ (1979) Phospho*enol*pyruvate carboxylase from soybean nodule. Evidence for isoenzymes and kinetics of the most active component. Biochim Biophys Acta 567: 445-452

Petropoulou Y, Manetas Y, Gavalas NA (1990) Intact mcsophyll protoplasts from *Zea mays* as a source of phospho*enol*pyruvate carboxylase unaffected by extraction: Advantages and limitations. Physiol Plant **80:** 605-611

Pierre JN, Pacquit V, Vidal J, Gadal P (1992) Regulatory phosphorylation of phospho*enol*pyruvate carboxylase in protoplasts from sorghum mesophyll cells and the role of pH and Ca^{+} as possible components of the light-transduction pathway. Eur J Biochem **210:** 531-538

Podesta FE, Colombo SL, Andreo CS **(1995)** Purification and characterisation of the light and dark forms of phospho*enol*pyruvate carboxylase from the dicot plant *Amaranthus viridis* L. An examination of its kinetic and regulatory properties in the presence of water-alcohol binary solvents. Plant Cell Physiol 36: 1471-1476

Podesta FE, Andreo CS (1989) Maize leaf phospho*enol*pyruvate carboxylase. Oligomeric state and activity in the presence of glycerol. Plant Physiol 90: 427-433

Podesta FE, Gonzalez DH, Iglesias AA (1990) Phosphate activates phospho*enol*pyruvate carboxylase from the C_4 plant *Amaranthus viridis* L. Bot Acta **103:** 266-269

Podesta FE, Plaxton WC (1993) Activation of cytosolic pyruvate kinase by polyethylene glycol. Plant Physiol **103:** 285-288

Podesta FE, Plaxton WC (1994a) Regulation of cytosolic carbon metabolism in germinating *Ricinus communis* cotyledons. I. Developmental profiles for the activity concentration and molecular structure of the pyrophosphate and ATP-dependent phosphofructokinases. phospho*enol*pyruvate carboxylase and pyruvate kinase. Planta **194:** 374-380

Podesta FE, Plaxton WC (1994b) Regulation of cytosolic carbon metabolism in germinating *Ricinus communis* cotyledons. II. Properties of phospho*enol*pyruvate carboxylase and cytosolic pyruvate kinase associated with the regulation of glycolysis and nitrogen assimilation. Planta **194:** 381-387

Poetsch W, Hermans J, Westhoff P (1991) Multiple cDNA of phospho-*enol*pyruvate carboxylase in the C_4 dicot *Flaveria trinervia.* FEBS Lett **292:** 133-136

Pollard A, Wyn Jones RG (1979) Enzyme activities in concentrated solutions of glycine-betaine and other solutes. Planta **144:** 291-298

Polya GM, Micucci V (1985) Interaction of wheat germ Ca^+-dependent protein kinases with calmodulin antagonists and polyamines. Plant Physiol 79: 968-972

Pooviah BW, Reddy ASN (1993) Calcium and signal transduction in plants. Crit Rev Plant Sci **12:** 185-211

Raghavendra AS, Das VSR (1976) Phospho*enol*pyruvate carboxylase from *Setaria italica*: inhibition by oxalacetate and malate. Z Pflanzenphysiol 78: 434-437

Raghavendra AS, Das VSR (1993) C_4 photosynthesis and C_3-C_4 intermediacy: Adaptive strategies for semiarid tropics, *In* YP Abrol, P Mohanty, Govindjee, eds, Photosynthesis: Photoreactions to Plant Productivity, Kluwer Academic Publishers, Dordrecht, pp 317-338.

Raghavendra AS, Yin Z-H, Heber U **(1993)** Light-dependent pH changes in leaves of C_4 plants. Comparison of the pH response to carbon dioxide and oxygen with that of C_3 plants. Planta **189:** 278-287

Rajagopalan AV, Devi MT, Raghavendra AS (1993) Patterns of phospho*enol*pyruvate carboxylase activity and cytosolic pH during light activation and dark deactivation in C_3 and C_4 plants. Photosynth Res 38: 51-60

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

Raschke H (1979) Movements of stomata. *In* W Haupt, ME Feinleib, eds, Encyclopedia of Plant Physiology, New Series, Vol. 7. Springer-Verlag. Berlin, pp 383-441.

