The Regulation of Phosphoenolpyruvate Carboxylase in *Bryophyllum Fedtschenkoi*

by

Pamela J. Carter

Thesis submitted for the degree of doctor of philosophy

Department of Biochemistry

University of Glasgow

1991

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Part of the work described in this thesis has been submitted for publication:

Carter, P.J., Nimmo, H.G., Fewson, C.A. and Wilkins, M.B. (1990) FEBS Lett. 263, 233-236

Carter, P.J., Nimmo, H.G., Fewson, C.A. and Wilkins, M.B. (1991) EMBO J. (in press)

Contents of Thesis

List of figures

List of tables

Abbreviations

The abbreviations listed in this thesis are those recommended by the Biochemical Society, London, except for those listed below;

B. fedtschenkoi	*Bryophyllum fedtschenkoi*
BSA	Bovine serum albumin
CAM	Crassulacean acid metabolism
DTT	Dithiothreitol
FPLC	Fast protein liquid chromatography
MDH	Malate dehydrogenase
OAA	Oxaloacetate
PEP	Phosphoenolpyruvate
P_i	Orthophosphate, inorganic phosphate
PPDK	Pyruvate, phosphate dikinase
PMSF	Phenylmethylsuphonyl fluoride
RuBP	Ribulose 1,5-bisphosphate
SDS	Sodium dodecyl sulphate
TCA	Trichloracetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine

Acknowledgements

I wish to express my sincere thanks to my supervisors, Dr H.G. Nimmo and Prof. C.A. Fewson of the Biochemistry Department , and Prof. M.B. Wilkins of the Botany Department, for their enthusiasm and expert guidance during the course of this study. I would like to thank Prof. M.D. Houslay and Prof. R.J. Cogdell for making the facilities of the Biochemistry and Botany Departments available to me, and the Agricultural and Food Research Council for funding the project.

I am grateful to Prof. P. Cohen and Dr C. MacKintosh for their generosity and expert advice in carying out studies on protein phosphatases. Thanks are also due to Mr R. Cuthbertson for ensuring the availability of a regular supply of plant material. I also wish to acknowledge Mrs Pam McEwan for typing part of this manuscript, and Mr Paul Nelson for helping to proof read it.

Finally I would like to thank all my colleagues in the Biochemistry Department past and present, for their friendship and help. Special thanks go to Chris for his endless encouragement.

SUMMARY

Phosphoenolpyruvate (PEP) carboxylase is the primary CO_2-fixing enzyme in plants which exhibit crassulacean acid metabolism. The activity of this enzyme is under the control of an endogenous circadian rhythm. A reversible phosphorylation event was proposed to control PEP carboxylase activity by altering its sensitivity to inhibition by the allosteric inhibitor, malate. The work described in this thesis aimed to investigate the regulation of PEP carboxylase and to gain further insight into the biochemical nature of this circadian clock.

PEP carboxylase was purified in two forms, namely a phosphorylated 'night' form and a dephosphorylated 'day' form, by a method that had been developed previously. Inclusion of an additional desalting step before chromatography on hydroxylapatite, and rapid desalting (instead of overnight dialysis) of the DEAE-cellulose pool prior to chromatography on Superose 6 yielded homogenous forms of the enzyme. The enzyme was stable, as assessed by total activity and allosteric properties, for several weeks when stored at -20°C. The stability of PEP carboxylase was assessed in terms of its allosteric properties. Malate is a feedback inhibitor of PEP carboxylase *in vivo*. The phosphorylated form of the enzyme has an apparent K_i for malate of approx. 3.0mM whereas the dephosphorylated form has an apparent K_i of approx. 0.3mM. Proteolysis of PEP carboxylase results in an irreversible increase in the malate sensitivity. The purified enzyme is tetrameric. It has subunit M_r values of 123kDa and 112kDa which are present in a ratio of 1:10 respectively. This subunit ratio is observed in both purified enzyme forms and in enzyme which was rapidly immunoprecipitated from extracts.

Purified PEP carboxylase was used as a substrate for *in vitro* studies directed at investigating the putative protein kinase(s) and protein phosphatase(s) responsible for bringing about the covalent modification of the enzyme *in vivo*. An endogenous protein kinase activity was found in desalted extracts of leaves of *B. fedtschenkoi* which were prepared in the middle of the night period. Subsequent studies showed that this activity appears and disappears throughout the 24h period. The PEP carboxylase kinase activity was present for about 8-10h during the 16h dark period and was absent from leaves prepared

during the 8h day period. The protein kinase was partially purified from 'night' leaves by ammonium sulphate fractionation, blue dextran-agarose chromatography and Mono Q chromatography. The dialysed Mono Q pool retained PEP carboxylase kinase activity for several weeks if stored at -20°C. The maximum stoichiometry of phosphorylation of PEP carboxylase obtained by *in vitro* phosphorylation, using the partially purified protein kinase was 0.9 mol P_i / mol subunit. A linear relationship was observed between the relative changes in the phosphorylation state and in the apparent K_i for malate of PEP carboxylase The maximum extent of phosphorylation resulted in an almost tenfold decrease in the malate sensitivity of the enzyme, which reflects the day/night change that is found *in vivo* .

'Cleveland map' analysis demonstrated that the site(s) which were phosphorylated *in vitro* were the same as those site(s) phosphorylated *in vivo* using ^{32}P-inorganic phosphate. Several compounds were tested as putative effectors of PEP carboxylase kinase and L-malate(5mM) and glucose 6-phosphate (10mM) showed substantial inhibition (70-80%). It is not known whether these compounds interact with the protein kinase directly , exhert their inhibitory effect via the PEP carboxylase, or both.

The 'night' form of PEP carboxylase was dephosphorylated by a mammalian protein phosphatase type 2A, but a type 1 protein phosphatase had no effect. A plant protein phosphatase from *B. fedtschenkoi,* identified as being type 2A dephosphorylated the enzyme *in vitro.* The catalytic subunit of the plant protein phosphatase was partially purified by ammonium sulphate fractionation, ethanol precipitation and Mono Q chromatography. The type 2A protein phosphatase showed no diurnal variation in activity in desalted extracts prepared over a 16h dark and 8h light period. The dephosphorylation of PEP carboxylase resulted in an increase in the malate sensitivity of the enzyme, such that the apparent K_i decreased from 3.0mM to 0.6mM in one experiment. These studies indicate the reversibility of the phosphorylation of PEP carboxylase.

The diurnal oscillation observed in the protein kinase activity but not in the protein phosphatase activity, suggests that circadian control of PEP carboxylase is mediated at the level of phosphorylation. No PEP carboxylase kinase activity was found in desalted leaf extracts in which the endogenous PEP carboxylase was in the low K_i form. The phosphorylation of PEP carboxylase is concomitant with the

appearance of PEP carboxylase kinase during the night. The nocturnal appearance of protein kinase activity may be due to *de novo* synthesis of the enzyme or to periodic activation of protein kinase activity. Cycloheximide and puromycin, inhibitors of protein synthesis prevented the nocturnal appearance of protein kinase activity and also inhibited the circadian rhythm of CO_2-fixation in detached leaves of *B. fedtschenkoi.* Judging by the apparent dissimilarity in the structure and mechanism of action of these two drugs, it is likely that protein synthesis is involved in regulating PEP carboxylase kinase activity and in generating the rhythm of CO_2-fixation. Nevertheless, it remains unknown whether it is the protein kinase itself which is synthesised at night because some protein kinase activity can be partially purified from leaves taken during the 'day' period, although it was not detectable in the desalted extracts. The possible existence of a tight-binding inhibitor of the protein kinase was highlighted by the presence of a specific PEP carboxylase kinase inhibitor in desalted 'day' extracts. These results illustrate the complexity of the regulation of PEP carboxylase and hence of CO_2-fixation in *B. fedtschenkoi.* In conclusion, it appears that regulation of PEP carboxylase activity occurs predominantly via the protein kinase. The protein kinase is in turn regulated by several possible mechanisms, involving protein synthesis of some key component and the presence of inhibitor/activator compounds.