Rathnam CKM (1978) Heat inactivation of leaf phospho*enol*pyruvate carboxylase: Protection by aspartate and malate in C_4 plants. Planta **141:** 289-295

Reibach PH, Benedict CR (1977) Fractionation of stable carbon isotopes by phospho*enol*pyruvate carboxylase from C_4 plants. Plant Physiol **59:** 564-568

Reinhart GD (1980) Influence of polyethylene glycols on the kinetics of rat liver phosphofructokinase. J Biol Chem **255:** 10576-10578

Rhodes D, Hanson AD (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu Rev Plant Physiol Plant Mol Biol **44:** 357-384

Roberts AW, Haigler CH (1990) Trachery-element differentiation in suspension-cultured cells *of Zinnia* requires uptake of extracellular Ca^{2+}. Experiments with calcium channel blockers and calmodulin inhibitors. Planta **180:** 502-509

Rustin P, Meyer C, Wedding R (1988) The effect of adenine nucleotides on purified phospho*enol*pyruvate carboxylase from the CAM plant *Crassula argentea.* Plant Physiol **88:** 153-157

Saitou K, Agata W, Masui Y, Asakura M, Kubota F (1994) Isoforms of NADP-malic enzyme from *Mesembryanthemum* ***crystallinum*** L. that are involved in C_3 photosynthesis and Crassulacean acid metabolism. Plant Cell Physiol **35:** 1165-1171

Salahas G, Kakoulidou K, Gavalas NA (1994) Chymostatin as an effector of C_4 phospho*enol*pyruvate carboxylase. Photosynthetica **30:** 447-454

Salahas G, Manetas Y, Gavalas NA (1990) Effects of glycerol on the *in vitro* stability and regulatory activation/inactivation of pyruvate, orthophosphate dikinase of *Zea mays* L. Photosynth Res **26:** 9-17

Salvucci ME (1992) Subunit interactions of Rubisco activase: polyethylene glycol promotes self-association, stimulates ATPase and activation activities, and enhances interactions with rubisco. Arch Biochem Biophys **298:** 688-696

Sangwan RS, Singh N, Plaxton WC (1992) Phospho*enol*pyruvate carboxylase activity and concentration in the endosperm of developing and germinating castor oil seeds. Plant Physiol 99: 445-449

Schnabl H, Denecke M, Schulz M (1992) *In vitro* and *in vivo* phosphorylation of stomatal phospho*enol*pyruvate carboxylase from *Viciafaba* L. Bot Acta **105:** 367-369

Schnabl H, Klockenbring T, Huhn M, Schulz M (1993) Purification of phospho*enol*pyruvate carboxylase subunits and isoforms from *Vicia faba* L. by preparative gel electrophoresis and their detection by enzyme-linked immunosorbent assay. Electrophoresis 14: 817-819

Schaffner AR, Sheen J (1992) Maize C_4 photosynthesis involves differential regulation of phospho*enol*pyruvate carboxylase genes. Plant J **2:** 221-232

Schuller KA, Werner D (1993) Phosphorylation of soybean (*Glycine max* L) nodule phospho*enol*pyruvate carboxylase ***in*** *vitro* decreases sensitivity to inhibition by L-malate. Plant Physiol 101:1267-1273

Schulz M, Hunte C, Schnabl H (1992) Multiple forms of phospho*enol*pyruvate carboxylase in mesophyll, epidermal and guard cells of *Viciafaba.* Physiol Plant 86: 315-321

Selinioti E, Karabourniotis G, Manetas Y, Gavalas NA (1985) Modulation of phospho*enol*pyruvate carboxylase by 3-phosphoglycerate. Probable physio-logical significance for C_4-photosynthesis. J Plant Physiol **121:** 353-360

Selinioti E, Manetas Y, Gavalas NA (1986) Cooperative effects of light and temperature on the activity of phospho*enol*pyruvate carboxylase from *Amaranthuspaniculatus* L. Plant Physiol **82:** 518-522

Selinioti E, Nikolopoulos D, Manetas Y (1987) Organic cosolutes as stabilizers of phospho*enol*pyruvate carboxylase in storage: An interpretation of their action. Aust J Plant Physiol 14: 203-210

Shi J-J, **Wu** M-X, **Zha** J-J (1981) Studies on plant phospho*enol*pyruvate carboxylase. V. A reversible cold-inactivation of sorghum leaf PEP carboxylase. Acta Phytophysiol Sinica 7: 317-326

Shimazaki K, Kinoshita T, Nishimura M (1992) Involvement of calmodulin-dependent myosin light kinase in blue light-dependent H^+ pumping by guard cell protoplasts from *Viciafaba* L. Plant Physiol **99:** 1416-1421

Shirahashi K, Hayakawa S, Sugiyama T (1978) Cold lability of pyruvate, orthophosphate dikinase in the maize leaf. Plant Physiol **62:** 826-830

Sims TL, Hague DR (1981) Light-stimulated increase of translatable mRNA for phospho*enol*pyruvate carboxylase in leaves of maize. J Biol Chem **256:** 8252-8255

Slack CR (1968) The photoactivation of phospho*enol*pyruvate synthase in leaves of *Amaranthus palmeri.* Biochem Biophys Res Commun 30: 483-488

Slocombe SP, Whitelam GC, Cockburn W (1993) Investigation of phospho*enol*pyruvate carboxylase (PEPcase) in *Mesembryanthemum crystallinum* L. in C_3 and CAM photosynthetic states. Plant Cell Environ 16:403-411

Smith AM, Woolhouse HW (1984) Occurrence of unstable PEP carboxylase in C_4 grasses in the genus *Spartina schreb.* Plant Cell Environ 7: 491-498

Stadtman ER, Chock PB (1977) Superiority of interconvertible enzyme cascades in metabolic regulation: analysis of monocyclic systems. Proc Natl Acad Sci USA 74: 2761-2765

Stamatakis K, Gavalas NA, Manetas Y (1988) Organic cosolutes increase the catalytic efficiency of phospho*enol*pyruvate carboxylase, from *Cynodon dactylum* (L.) Pers, apparently through self-association of the enzymic protein. Aust J Plant Physiol 15: 621-631

Stiborova M (1988) Phospho*enol*pyruvate carboxylase: the key enzyme of C_4-photosynthesis. Photosynthetica **22:** 240-263

Stockhaus J, Poetsch W, Steinmüller K, Westhoff P (1994) Evolution of the C_4 phospho*enol*pyruvate carboxylase promoter of the C_4 dicot *Flaveria trinervia*: an expression analysis in the C_3 plant tobacco. Mol Gen Genet **245:** 286-293

Sugiharto B, Miyata K, Nakamoto H, Sasakawa H, Sugiyama T (1990) Regulation of expression of carbon-assimilating enzymes by nitrogen in maize leaf. Plant Physiol **92:** 963-969

Sugiharto B, Sugiyama T (1992) Effects of nitrate and ammonium on gene expression of phospho*enol*pyruvate carboxylase and nitrogen metabolism in maize leaf tissue during recovery from nitrogen stress. Plant Physiol **98:** 1403-1408

Sugiharto B, Suzuki I, Burnell JN, Sugiyama T (1992) Glutamine induces the N-dependent accumulation of mRNAs encoding phospho*enol*pyruvate carboxylase and carbonic anhydrase in detached tissue. Plant Physiol **100:** 2066-2070

Sugimoto K, Kawasaki T, Kato T, Whittier RF, Shibata D, Kawamura Y (1992) cDNA sequence and expression of a phospho*enol*pyruvate carboxylase gene from soybean. Plant Mol Biol **20:** 743-747

Sugiyama T, Mizuno M, Hayashi M (1984) Partitioning of nitrogen among ribulosel,5-bisphosphate carboxylase/oxygenase, phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase as related to biomass productivity in maize seedlings. Plant Physiol 75: 665-669

Taybi T, Sotta B, Gehrig H, Güelü S, Kluge M, Brulfert J (1995) Differential effects of abscisic acid on phospho*enol*pyruvate carboxylase and CAM operation in *Kalanchoe blossfeldiana*. Bot Acta **108:** 240-246

Terada K, Kai K, Okuno S, Fujisawa H, Izui K (1990) Maize leaf phospho*enol*pyruvate carboxylase: phosphorylation of ser-15 with a mammalian cyclic AMP-dependent protein kinase diminishes sensitivity to inhibition by malate. FEBS Lett **259:** 241-244

Terada K, Murata T, Izui K (1991) Site-directed mutagenesis of phospho*enol*pyruvate carboxylase from *E.coli*: the role of his^{579} in the catalytic and regulatory functions. J Biochem **109:** 49-54

Thomas MC, Cretin C, Keryer E, Vidal J, Gadal P (1987) Photocontrol of sorghum leaf phospho*enol*pyruvate carboxylase. Characterization of messenger RNA and of photoreceptor. Plant Physiol 85: 243-246

Timasheff SN (1992) A physicochemical basis for the selection of osmolytes by nature. *In* GN Somero, CB Osmond, CL Bolis, eds, Water and Life. Springer-Verlag, Berlin-Heidelberg-New York, pp 70-84.

Timasheff SN, Arakawa T, Inoue H, Gekko K, Gorbunoff MJ, Lee JC, Na GC, Pirtz ER, Prakash V (1982) The role of solvation in protein structure stabilization and unfolding. *In* F Franks, SF Mathias, eds, In Biophysics of Water. John Wiley and Sons, New York, pp 48-50

Ting IP, Osmond CB (1973a) Photosynthetic phospho*enol*pyruvate carboxylases. Characteristics of alloenzymes from leaves of C_3 and C_4 plants. Plant Physiol 51: 439-447

Ting IP, Osmond CB (1973b) Multiple forms of plant phospho*enol*pyruvate carboxylase associated with different metabolic pathways. Plant Physiol **51:448-453**

Toh H, Kawamura T, Izui K (1994) Molecular evolution of phospho*enol*pryuvate carboxylase. Plant Cell Environ **17:** 31-43

Towbin H , Staehlin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354

Uedan K, Sugiyama T (1976) Purification and characterization of phospho*enol*pyruvate carboxylase from maize leaves. Plant Physiol 57: 906-910

Utter MF, Kolenbrander HM (1972) Formation of oxalacetate by CO_2 fixation on phospho*enol*pyruvate. *In* PD Boyer, ed, The Enzymes, 3rd ed. Vol 6. pp 117-136

Vance CP, Gantt JS (1992) Control of nitrogen and carbon metabolism in root nodules. Physiol Plant 85: 266-274