Chapter 1

INTRODUCTION

1.1. Crassulacean acid metabolism

1.1.1. CO_2 assimilation in higher plants

Green plants utilise atmospheric CO_2 for the synthesis of organic molecules in the process of photosynthesis. The ubiquitous CO_2-fixing enzyme, ribulose-1,5-bisphosphate (RuBP) carboxylase accounts for up to 50% or more of the total soluble leaf protein in many plants (Ku *et al.*, 1979). This enzyme is located in the chloroplast and catalyses the fixation of CO_2 into triose phosphate in a process called the reductive pentose phosphate pathway (also called the C_3 or Calvin cycle) (Figure 1.1). Triose phosphate produced by this pathway can be used to synthesise sucrose or starch. In addition to the carboxylase activity of RuBP carboxylase, the enzyme exhibits a competitive oxygenase activity (Bowes *et al.*, 1971). The oxygenase reaction results in the synthesis of phosphoglycolate (Figure 1.1) which is ultimately converted into 3-phosphoglycerate (3-PGA) at the expense of ATP and NADH in the process of photorespiration. The physiological benefits of photorespiration are unclear, yet the majority of higher plants can lose a significant amount of their valuable carbon by this process, especially during periods of high light intensity. The rate of photorespiration is also enhanced by elevated temperature and high O_2 concentration. Certain species of plants growing in tropical climates have evolved mechanisms of limiting photorespiraton. These plants can be divided into two main categories: (i) C_4 plants and (ii) those that perform Crassulacean acid metabolism (CAM) (discussed in section 1.1.2). Both types of plant possess another CO_2-fixing enzyme, phosphoenolpyruvate (PEP) carboxylase. While the pathways of CO_2-assimilation in C_4 and CAM plants are similar, their anatomical features are quite diverse.

PEP carboxylase catalyses the fixation of atmospheric CO_2 and constitutes the initial step in the C_4 (Figure 1.2) and CAM (Figure 1.3) pathways. The enzyme uses HCO_3^- as a substrate and this is formed from CO_2 by carbonic anhydrase (Coombs *et al.*, 1975). The product of the carboxylase reaction is oxaloacetate (OAA) which is rapidly reduced to malate by malate dehydrogenase (MDH). The

Figure 1.1. **The reductive pentose phosphate pathway**. The abbreviations are: RuBP, ribulose 1,5-bisphosphate; 3-PGA, 3-phosphoglycerate; 2-PG, 2-phosphoglycolate; GBP, glycerate 1,3,-bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; Xu5P, xylulose 5-phosphate; E4P, erythrose 4-phosphate; SBP, sedoheptulose 1,7-bisphosphate; S7P, sedoheptulose 7-phosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate. The light regulated enzymes in the pathway are as follows : 1,6, RuBP carboxylase/oxygenase; 2, NADP-glyceraldehydephosphate dehydrogenase; 3, fructose-1,6-bisphosphatase; 4, sedoheptulose-1,7-bisphosphatase; 5, ribulose --5-phosphate kinase.

Reaction 6 catalyses the initial step in photorespiration whereby oxygenation of RuBP (5C) yields 1 molecule of 2-PG (2C) and 1 molecule of 3-PGA (3C) . In the absence of photorespiration the carboxylation of three molecules of RuBP (5C) yields six molecules of 3-PGA (3C). These are then converted to DHAP and GAP (triose phosphate) using six molecules of ATP and six molecules of NADPH and releasing six molecules of P_i in the process. Under steady-state conditions of photosynthesis, five of these 3C molecules are used to produce three molecules of Ru5P (5C) with two further P_i molecules being released by the two bisphosphatase reactions. The Ru5P can then be converted to RuBP at the expense of three molecules of ATP. One molecule of triose phosphate, the product can be converted to sucrose in the cytosol or starch in the chloroplast. When necessary, all of the triose phosphate can be fed back into the cycle to replenish intermediates and no product is formed (autocatalysis).

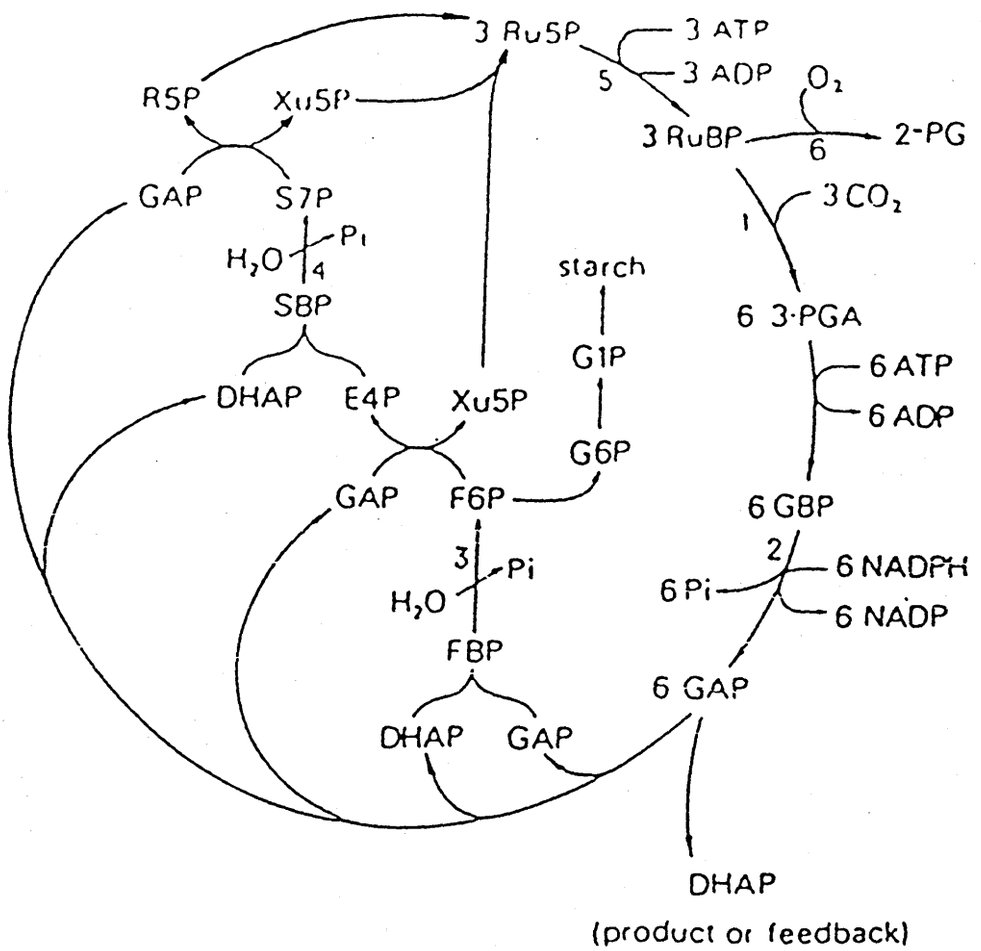
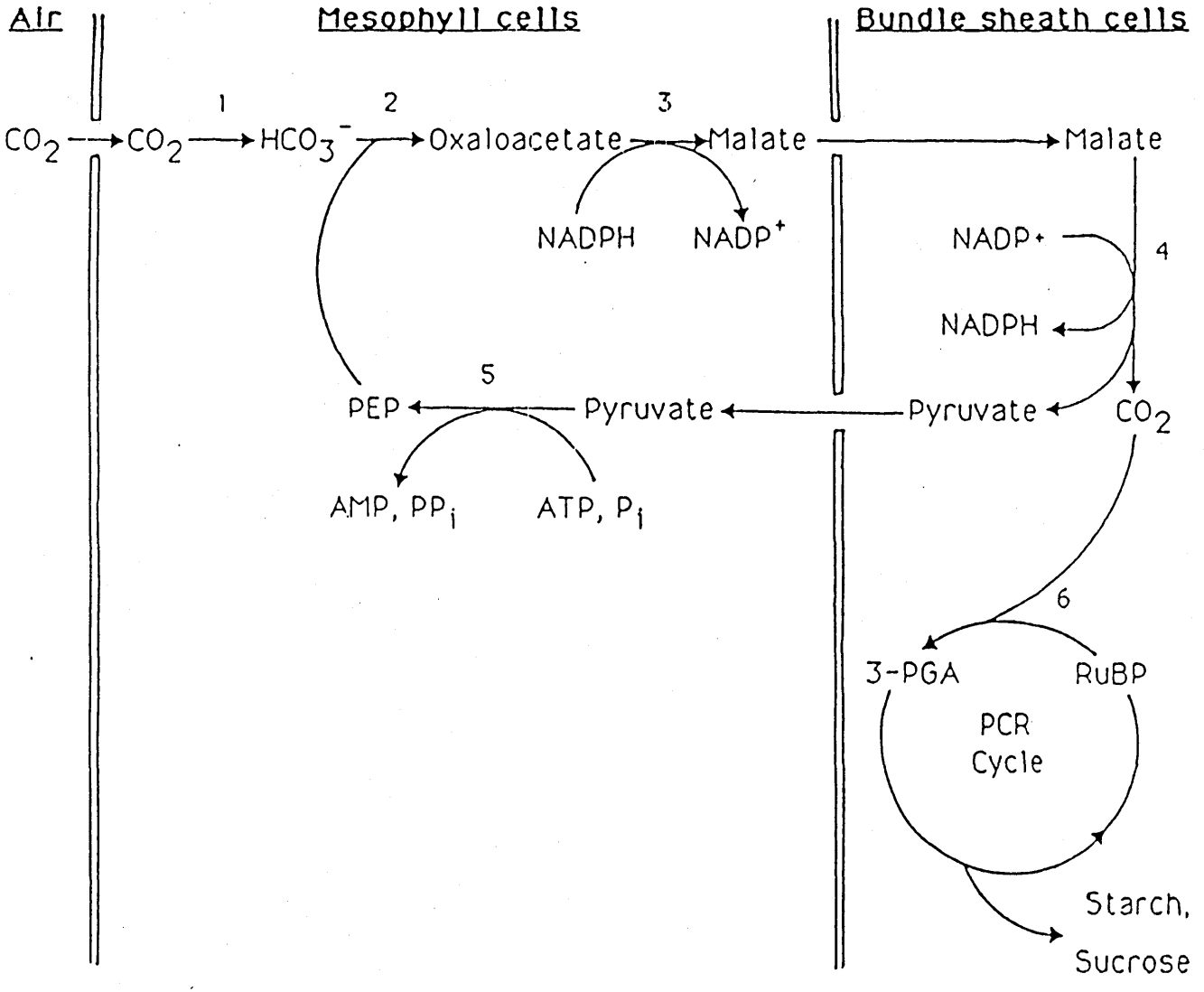