Vance CP, Stade S (1984) Alfalfa root nodule carbon dioxide fixation. II. Partial purification and characterization of root nodule phospho*enol*pyruvate carboxylase. Plant Physiol 75: 261-264

Vanlerberghe GC, Schuller KA, Smith RG, Feil R, Plaxton WC, Turpin DH (1990) Relationship between NH_4^+ assimilation rate and ***in*** *vivo* phospho*enol*pyruvate carboxylase activity. Plant Physiol **94:** 284-290

Van Quy L, Champigny M-L (1992) NO_3^- enhances the kinase activity for phosphorylation of phospho*enol*pyruvate carboxylase and sucrose phosphate synthase proteins in wheat leaves. Plant Physiol **99:** 344-347

Van Quy L, Foyer C, Champigny M-L (1991a) Effect of light and NO_3 on wheat leaf phospho*enol*pyruvate carboxylase activity. Evidence for covalent modification of the C_3 enzyme. Plant Physiol 97: 1476-1482

Van Quy L, Lamaze T, Champigny **M-L (1991b)** Short-term effects of nitrate on sucrose synthesis in wheat leaves. Planta **185:** 53-57

Vaughan MA, Mulkey TJ, Goff CW (1984) Effect of calmodulin antagonists and calcium entry blockers on ATP-dependent Ca^{2+} uptake in maize root microsomes. Plant Physiol 75: suppl., 2 (Abstr)

Vidal J, Gadal P (1983) Influence of light on phospho*enol*pyruvate carboxylase in sorghum leaves. Identification and properties of two isoforms. Physiol Plant 57: 119-123

Vidal J, Godbillon G, Gadal P (1980) Recovery of active, highly purified phospho*enol*pyruvate carboxylase from specific immunoadsorbent •column. FEBS Lett. **118:** 31-34

Vidal J, Godbillon G, Gadal P (1983) Influence of light on phospho*enol*pyruvate carboxylase in sorghum leaves. II. Immunochemical study. Physiol Plant 57: 124-128

Vidal J, Echevarría C, Bakrim N, Arrio M, Pierre JN, Cretin C, Gadal P, (1990) Regulation of phospho*enol*pyruvate carboxylase in sorghum

leaves: Study of the phosphorylation process **of the enzyme. Curr** Top Plant Biochem Physiol **9: 1-6**

Wagner R, Gonzalez DH, Podesta FE, Andreo CS (1987) Changes in the quaternary structure of phosphoeno/pyruvate carboxylase induced by ionic strength affect its catalytic activity. Eur J Biochem **164:** 661-666

Walker GH, Ku MSB, Edwards GE (1986) Catalytic activity of maize leaf phosphoeno/pyruvate carboxylase in relation to oligomenzation. Plant Physiol **80:** 848-855

Walker GH, Ku MSB, Edwards GE (1988) The effect of phosphorylated metabolites and divalent cations on the phosphatase and carboxylase activity of phosphoeno/pyruvate carboxylase. J Plant Physiol **133:** 144-151

Wang Y-H, Chollet R (1993) *In vitro* phosphorylation of purified tobacco-leaf phosphoeno/pyruvate carboxylase. FEBS Lett **328:** 215-218

Wang Y-H, Duff SMG, Cretin C, Lepiniec L, Sarath G, Condon SA, Vidal J, Gadal P, Chollet R (1992) Site-directed mutagenesis of the phosphorylatable serine (Ser^8) in C_4 phosphoeno/pyruvate carboxylase from sorghum. The effect of negative charge at position 8. J Biol Chem **267:** 16759-16762

Wedding RT, Black MK (1986) Malate inhibition of phosphoeno/pyruvate carboxylase from *Crassula*. Plant Physiol **82:** 985-990

Wedding RT, Black MK, Meyer CR (1989) Activation of higher plant phosphoeno/pyruvate carboxylases by glucose-6-phosphate. Plant Physiol **90:** 648-652

Wedding RT, O'Brein CE, Kline K (1994) Oligomenzation and its affinity of maize phosphoeno/pyruvate carboxylase for its substrate. Plant Physiol **104:** 613-616

Wedding RT, Dole P, Chardot TP, Wu M-X (1992) Inactivation of maize phospho*enol*pyruvate carboxylase by urea. Plant Physiol **100:** 1366-1368

Wedding RT, Rustin P, Meyer CR, Black MK (1988) Kinetic studies of the form of substrate bound by phospho*enol*pyruvate carboxylase. Plant Physiol **88:** 976-979

Weigend M, Hincha DK (1992) Quaternary structure of phospho*enol*pyruvate carboxylase from CAM, C_4- and C_3- plants: No evidence for diurnal changes in the oligomeric state. J Plant Physiol **140:** 653-660

WHO (1986) Ammonia. World Health Organization, Geneva.

Williams LE, Kennedy RA (1978) Photosynthetic carbon metabolism during leaf ontogeny in *Zea mays L.* Enzyme studies. Planta **142:** 269-274

Willeford KO, Wedding RT (1992) Oligomerization and regulation of higher plant phospho*enol*pyruvate carboxylase. Plant Physiol **99:** 755-758

Willeford KO, Wu M-X, Meyer CR, Wedding RT (1990) The role of oligomerization in regulation of maize leaf phospho*enol*pyruvate carboxylase activity . Influence of Mg-PEP and malate on the oligomeric equilibrium of PEP carboxylase. Biochem Biophys Res Commun **168:** 778-785

Willmer CM (1983) Phospho*enol*pyruvate carboxylase activity and stomatal operation. Physiol Veg. **21:** 943-953

Willmer CM, Petropoulou Y, Manetas Y (1990) No light activation and high malate sensitivity of phospho*enol*pyruvate carboxylase in guard cell proto-plasts of *Commelina communis* L. J Exp Bot **41:**1103-1107

Wong KF, **Davies** DD **(1973)** Regulation of phospho*enol*pyruvate carboxylase of *Zea mays* by metabolites. Biochem J **131:** 451-458

Wu M-X, Meyer CR, Willeford KO, Wedding RT (1990) Regulation of the aggregation state of maize phospho*enol*pyruvate carboxylase: Evidence from dynamic light scattering measurements. Arch Biochem Biophys **281:** 324-329

Wu M-X, Wedding RT (1985) Regulation of phospho*enol*pyruvate carboxylase from *Crassula* by interconversion of oligomeric forms. Arch Biochem Biophys 240: 655-662

Wu M-X, Wedding RT (1987) Temperature effects on phospho*enol*pyruvate carboxylase from a CAM and a C_4 plant. A comparative study. Plant Physiol **85:** 497-501

Wu M-X, Wedding RT (1994) Modification of maize leaf phospho*enol*pyruvate carboxylase with fluorescein isothiocyanate. Plant Cell Physiol **35: 569-574**

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: Evolution of osmolyte systems. Science **217:** 1214-1222

Yin Z-H, Heber U, Raghavendra AS (1993) Light-dependent pH changes in leaves of C_4 plants. Comparison of cytosolic alkalization and vacuolar acidification with that of C_3 plants. Planta **189:** 267-277

Zhang XQ, Li B, Chollet R (1995) ***In*** *vivo* regulatory phosphorylation of soybean nodule phospho*enol*pyruvate carboxylase. Plant Physiol **108:** 1561-1568

Zhang XQ, Outlaw WH Jr, Chollet R (1994) Lessened malate inhibition of guard-cell phospho*enol*pyruvate carboxylase velocity during stomatal opening. FEBS Lett **352:** 45-48

Appendix

Appendix

Paper published

Vol. 33, No. 2, May 1994 BIOCHEMISTRY *anti* MOLECULAR BIOLOGY INTERNATIONAL
Pages 337-344

AMMONIUM IONS STIMULATE *IN VITRO* THE ACTIVITY OF PHOSPHO*ENOL*PYRUVATE CARBOXYLASE FROM LEAVES OF *AMARANTHUS HYPOCHONDRIACUS*, A C4 PLANT: EVIDENCE FOR ALLOSTERIC ACTIVATION

J. Gayathri, A.S. Raghavendra[1]

School of Life Sciences, University of Hyderabad,

Hyderabad 500 134, India

Received February 14, 1994
Received after revision. April 6, 1994

SUMMARY: Ammonium ions stimulated *in vitro* the activity of PEP carboxylase (PEPC) extracted from dark-adapted leaves of *Amaranthus hypochondriacus*. Maximum stimulation of 80 to 85 % occured at 50 μM ammonium chloride. There was a marginal inhibition of PEPC at 5 mM ammonium chloride. Among several ions tested, potassium ions stimulated PEPC to a limited extent of about 30 %. In presence of ammonium, there was no change either in the sensitivity of enzyme to malate or in the affinity for substrate, PEP. On the other hand, glucose-6-phosphate, an allosteric activator, which stimulated the enzyme by two-fold, could enhance PEPC activity by <20 % in the presence of ammonium. The light-activated form of PEPC from leaves of *Amaranthus hypochondriacus* was not stimulated, but was inhibited in the presence of ammonium. Our results demonstrate that ammonium ions stimulate PEPC by acting at the allosteric site. Ammonium ion being a component of plant metabolism could be an important regulator of PEPC, particularly in C4 plants.

INTRODUCTION

PhosphoenoJpyruvate carboxylase (PEPC, EC 4.1.1.31), a key enzyme of photosynthetic C02 fixation in C4 and CAM plants, catalyses the β-carboxyla-tion of PEP to yield oxalacetate and Pi (1). The properties of the enzyme varies depending on the source (2,3). The C4 enzyme is subject to typical feed-back inhibition by oxalacetate and malate and allosteric activation by G-6-P and glycine (2,4,5).

Ammonia is a toxic metabolite and inhibits several enzymes of plant and animal metabolism (6). Nevertheless, a few enzymes such as pyruvate kinase are stimulated by ammonium (7-9). Ammonium ions enhance assimilation of carbon into C4 acids in higher plants, algal cells and cyanobacteria (10-12).