3 Ru5P
3 ATP
5
3 ADP
R5P
Xu5P
O2
3 RuBP
6
2-PG
GAP
S7P
3 CO2
1
H2O
Pi
4
starch
SBP
6 3-PGA
G1P
6 ATP
DHAP
E4P
Xu5P
6 ADP
G6P
6 GBP
GAP
F6P
2
6 NADPH
3
6 Pi
Pi
H2O
6 NADP
FBP
6 GAP
DHAP
GAP
DHAP
(product or feedback)

Figure 1.2. **The C_4 pathway.** The diagram illustrates the pathway in C_4 plants which synthesise malate as the C_4 acid. The enzymes which catalyse the numbered reactions are: 1, carbonic anhydrase; 2, PEP carboxylase; 3, malate dehydrogenase; 4, malic enzyme; 5, pyruvate, phosphate dikinase; 6, RuBP carboxylase.


Air
Mesophyll cells
Bundle sheath cells
CO_2
CO_2
HCO_3^-
Oxaloacetate
Malate
Malate
1
2
3
NADPH
$NADP^+$
$NADP^+$
4
NADPH
5
PEP
Pyruvate
Pyruvate
CO_2
AMP, PP_i
ATP, P_i
6
3-PGA
RuBP
PCR
Cycle
Starch,
Sucrose

immediate fate of malate differs in C_4 and CAM plants.

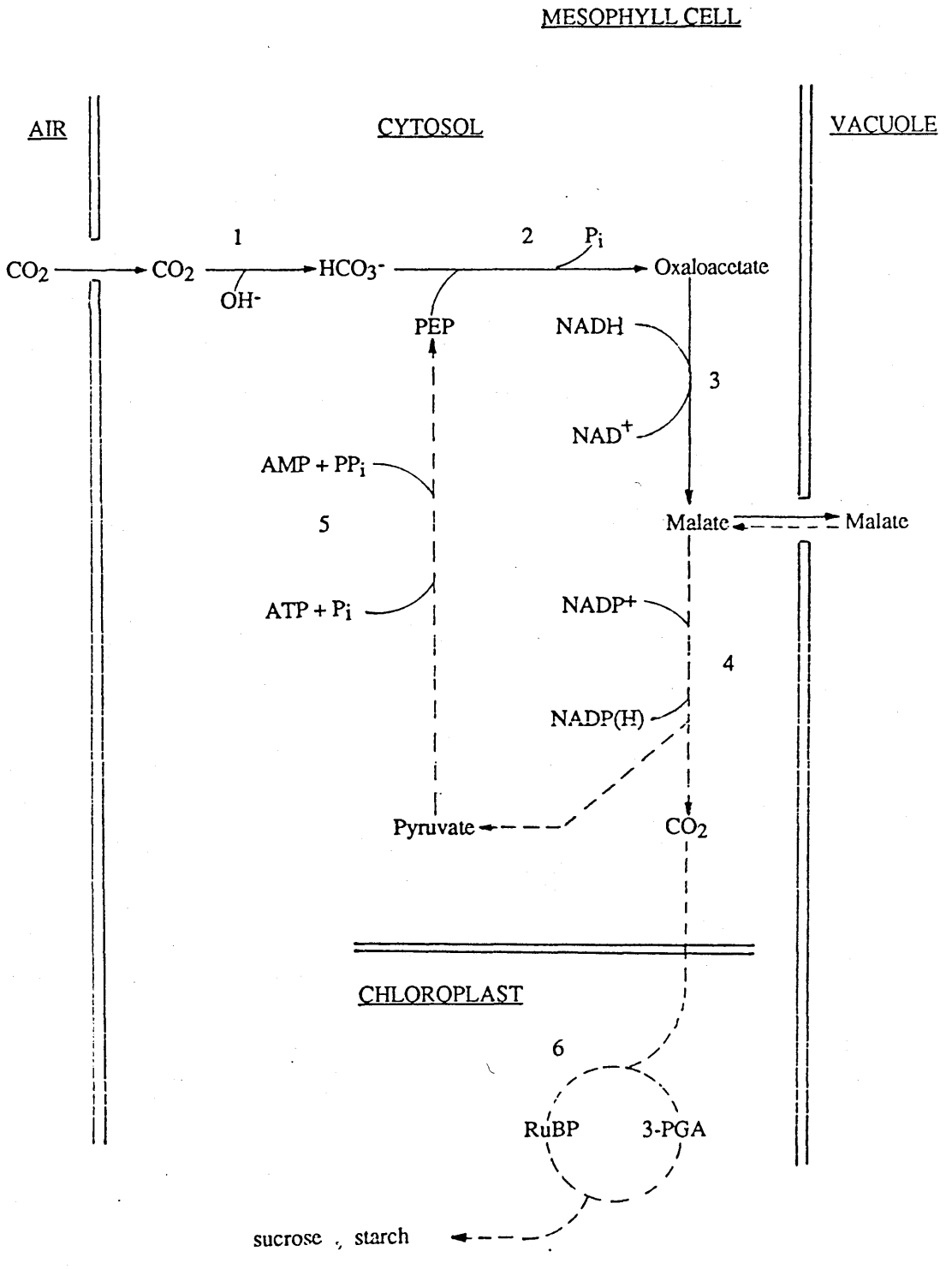
C_4 plants have a unique leaf structure (Kranz anatomy). Two photosynthetic cell types are found, one of which contains PEP carboxylase (mesophyll cells) while the other contains RuBP carboxylase (bundle sheath cells). Many tropical grasses and important crops such as maize and sugar cane exhibit C_4 metabolism. In the C_4 pathway malate is synthesised in the mesophyll cells and migrates to the bundle-sheath cells where it is decarboxylated by malic enzyme. This decarboxylation yields high concentrations of CO_2 for fixation by RuBP carboxylase (Hatch, 1977). C_4 photosynthesis is effective in reducing photorespiration for three main reasons; (i) high CO_2 levels in the bundle-sheath cells diminish the oxygenase activity of RuBP carboxylase (ii) any CO_2 that is produced by photorespiration can be retained within the bundle-sheath cells and recycles, and (iii) at least in the case of maize, the bundle-sheath chloroplasts appear to minimize non-cyclic electron transport and therefore do not produce much oxygen (Gregory, 1989). The process of C_4-photosynthesis has been reviewed by Hatch (1977), Edwards and Huber (1981), and Edwards and Walker (1983). In comparison to C_4 plants, the activities of PEP carboxylase and RuBP carboxylase in CAM plants are separated in time rather than in space. This is achieved by the temporary storage of malate in large vacuoles which are present in the leaf mesophyll cells.