Abbreviations used: PEP, PhosphoenoJpyruvate; PEPC, PhosphoenoJpyruvate carboxylase; G-6-P, Glucose-6-Phosphate; CAM, Crassulacean acid metabolism.

To whom correspondence should be sent-.

1039-9712/94/020337-07$05.00/0

Further, ammonia assimilation rates in *vivo* are well correlated with PEPC activity in green alga, *Selanastrum minutum* (12). Although an increase in the activity of PEPC is assumed to be the reason for elevated rates of dark carbon fixation, the direct effects of ammonium salts on PEPC activity *in vitro* are not assessed.

Biosynthesis of PEPC in leaves is affected by the extent and form of nitrogen available to the plant. For e.g., the levels of PEPC increased when leaves of maize were fed with NO_3^- or NH_4^+ (13,14). The effectiveness of ammonium salt as an inducer of PEPC biosynthesis is two fold greater than that of nitrate (14). However these effects *in vivo* of nitrate/ammonia are long-term ones on biosynthesis of PEPC protein.

The present article is an attempt to study the effect *in vitro* of ammonium ions on PEPC from light/dark-adapted leaves of *Amaranthus hypochondriacus,* a NAD-malic enzyme type C_4 plant. Ammonium ions at low concentrations could stimulate markedly the activity of PEPC *in vitro.* The effect of ammonium on PEPC was at the regulatory allosteric site on the enzyme.

MATERIALS AND METHODS

Plant material: Plants of *Amaranthus hypochondriacus* L. (cultivar AG-67)were grown from seed in field (approximate photoperiod 12 h and temperature of 30-40°C day/ 25-30 °C night). Leaves were excised from the plant between 9:00 to 10:00 A.M., approximately 3 to 4 h after sunrise. Discs of ca. 0.2 cm were cut from leaves under water. Twenty discs were kept in a Petri dish containing water and left in darkness for 2 h. If necessary, the discs were illuminated at 1500 µE • s for 20 min (after pre-darkening).
Extraction: Leaf discs were extracted in chilled mortar and pestle with100 mM HEPES-KOH pH 7.3, containing 10 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM EDTA, 20% (v/v) glycerol and 10 mM β-mercaptoethanol. The homogenate was passed through four layers of cheese cloth and centrifuged at 7000 *g* for 10 min. The supernatant was used for enzyme assays. An aliquot of homogenate (before centrifugation) was examined for chlorophyll by extraction into 80% (v/v) acetone (15).
Assay of phospho*enol*pyruvate carboxylase (EC 4.1.1.31): The reaction of PEPC was coupled to NAD malic dehydrogenase and the enzyme activity was determined by monitoring NADH oxidation at 340 nm in a Shimadzu UV-spectrophotometer at 30 °C. The assay mixture (1 ml) contained 50 mM HEPES-KOH pH 7.3, 5 mM $MgCl_2$ and 2.5 mM PEP. The test salts were included in the reaction medium, as indicated. The reaction was started by addition of PEP.

The experiments were repeated on several days, with each assay done at least three times. The average values ± SE are presented.

RESULTS AND DISCUSSION

This is the first report on direct activation by ammonium ions *in vitro* of PEPC, a key enzyme of *Ci* pathway. The activity of PEPC was stimulated markedly by ammonium chloride, particularly at low concentrations. The enzyme activity was enhanced by 80% (over control) at SO μm NH_4Cl (Fig. 1). However, the extent of stimulation declined as NH_4Cl concentration was raised above 100 μm. The effect of NH_4Cl on PEPC was only marginal at 3-4 mM NH4C1 and the enzyme activity decreased at 5 mM NH_4Cl.

When a range of different salts were tested, PEPC was stimulated only by ammonium salts and to some extent by potassium ions (Table 1). Besides chloride, acetate and sulphate salts of ammonium also stimulated the activity of PEPC. On the other hand, monovalent ions like lithium, sodium and rubidium had only a marginal effect on PEPC. The presence of calcium chloride decreased the activity of PEPC (data not shown). The inhibition of PEPC by calcium

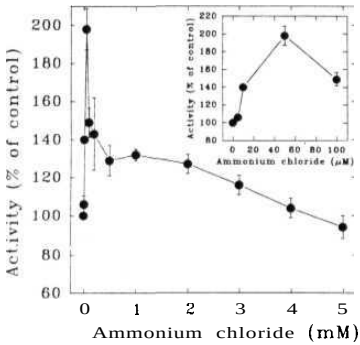

Figure 1. Stimulation by NH_4Cl in *vitro* of PEPC extracted from dark-adapted leaves of *Amaranthus hypochondriacus*. Leaf discs were left in darkness for 2 h before extraction. The enzyme assay was done at pH 7.3 and the reaction was started by 2.5 mM PEP. Ammonium chloride was included in the assay medium to get the required final concentration. The inset shows the response of the enzyme to low concentrations of ammonium chloride. Maximum stimulation of about 80 % (over control) occured at 50 μM ammonium chloride. Enzyme activity in the absence of ammonium chloride (control) was 544 i 10 μmol mg chl h . Data are averages ± SE of at least three separate experiments. If not seen, the errors are within the symbols.

Table 1. Effect of different salts *in vitro* on the activity PEP carboxylase extracted from leaves of *Amaranthus hypochondriacus*

Salt	Optimal concentration	Enzyme activity	Activity compared to control
	µM	µmol mg chl^{-1} h^{-1}	%
None (control)	0	627 ± 23	100
Ammonium chloride	50	1130 ± 10	180
Ammonium sulphate	50	1150 ± 5	183
Ammonium acetate	50	1121 ± 9	179
Potassium chloride	500	821 ± 1	131
Sodium chloride	500	730 ± 1	116
Lithium chloride	500	697 ± 1	111
Rubidium chloride	500	697 ± 1	111

has been reported earlier (16). The stimulation by K^+ was not as marked as that by NH_4^+. At 0.5 mM KCl, the activity of PEPC was stimulated by 35 % over control. However, the activity of PEPC was slightly decreased at higher concentrations of K^+ (about 20-25 X inhibition at 2-5 mM KCl). K^+ ions, which promote stomatal opening (17) and swelling of guard cell protoplasts (18) are reported to stimulate PEPC activity in guard cell protoplasts (19) and epidermal strips (20).

In the presence of NH_4Cl, the affinity to PEP was not much altered, although V_{max} increased by 85 '/.. The double reciprocal plots revealed similar K_m for PEP in the presence or absence of NH_4Cl (Table 2). Also, there was no significant change in the sensitivity of the enzyme to malate in the presence of NH4C1 (Table 2).

Glucose-6-phosphate is an allosteric activator of PEPC from C_4 plants (1). However, the respone to G-6-P was quite different in the presence of NH4C1 (Fig. 2). G-6-P activated PEPC markedly in the absence but not in the presence of NH_4Cl. The enzyme was stimulated by more than two fold (>215 '/.) by G-6-P. But there was only a marginal stimulation (<20 %) by G-6-P in the presence of NH_4Cl. Further, there was a decrease in PEPC activity at higher concentrations of G-6-P (>3 mM) in the presence of NH_4Cl. The K_A of PEPC for G-6-P was 0.17 mM in the absence (control) and 0.94 mM in the presence of NH_4Cl (Table 2). A major reason for ineffectiveness of G-6-P appears to be that the enzyme is already activated by NH4C1. Similarly, the decrease in the enzyme activity at higher concentrations of G-6-P may be due to the

Table 2. Kinetic characteristics of PEP carboxylase from leaves of *Amaranthus hypochondriacus* in the presence or absence of 50 μM ammonium chloride

Parameter	Control	NH_4Cl
V_{max} (μmol mg chl^{-1} h^{-1})	1316	2436
K_m PEP (mM)	0.60	0.70
K_i malate (mM)	0.20	0.25*
K_A G-6-P (mM)	0.17	0.94*

*Apparent, due to non typical kinetics.

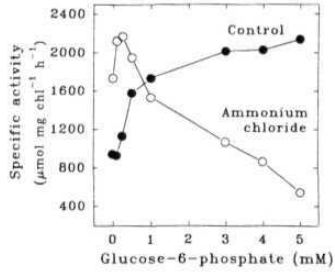

Figure 2. Response to G-6-P of PEPC from dark-adapted leaves of *Amaranthus hypochondr iacus.* The enzyme was assayed at pH 7.3 in the presence (•) or absence of 50 μM ammonium chloride (o) and different concentrations of G-6-P. PEPC was activated markedly (>two fold) by G-6-P only in the absence of ammonium chloride. The stimulation by G-6-P was marginal (<20 %) in the presence of ammonium chloride. If not seen, the errors were within symbols and did not exceed 3 %. Further details were as in Fig. 1.

marked alteration of regulatory site by NH_4 . Therefore, NH_4Cl appears to modulate PEPC *in vitro* by changing the sensitivity of the allosteric regulatory site.

On illumination, the activity of PEPC is stimulated two to three fold in leaves of C_4 plants (3,4). We have therefore tested the response to ammonium of enzyme extracted from illuminated leaves. However, unlike the dark-adapted enzyme, the light-activated form of PEPC was not stimulated, but was inhibited (Fig. 3). The light-activated form of PEPC differs from the dark-

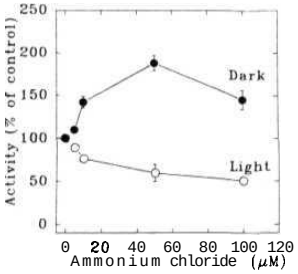

Figure 3. Response to ammonium chloride of PEPC extracted from dark-adapted (•) or illuminated (o) leaves of *Amaranthus hypochondr iacus*. Leaves which were kept in darkness for 2 h, were either retained in darkness or illuminated at 1200 μE m^{-2} s^{-1} for 20 min. After extraction the assay was done at pH 7.3 and the reaction was started with 2.5 mM PEP. Ammonium chloride was included in the assay medium at the indicated concentrations. The activities of PEPC in the control sets 'i.e.without NH4C1) were 836 + 4 μmol mg chl^{-1} h^{-1} (dark-adapted leaves) and 2090 *t* 10 μmol mg chl^{-1} h^{-1} (illuminated leaves). Further details were as in Fig. 1.

adapted form in several characteristics: malate sensitivity, phosphorylation status and K_m for PEP (3). When leaves are illuminated, conformational changes are likely to occur in PEPC. The absence of stimulation by NH_4Cl of PEPC from illuminated leaves indicates that the dark-form of the enzyme alone has the conformational status capable of responding to ammonium ions.