1.1.2. Physiological and biochemical aspects of CAM

Ecologically, CAM is best described as an adaption to water stress. CAM plants generally grow in arid environments and display succulent leaves or stems, which have a low surface/volume ratio. Apart from having large vacuoles the photosynthetic cells of CAM plants exhibit no apparent unique ultrastructure or differentiation into bundle-sheath and mesophyll cell types (Kluge and Ting, 1978; Ting, 1985).

Plants that perform CAM fall into two categories; those that perform CAM at all times (obligate or constitutive) and those in which CAM can be induced by environmental conditions (facultative). Several species of CAM plant have been studied including *Bryophyllum fedtschenkoi*, *Kalanchoë daigremontiana* and *Kalanchoë blossfeldiana* (Holdsworth, 1971; Knopf and Kluge, 1979).

Figure 1.3. **The Crassulacean acid metabolism pathway.** The enzymes which catalyse the numbered reactions are: 1, carbonic anhydrase; 2, PEP carboxylase; 3, malate dehydrogenase; 4, malic enzyme; 5, pyruvate, phosphate dikinase; 6, RuBP carboxylase. The reactions indicated by broken lines and black lines signify reactions occuring in the light and dark respectively. The mechanism and function of the pathway is discussed in the text.


MESOPHYLL CELL
AIR
CYTOSOL
VACUOLE
CO_2
CO_2
1
OH^-
HCO_3^-
2
P_i
Oxaloacetate
PEP
NADH
3
NAD^+
$AMP + PP_i$
5
$ATP + P_i$
Malate
Malate
$NADP^+$
4
NADP(H)
Pyruvate
CO_2
CHLOROPLAST
6
RuBP
3-PGA
sucrose , starch

Water stress can induce CAM in some species (Kluge and Ting, 1978) including *Sedum rupestre* (Pilon-Smits *et al.*, 1990) and *Mesembryanthemum crystallinum* (Cushman *et al.*, 1990), although in the latter case photoperiod also plays a role (von Willert *et al.*, 1976). High day and low night temperature favour CAM in *Ananas comosus* (Neales, 1973).

The characteristics of CAM can be summarised as follows:

(i) Net uptake of atmospheric CO_2 occurs at night and ceases during the day. This event is accompanied by an inverse rhythm of stomatal opening in comparison with non-CAM plants; the stomata opening at night.

(ii) The malate content of the photosynthetic tissue fluctuates in a diurnal rhythm; malate accumulates during the night and disappears during the day.

(iii) The content of the storage carbohydrates of the photosynthetic tissues fluctuate in an inverse fashion to the malate concentration.

The pathway of CAM is illustrated in Figure 1.3 (reviewed in Osmond, 1978; Osmond and Holtum, 1981). The most notable feature of the pathway is the temporal separation in malate synthesis and breakdown, which is accomplished by the transport and storage of malate in the vacuole. Atmospheric CO_2 is fixed during the night by PEP carboxylase, thereby allowing malate to be synthesised. The malate is transported into the vacuole where it accumulates until the following day. The malate then moves back into the cytoplasm where it is decarboxylated by malic enzyme yielding CO_2 and pyruvate. The pyruvate is converted back into storage carbohydrate, while the CO_2 is utilised by RuBP carboxylase in photosynthesis.

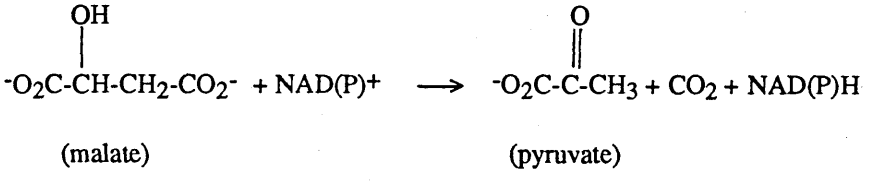
PEP carboxylase plays a pivotal role in the overall maintenance of CAM, since it catalyses the first committed step in the pathway. The regulation of PEP carboxylase is discussed in Section 1.2.3 and in Chapters 4 and 5. Despite its importance, the intracellular location of PEP carboxylase is controversial.

Biochemical techniques based on the isolation of organelles from CAM plants suggested that PEP carboxylase was located primarily in the chloroplast (Schnarnenberger *et al.*, 1980; Perrot-Rechenmann *et al.*, 1982). Immunocytochemical visualization studies, however, revealed that PEP carboxylase in *Kalanchoë blossfeldiana* was present in the cytosol (Kluge and Ting, 1978; Gadal *et al.*, 1983).

Immunocytochemical studies also revealed that the enzyme was present in the cytosol of C_4 plants, and in both the cytosol and chloroplasts of the C_3 plant spinach (Gadal *et al.*, 1983). Further experiments are, therefore, required to unambiguously determine the intracellular location of PEP carboxylase. It also remains to be established whether the enzyme activity associated with the chloroplasts is in the stroma or merely adhered to the outer envelope of the chloroplast.

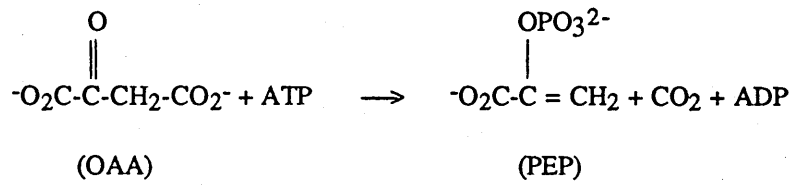
The reaction catalysed by the NAD-dependent MDH is reversible, but the equilibrium constant strongly favours reduction of OAA (Kluge and Ting, 1978). Succulent plants are known to accumulate large quantities of malate at night; for example up to 80 μmol per g fresh weight has been found in *B. fedtschenkoi* (Nimmo *et al.*, 1984). The regulation of plant NAD^+-dependent MDH is not understood. An NADP-dependent MDH is also known to occur in plants. This latter chloroplastic enzyme is light-activated, its activity being dependent on the photosynthetic electron transport chain (Edwards *et al.*, 1985). The NADP-dependent MDH cannot therefore play a role in the nocturnal synthesis of malate. Three isoforms of the NAD^+-dependent MDH have been identified (Rocha and Ting, 1970) in C_3 plants. The activity of a cytosolic form of the enzyme does not appear to be light modulated, and is thought to be controlled by metabolites (Doncaster and Leegood, 1990).

Two different decarboxylation systems are known to occur in CAM plants. Some species utilize an NAD- or NADP- dependent malic enzyme (e.g. *B. fedtschenkoi).* An NAD-malic enzyme has been purified from *Crassula argentea* (Wedding and Black, 1983) and in *Kalanchoë daigremontiana* both NAD- and NADP-dependent activities have been observed (Dardart and Queiroz, 1974; Dittrich *et al.*, 1975). Decarboxylation of malate by malic enzyme yields CO_2 and pyruvate:



Other CAM species utilize PEP carboxykinase instead of malic enzyme. In such species malate is

oxidised to OAA by MDH. The OAA is subsequently decarboxylated yielding PEP and CO_2 as follows:



Many plants in the Bromeliaceae, Euphorbiaceae and Liliacea succulent families contain PEP carboxykinase activity and show little or no malic enzyme (Dittrich *et al.*, 1973), indicating that the decarboxylation pathway occurs exclusively by PEP carboxykinase.

The CAM cycle is completed by the regeneration of the substrate PEP. In 'malic enzyme' type CAM plants, the enzyme pyruvate, phosphate dikinase (PPDK) converts pyruvate into PEP (Kluge and Osmond, 1971; Sugiyama and Laetsch, 1975). Studies on PPDK in C_4 plants show it to be a light-modulated enzyme which is regulated by reversible phosphorylation (see Section 1.3.2) (Edwards *et al.*, 1985; Chollet, 1990). PPDK is unnecessary in plants which use PEP carboxykinase.