Light dependent phosphorylation of PEPC in mesophyll protoplasts of sorghum was promoted by weak bases such as ammonium chloride and methylamine (21). These ions were expected to increase the cytosolic pH, raise the levels of calcium and activate either PEPC or PEPC protein kinase or both. Our present observations demonstrate for the first time that ammonium ion is an allosteric activator in *vitro* of PEPC from C_4 plants.

In C4 plants, the reactions of carbon and nitrogen metabolism are spatially separated between the mesophyll and bundle sheath cells (22). PEPC is located in cytosol of mesophyll cells in leaves of C_4 plants. The reduction of nitrate to ammonium also occurs in C_4 mesophyll cells (22). Allosteric activation by ammonium of PEPC fits well with intercellular enzymic distribution. An increase in the availability of ammonium ions can stimulate PEPC and thus promote carbon and amino acid metabolism. Our observations can therefore form an additional basis of a better nitrogen use efficency in C_4 plants than that in C3 species (22).

Acknowledgements: A part of this work is supported by a Research grant (No. 38(0862)/94/EMR-II) from Council of Scientific and Industrial Research, New Delhi. We thank Dr. Mohinder Pal, Head, Cytogenetics Division, National Botanical Research Institute, Lucknow for kindly providing us the seeds of *Amaranthus hypochondriacus*. G.J. is recipient of a Senior Research Fellowship from the University Grants Commission, New Delhi.

REFERENCES

1. O'Leary, M.H. (1982) *Ann. Rev. Plant Physiol.* 33, 297-315.
2. Andreo, C.S., Gonzalez, D.H. and Iglesias, A.A. (1987) *FEBS Lett.* 213, 1-8.
3. Rajagopalan, A.V., Devi, M.T. and Raghavendra, A.S. (1994) *Photosyn. Res.* in press.
4. Doncaster, H.D. and Leegood, R.C. (1987) *Plant Physiol.* 84, 82-87.
5. Bandarian, V., Pochner, W.J. and Grover, S.D. (1992) *Plant Physiol.* 100, 1411-1416.
6. WHO (1986) *Ammonia.* World Health Organization, Geneva.
7. Kanazawa, T., Kirk, M.R. and Bassham, J.A. (1972) *Biochim. Biophys. Acta* 256, 656-669.
8. Hiller, R.G. (1970) *J. Exp. Bot.* 21, 628-638.
9. Peterson, J.B. and Evans, H.J. (1978) *Plant Physiol.* 61, 909-914.
10. Ohmori, M., Oh-hama, T., Furihata, K. and Miyachi, S. (1986) *Plant Cell Physiol.* 27, 563-566.
11. Muller, R., Steigner, W., Gimmler, H. and Kaiser, W.M. (1990) *J. Exp. Bot.* 41, 441-448.
12. Vanlerberghe, G.C., Schuller, K.A., Smith, R.G., Foil, R., Plaxton, W.C. and Turpin, D.H. (1990) *Plant Physiol.* 94, 284-290.
13. Sugiharto, B., Miyata, K., Nakamoto, H., Sasakawa, H. and Sugiyama, T. (1990) *Plant Physiol.* 92, 963-969.
14. Sugiharto, B., and Sugiyama, T. (1992) *Plant Physiol.* 98, 1403-1408.
15. Arnon, D.I. (1949) *Plant Physiol.* 24, 1-16.
16. Gavalas, N.A. and Manetas, Y. (1980) *Z. Pflanzenphysiol.* 100, 179-184.
17. Raschke, H. (1979) in *Encyclopedia of Plant Physiology, New Series* (Haupt, W. and Feinleib, M. E., Eds.), Vol. 7, pp 383-441, Springer-Verlag, Berlin.
18. Hampp, R. and Schnabl, H. (1984) *Plant cell Physiol.* 25, 1233-1239.
19. Michalke, B. and Schnabl, H. (1990) *Planta* 180, 188-193.
20. Willmer, C.M., Petropoulou, Y. and Manetas, Y. (1990) J. *Exp. Bot.* 41, 1103-1107.
21. Pierre, J.N., Pacquit, V., Vidal, J. and Gadal, P. (1992) *Ear. J. Biochem.* 210, 531-537.
22. Hatch, M.D. (1987) *Biochim. Biophys. Acta*895, 81-106.