The carbon flow to and from storage glucan in CAM plants has been the subject of several studies (Sutton, 1975a, b; Black, 1973; Kluge and Fischer, 1984). The factors integrating carbon flow with diurnal CO_2 fixation are not understood. There appears to be increased glycolysis from glucans rather than from soluble sugars during the night to supply the high demand for PEP (Sutton, 1975a). Both α-amylase and phosphorylase activity are thought to participate in starch mobilization (Kluge and Ting, 1978). The glucose derived from starch hydrolysis is converted into triose-phosphate and subsequently into PEP by glycolysis. Analysis of the activities of the glycolytic enzymes in *Kalanchoë daigremontiana* and *Kalanchoë tubiflorum* showed no significant variation from those enzyme activities reported in leaves of C_3 or C_4 plants (Osmond and Holtum, 1981). A different conclusion was reached by Pierre and Queiroz (1979), who reported a threefold increase in the activity of several glycolytic enzymes upon induction of CAM in *Kalanchoë blossfeldiana.* This increase in activity may not be directly related to CAM, but may be a consequence of normal leaf development. The relationship between glucose utilized for the production of malate and that consumed in respiration is not understood.

The possibility of parallel pathways of glycolysis in CAM plants has been proposed (Osmond, 1978). However, the cell could only maintain the regulation of such pathways if the appropriate enzymes were located in different cellular compartments or formed multi-enzyme aggregates. In C_3 plants, enzymes of glucan hydrolysis and enzymes of glycolysis converting glucose 6-phosphate to PEP, have been found in the chloroplast (Kelly *et al.*, 1976). If such a pathway existed in CAM plants, then the PEP would have to be transported to the cytoplasm (asssuming PEP carboxylase is cytoplasmic). Export of PEP from the chloroplast via a P_i-translocator has been observed in mesophyll cells of C_4 plants (Hatch and Osmond, 1976). Apart from observations of increased glycolysis at night and increased gluconeogenesis during the day, the regulation of carbohydrate metabolism in CAM plants remains to be explored.

In order for CAM to proceed efficiently, CO_2 released during decarboxylation must not be refixed by PEP carboxylase. Hence the CAM pathway must be controlled in order to allow maximum CO_2-assimilation via RUBP carboxylase during the day. This could be achieved if PEP carboxylase activity was absent or limited during the period of photosynthesis. In recent years a number of studies on the regulation of PEP carboxylase have been carried out (see Section 1.3). It is evidently a key metabolic enzyme in C_4 and CAM plants. A number of factors including compartmentation of metabolites and environmental conditions also exert a regulatory role in CAM. In addition, detailed investigation of the physiological aspects of CAM in *B. fedtschenkoi* (Wilkins, 1960, 1962, 1984; Anderson and Wilkins, 1989a, b) revealed that there is a strong temporal regulatory mechanism, which operates through an endogenous circadian rhythm.

1.1.3. Circadian rhythms in CAM

A circadian rhythm is a biological rhythm with a period of about 24 hours that persists in constant enviromental conditions. Circadian rhythms are often synchronised to daily light/dark cycles. Such rhythms are said to be endogenous. In addition to their persistence in constant conditions, all circadian rhythms share two other distinguishing characteristics. First, the period length of the free-running system is temperature compensated, that is, the period length remains nearly constant over a wide range of temperatures. Second, endogenous rhythms can be re-set or phase-shifted by various

stimuli. How these biological rhythms are generated and maintained is not understood, despite their widespread occurrence and importance in plants, fungi, animals and man.

Many physiological and metabolic activities in higher plants show circadian rhythms. Among the best studied are, leaf and petal movements (Bunslow, 1953; Bunning, 1958; Satter and Watson, 1981), photosynthesis (Meyer *et al.*, 1989; Kloppstech, 1985; Nagy *et al.*, 1988; Tavladosrki, 1989), respiration (Endo and Ikusima, 1989), stomatal movement (Lüttge and Ball, 1978; Gordon *et al.*, 1989) and dark CO_2-fixation in some CAM species (Wilkins, 1959, 1961; Buchanan-Bollig, 1984). Some well studied circadian rhythms occur in unicellular organisms. Circadian rhythms of bioluminescence, cell division and protein synthesis occur in the marine dinoflagellate *Gonyaulax polyedra* (Sweeney, 1974; Knoetzel and Rensing, 1990) and of photosynthetic capacity in *Acetabularia* (Berger *et al.*, 1987).

Detached leaves of *B. fedtschenkoi* exhibit circadian rhythms of CO_2-fixation under constant environmental conditions. If the leaves are maintained in continuous darkness and initially in CO_2-free air at 15°C, they display a rhythm of CO_2 output which persists for about four days and has a period of about 23 hours (see Figure 1.4). No rhythm is observed in the dark if normal air replaces the CO_2-free air (Wilkins, 1959, 1960, 1961, 1962). A free-running rhythm is also observed if *B. fedtschenkoi* leaves are maintained in continuous light and normal air at 15°C (see Figure 1.5). This rhythm persists for up to 10 days and has a short period of about 16 hours (Wilkins, 1983, 1984; Anderson and Wilkins, 1989a, b). Similar free-running rhythms of CO_2-metabolism have been studied in *Kalanchoë daigremontiana* (Buchanan-Bollig, 1984; Buchanan-Bollig *et al.*, 1984). The variation in periodicity and persistence of the CO_2-rhythms under light and dark conditions, suggest different underlying mechanisms. Wilkins (1983, 1984) has developed a hypothesis to explain these persistent rhythms. It is based on the cyclical presence and absence of malate in the cytoplasm. Malate is known to be an allosteric feedback inhibitor of PEP carboxylase (see Section 1.2). In the CO_2-rhythm generated in continuous darkness and CO_2-free air it was proposed that malate was moved from the cytoplasm to the vacuole in a periodic manner. PEP carboxylase fixes respiratory CO_2 produced by the leaves themselves (Wilkins, 1983) thereby allowing malate to accumulate in the cytoplasm. The cytoplasmic concentration of malate must reach a significant

Figure 1.4. **The circadian rhythm of CO_2 output from *B. fedtschenkoi* leaves held in continuous darkness.** Detached leaves of *B. fedtschenkoi* were maintained in continuous darkness and CO_2-free air at 15°C and the CO_2 output was montiored over several days (Wilkins 1984).

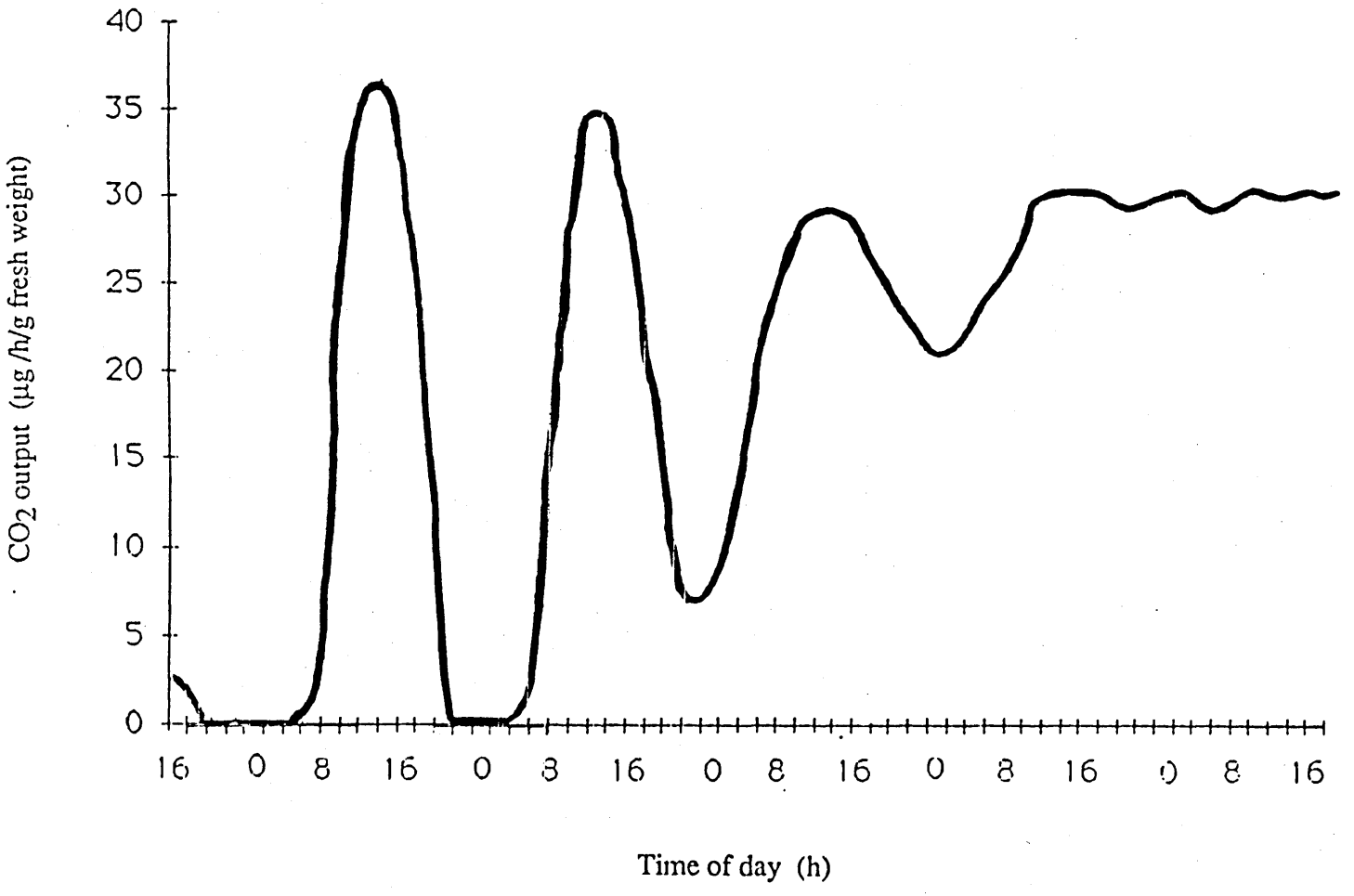

40
35
30
25
20
15
10
5
0
CO_2 output (µg /h/g fresh weight)
16 0 8 16 0 8 16 0 8 16 0 8 16 0 8 16
Time of day (h)

Figure 1.5. **The circadian rhythm of CO_2 exchange in *B. fedtschenkoi* leaves held in continuous light.** Detached leaves of *B. fedtschenkoi* were maintained in continuous light in a stream of normal air at 15°C and the CO_2 exchange was monitored over several days (Wilkins, 1984).

CO2 output (µg /h/g fresh weight)
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
Time of day (h)

level whereby it adequately inhibits PEP carboxylase activity and prevents further CO_2-fixation. The malate is subsequently transported to the vacuole allowing PEP carboxylase to be active once again. It was proposed that after about four cycles of CO_2-fixation no further malate could be pumped into the vacuole. From this interpretation it is clear that the permeability of the tonoplast to malate is a key factor in regulating the phase of the CO_2-rhythm in continuous darkness. The remarkable persistence of the CO_2-rhythm in continuous light and normal air is proposed to be due to the fact that there is no appreciable accumulation of malate in the leaf with time (Wilkins, 1984). After each period of CO_2-fixation and inhibition of PEP carboxylase, the cytoplasmic malate is metabolised by malic enzyme and photosynthesis. In contrast, Lüttge and Ball (1978) interpreted the rhythm of CO_2 exchange in *Kalanchoë daigremontiana* under continuous light and normal air to be due to a stomatal rhythm.

More recently, Wilkins (1990) showed that the CO_2-rhythm in continuous light was abolished when the epidermis was removed from *B. fedtschenkoi* leaves. However, the rhythm in continuous darkness persisted even when both the upper and lower parts of the epidermis were removed. These observations suggest that different regulatory mechanisms are induced under different environmental conditions.

The phase of the CO_2-rhythm persisting in continuous darkness can be altered by single exposures to light or high temperature (35°C) for a few hours (Wilkins, 1973, 1983; Harris and Wilkins, 1978a). The effectiveness of these treatments depends on (i) the time in the cycle at which they are applied and (ii) the duration of the treatment. In the case of light treatments the wavelength and radiant fluence rate are also important parameters (Harris and Wilkins, 1978a). Studies suggest that phytochrome is involved in the entrainment of the CO_2-rhythm. Harris and Wilkins (1978b) showed that phase-shifts induced by red light (600-700 nm) can be reversed by treatment with far-red light (720 nm). An hypothesis which accounts for the characteriestic phase-shifts induced by light and temperature stimuli has been proposed by Wilkins (1983). This hypothesis involves the notion of 'gated' channels in the tonoplast, which are light and temperature sensitive. Stimulation of these 'gates' causes malate leakage from the vacuole to the cytoplasm. This re-distribution of malate will have a direct effect on PEP

carboxylase activity and thereby will alter the phase of the CO_2-rhythm.

The rhythm of CO_2-fixation in *B. fedtschenkoi* shows little variation between 10-30°C, indicating its true circadian nature. However, at temperatures outside this range the rhythm is inhibited (Anderson and Wilkins, 1989a) under constant conditions of continuous light and normal air. Prolonged exposure of the leaves to 40°C or 2°C inhibits the rhythm by driving the basic oscillator to, and holding it at, fixed phase points in the cycle (adjusting the temperature from 2°C or 40°C to 15°C restarts the rhythm). The fixed phase-points have been identified in terms of the malate status of the leaf (Anderson and Wilkins, 1989a). High-temperature inhibition induces a low malate status in the leaf, while low temperature inhibition induces a high malate status. The differential temperature dependence of malic enzyme and PEP carboxylase is thought to be at least partly responsible for these observations (Brandon, 1967; Jones *et al.*, 1978; Ritz *et al.*, 1987).

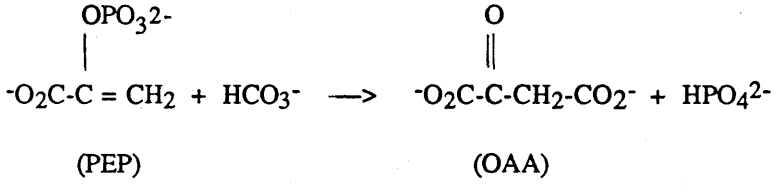
The role of membranes in the control of circadian rhythms has been highlighted by Njus *et al.* (1974). These authors have proposed a membrane model for the biological clock, which accounts qualitatively for many of its characteristic features. This model is based on ion transport channels and the adaptability of membrane lipids. The movement of malate across the tonoplast is clearly an important feature in maintaining CAM and in generating the circadian rhythm of CO_2-fixation in CAM species. Despite the importance of this membrane, little is known about its biochemical properties. Malate is thought to be transported against a large concentration gradient in an energy dependent process. This process is driven by an electrochemical proton gradient, which is driven by a tonoplast ATPase (Lüttge *et al.*, 1979; Smith *et al.*, 1984; Nishida and Tominaga, 1987) or a pyrophosphatase (Marquardt and Luttge, 1987) . $Malate^{2-}$ is transported along with H^+ in this process. Studies by Buser-Suter *et al.* (1982) on isolated vacuoles from a CAM plant suggested that ^{14}C-malate uptake reflected a catalysed exchange diffusion, which does not require an energy source. A permease was proposed to catalyse this facilitated exchange diffusion across the tonoplast. Malate efflux from the vacuole is thought to be a passive process (Lüttge *et al.*, 1981). Passive diffusion of malate was observed in non-energized tonoplast vesicles from *Kalanchoë daigremontiana* (Marquandt-Jarczyk and Lüttge, 1990). Studies using various chemical agents known to alter ion movement in biological membranes have also been shown to perturb circadian rhythms (Njus *et al.*, 1974). Apart from having a direct effect on processes such as

photosynthesis and respiration, ions may also act as secondary messenger systems which stimulate a number of cellular processes. Valinomycin, a highly specific K^+ carrier, was shown to cause phase-shifts in the photosynthetic capacity of *Phaseolus* (Bünning and Moser, 1972). Cycloheximide, a protein-synthesis inhibitor was shown to inhibit the rhythm of CO_2-fixation in *B. fedtschenkoi* (Bollig and Wilkins, 1979). This was postulated to be due to alteration of the tonoplast permeability, since several other studies have shown similar membrane-leakage effects with cycloheximide (Ellis and McDonald, 1970; McMahon, 1975). However, cyclohexmide may be directly blocking synthesis of key 'oscillator' proteins. The control of biological rhythms at the level of translation and transcription has been demonstrated (reviewed by Morse *et al.*, 1990; see also Chapters 6 and 7).

1.2. PEP carboxylase : A key enzyme in CAM

1.2.1. PEP carboxylase activity in higher plants

PEP carboxylase [orthophosphate:oxaloacetate carboxylase (phosphorylation) E.C.4.1.1.31] activity is widespread in higher plants, algae and bacteria, but is absent from mammalian tissues (O'Leary, 1982). This enzyme catalyses the highly exergonic reaction:



The reaction requires a divalent cation. Mg^{2+} and Mn^{2+} can activate PEP carboxylase activity, either of which may function *in vivo* (Nguyen *et al.*, 1988). Zn^{2+}, Ca^{2+}, Cu^{2+} and Cd^{2+} were shown to be inhibitory (Andreo *et al.*, 1987). The detailed reaction mechanism of PEP carboxylase has yet to be determined. Studies involving chemical modifications and substrate analogues suggest it is a two-step mechanism (Gonzalez and Andrew, 1989; O'Leary, 1990) involving a carboxyphosphate intermediate. PEP carboxylase is unique in that it is capable of activating HCO_3^-, by hydrolysis of a high energy phosphate bond. Other carboxylases which utilize HCO_3^- instead of CO_2 require biotin (Wood and Bardon, 1977).

Ting and Osmond (1973) have suggested that there are four different molecular species of PEP carboxylase in higher plants, namely a C_3, a C_4-photosynthetic, a CAM, and a non-autotrophic type. The main role of the C_3 and non-autotrophic types is thought to be an anaplerotic one. They serve to replenish TCA intermediates consumed in the assimilation of NH_4^+ into amino acids (Melzer and O'Leary, 1987; Schuller *et al.*, 1990). PEP carboxylase activity is also an important enzyme in the control of stomatal opening, whereby formation of malate (from OAA) alters the charge balance during guard-cell swelling (Willmer, 1983; Schnabl and Kottmeier, 1984). Both the C_4 and CAM type PEP carboxylase are responsible for the fixation of atmospheric CO_2. In C_4 plants the activity is greatly enhanced during greening of the leaf tissue, due to *de novo* synthesis of the enzyme (Goatly and Smith, 1974; Perrot *et al.*, 1984). The C_4 PEP carboxylase can be separated chromatographically from the C_3-form. In addition, the two isozymes can be distinguished by several criteria, including their molecular weight, immunochemical properties, tryptic digest peptide maps and their kinetic properties (Ting and Osmond, 1973a; Goatly and Smith, 1974; Vidal *et al.*, 1983). PEP carboxylase has been studied in several CAM species, including *B. fedtschenkoi* (Jones *et al.*, 1978, 1981; Pays *et al.*, 1980; Nimmo *et al.*, 1984, 1987) and the inducible CAM plants *Mesembryanthemum crystallinum* (Winter, 1981, 1982). The properties of this form of the enzyme are discussed in the following sections.

1.2.2. Structure

The induction of CAM in *Mesembryanthemum crystallinum* by salt stress provided a mechanism of identifying and isolating the structural gene for CAM PEP carboxylase (Bohnert *et al.*, 1988). An increase in PEP carboxylase activity (approx. 40-fold) and protein (increased from 1% to 10% of the soluble leaf protein) occurs after two weeks of salt stress (Rickers *et al.*, 1989). This increase correlates with increases in the steady state levels of PEP carboxylase mRNA (Ostrem *et al.*, 1987). In addition, anti-PEP carboxylase antibodies reacted with four apparent isoforms of the enzyme, but only one of these (M_r 110,000) increased in response to stress (Cushman *et al.*, 1990). The four isoforms may represent members of a gene family, since at least five members of the PEP carboxylase gene family were identified in the C_4 plant maize (Grula and Hudspeth, 1987). The gene encoding the active form of

CAM PEP carboxylase has been sequenced in *Mesembryanthemum crystallinum* (Rickers *et al.*, 1989). The cDNA sequence comprises 966 codons which specified a protein of M_r 110,533. The deduced amino acid sequence indicates 75% identity to the C_4 maize sequence (Hudspeth and Grula, 1989), but only 41% identity to *E. coli* and 34% identity to *Anacystis nidulans* PEP carboxylase gene sequences. (Ishijima *et al.*, 1985).

Ishijima *et al*. (1985) have proposed a conserved 14 amino acid sequence as the PEP binding site, this region is absolutely conserved between the CAM sequence and the C_4 maize sequence and is highly conserved in the *E. coli* and *Anacystis nidulans* sequences. The sequence is rich in glycine (6/14) and contains basic histidine and arginine residues which are conserved in all four known sequences (Rickers *et al.*, 1989). Andreo *et al.* (1987) indicated the importance of histidine and arginine residues in a speculative model of the active site. In addition, cysteine and lysine residues are proposed to be involved in the binding of PEP (Stiborova and Leblova, 1983; Rustin *et al.*, 1988; Gonzalez and Andreo, 1989).

PEP carboxylase has been purified from several CAM species (Jones *et al.*, 1978; Nott and Osmond, 1982; Wu and Wedding, 1985; Nimmo *et al.*, 1986). It is an oligomeric protein with subunit molecular mass values in the range 103-123 kDa, depending on the species. Several CAM and C_4 (McNaughton *et al.*, 1989) PEP carboxylase enzymes contain four identical subunits per holoenzyme. However, *B. fedtschenkoi* and some species of *Sedum* and *Kalanchoë* were shown to contain dissimilar subunits with regard to their molecular mass. Muller *et al.* (1981) found monomers of 105 kDa and 115 kDa in *Sedum* and *Kalanchoë* , while Nimmo *et al.* (1986) showed the presence of 123 kDa and 112 kDa subunits in *B. fedtschenkoi*, the subunits in the latter being present in a ratio of approximately 1:5-10 respectively. Proteolytic cleavage of the two subunits of PEP carboxylase from *B. fedtschenkoi* with V8 protease revealed very similar peptide-map patterns (Nimmo *et al.*, 1986). The subunits could not be separated chromatographically and gave a single band on native polyacrylamide gels. The physiological function of dissimilar subunits is unknown. They may originate from separate gene products or they may arise as a result of post-translational modification.

The oligomerization state of PEP carboxylase varies not only amongst species but in some cases has been shown to vary within a given species. In *Kalanchoë daigremontiana* and *Annus comosus*, PEP

carboxylase was found to be an active dimer on polyacrylamide gel electrophoresis, while gel filtration studies showed it to be a tetramer (Nott and Osmond, 1982). Dimer and tetramer states were also observed in *Mesembryanthemum crystallinum* (von Willert *et al.*, 1976) and in earlier studies on *B. fedtschenkoi* at high dilution (Jones *et al.*, 1978). However, later studies in *B. fedtschenkoi* showed that the quaternary structures of the purified day and night forms (see Section 1.2.3) of PEP carboxylase were identical, i.e. tetramers (Nimmo *et al.*, 1986). Wu and Wedding (1985) observed diurnal changes in the ratio of dimer:tetramer in *Crassula*, suggesting that a regulatory role was involved. Kruger and Kluge (1988) found no diurnal changes in quaternary structure of PEP carboxylase in *Kalanchoë daigremontiana*. There have also been conflicting reports on the role of oligomerization in C_4 plants (McNaughton *et al.*, 1989; Budde and Chollet, 1986; Huber *et al.*, 1986; Podesta and Andreo, 1989). It remains unclear whether dissociation/association of PEP carboxylase is a regulatory factor in CAM.

1.2.3. Regulation

CAM PEP carboxylase can be distinguished from C_3 and C_4 PEP carboxylase by its low K_m for PEP and high V_{max} (Ting and Osmond, 1973a). Both V_{max} and K_m in *Mesembryanthemum crystallinum* were found to be very pH dependent (Winter, 1982), while only the K_m showed significant sensitivity to change in pH, in *Kalanchoë daigremontiana* (Nott and Osmond, 1982). Diurnal changes in the K_m for PEP and pH optima were found in *Crassula* (Wu and Wedding, 1985) and also in *Mesembryanthemum crystallinum* (Winter, 1982). The V_{max} for PEP carboxylase in *B. fedtschenkoi* was unusual in that it appeared to show two pH optima, at 5.8 and 7.5 (Jones *et al.*, 1978).

PEP carboxylase activity is regulated allosterically. Glucose 6-phosphate and L-malate are the two principal metabolic effectors of the enzyme in higher plants (O'Leary, 1982). Glucose 6-phosphate, a precursor of starch and sucrose, accumulates in the early night period of CAM (Cockburn and McAulay, 1977) and was shown to activate PEP carboxylase. The activation by glucose 6-phosphate is complex. Evidence suggests that PEP can also bind to the glucose 6-phosphate site (Rustin *et al.*, 1988) and induce activation. The maximal response to glucose 6-phosphate is somewhat dependent on the concentration of substrate (PEP) present. A similar type of activation is thought to occur in several CAM species studied,

including *B. fedtschenkoi*, *Kalanchoë daigremontiana* and *Crassula* (Pays *et al.*, 1981, Nott and Osmond, 1982; Wedding *et al.*, 1989). Little is known about the binding site of glucose 6-phosphate, but studies have suggested the role of cysteine residue(s) in the process. The role of thiol groups in the binding of PEP by PEP carboxylase has been demonstrated (Rustin *et al.*, 1988; Gonzalez and Andreo, 1989) and Wedding *et al.*, (1989) have shown that glucose 6-phosphate protects PEP carboxylase against inactivation by thiol reagents. Glucose 6-phosphate also effectively reverses the inhibition caused by L-malate (Pays *et al.*, 1980).

Inhibition of PEP carboxylase by the allosteric effector L-malate undoubtedly plays a highly significant role in the regulation of the enzyme *in vivo*. The nocturnal accumulation of malate in CAM has a negative feedback effect on PEP carboxylase (O'Leary, 1982; Nimmo *et al.*, 1986; Wedding *et al.*, 1990). Large diurnal fluctuations in malate concentration occur in CAM (see Section 1.1). In addition, diurnal cycles in the malate sensitivity of PEP carboxylase have been demonstrated in *B. fedtschenkoi* (Nimmo *et al.*, 1984, 1986), *Mesembryanthemum crystallinum* (Winter, 1980, 1982), *Crassula* (Wu and Wedding, 1985; Wedding *et al.*, 1990), and *Sedum* (Manetas, 1982). Variation in the malate sensitivity of PEP carboxylase persisted in some cases even under constant environmental conditions (Nimmo *et al.*, 1987), indicating an endogenous control mechanism. In all CAM species studied, malate acts as a competitive inhibitor at high pH. At lower pH values, the type of inhibition varies, but malate clearly has a more potent effect (Pays *et al.*, 1980; Nott and Osmond, 1982; Winter, 1982). In *B. fedtschenkoi*, PEP carboxylase has an apparent K_i for malate of 3.0 mM during the 'night' and of 0.3 mM during the 'day' (Nimmo *et al.*, 1986). This tenfold change in the malate sensitivity is attributed to a covalent modification. Nimmo *et al.* (1984, 1986) identified a diurnal variation in the phosphorylation state of PEP carboxylase and subsequently purified two kinetically distinct forms of the enzyme (discussed in Section 1.3). This covalent modification allows PEP carboxylase to be physiologically 'active' at night while cytoplasmic malate levels are increasing. In contrast, the enzyme is 'inactive' during the day (due to its high malate sensitivity), while cytoplasmic malate is being decarboxylated. Further analysis of the diurnal variation in the malate sensitivity of PEP carboxylase revealed that the interconversion from the low to high K_i form and *vice versa* occurs during the dark period. The K_i increases 4-5 hours after the onset of the 'night' period and decreases 2-3 hours before the onset of the 'day' period (Nimmo *et al.*,

1984). This observation shows that light does not directly affect the covalent modification (see also Chapter 6), and is likely, therefore, to be under an endogenous control mechanism. Phosphorylation of PEP carboxylase has also been found to occur in other CAM and C_4 species (Wu and Wedding, 1985; Brulfert *et al.*, 1986; Jiao and Chollet, 1989; McNaughton *et al.*, 1989).

There is considerable variation in the reported malate sensitivities of PEP carboxylase. This variation arises for several reasons. Early investigators were unaware of the diurnal change in apparent K_i. In addition, a rapid loss in malate sensitivity of PEP carboxylase after extraction was reported, which was attributed to the conversion of the more malate sensitive, but less stable, 'day' form into the less sensitive 'night' form (Jones *et al.*, 1981; Winter, 1982; Buchanan-Bollig and Smith, 1984; Wu and Wedding, 1985). However, later studies highlighted that at least one factor in the decline of malate sensitivity was a proteolytic cleavage of the enzyme near one end of the polypeptide chain (Nimmo *et al.*, 1986; McNaughton *et al.*, 1989). Proteolysis of the enzyme does not alter the V_{max} and is only detectable on very lightly loaded polyacrylamide gels, the difference in the electrophoretic mobility being only a few kDa. Sequence analysis of PEPc carboxylase indicates the presence of a putative proteolytic-sensitive site near the N-terminus of the sequence (Chollet, 1990). McNaughton *et al.* (1989) showed that maize PEP carboxylase could be protected from proteolysis by chymostatin (a chymotrypsin inhibitor). Aromatic amino acids which are labile to chymotrypsin were identified around position 40 of the amino acid sequence (Chollet, 1990). Cleavage at these residues would release a short peptide of approximately 4 kDa.

Several other effectors of PEP carboxylase activity have been reported *in vitro*, but may not be of physiological significance. Glucose 1-phosphate and fructose 1-phosphate activate *B. fedtschenkoi* PEP carboxylase at pH 7.8, while fructose 1, 6-phosphate activates it at pH 5.8 (Pays *et al.*, 1980). P_i was found to be inhibitory in *B. fedtschenkoi* (Jones *et al.*, 1978) and maize (Doncaster and Leegood, 1987) PEP carboxylase, but Walker *et al.* (1988) observed P_i stimulation in maize. AMP and ADP stimulated PEP carboxylase activity by decreasing the K_m for PEP in *Crassula* , whereas ATP inhibited the activity (Rustin *et al.*, 1988). AMP stimulation and ATP inhibition of PEP carboxylase were also demonstrated in C_4 plants (Wong and Davis, 1973; Lavergne and Champigny, 1983; Walker *et al.*, 1988).

Regulation of PEP carboxylase activity in CAM, therefore, appears to involve covalent modification and/or association/dissociation of subunits, along with allosteric regulation by effector metabolites. There have been reports of diurnal variation in the extractable activity of PEP carboxylase (Brulfert *et al.*, 1975; von Willert and von Willert, 1979). This may be due to extraction of active and inactive forms of the enzyme, or more likely due to interference by varying malate concentration in the extracts. These authors suggested that PEP carboxylase may be synthesised nocturnally; however immunoprecipitation studies in *B. fedtschenkoi* (Nimmo *et al.*, 1984, 1987) showed that there was no diurnal variation in the amount of PEP carboxylase protein over the diurnal cycle. This observation in itself suggests that the enzyme activity is controlled by a post-translational mechanism. Evidence now suggests that the key regulatory mechanism controlling PEP carboxylase activity is reversible phosphorylation (Nimmo *et al.*, 1986; 1987; this thesis).

1.3. Protein phosphorylation as a control mechanism in higher plants

1.3.1. Protein kinases

It is well established that reversible phosphorylation of enzymes is a key regulatory mechanism in animal cells (Krebs and Beavo, 1979). In recent years, it has emerged that reversible phosphorylation is also an important feature in plant metabolic regulation (reviewed in Ranjeva and Boudet, 1987). Phosphorylation involves two reactions:

$$\text{Protein} + n\text{ATP} \longrightarrow \text{Protein-P}_n + n\text{ADP} \qquad (1)$$

$$\text{Protein-P}_n + n\text{H}_2\text{O} \longrightarrow \text{Protein} + n\text{P}_i \qquad (2)$$

Reaction 1 is catalysed by protein kinase(s) and reaction 2 by protein phosphatase(s). Most protein kinases utilize ATP as a phosphate donor. Phosphorylation occurs on serine, threonine or tyrosine residues, and affects the activity of the enzyme directly or indirectly via conformational changes. Protein kinases generally recognise the target amino acids by their surrounding amino acid motifs. Although peptide substrates have been successfully used to identify these recognition motifs, it is clear that

secondary and tertiary structures can have an overriding influence *in vivo* (Kemp *et al.*, 1975).

Protein kinases contain a regulatory domain and a highly conserved catalytic domain. The latter can extend to over 240 residues and bears ATP and substrate binding sites (Hanks *et al.*, 1988; Kemp and Pearson, 1990). Regulatory sites often contain pseudosubstrate sequence motifs which serve to inactivate protein kinases. Activation of protein kinase C (see below) is thought to occur by removal of the pseudosubstrate from the active site (House *et al.*, 1989).

Protein kinases are often classified in accordance with their mode of activation (Krebs and Beavo, 1979; Edelman *et al.*, 1987). The Ca^{2+}-dependent protein kinases constitute a large group, including Ca^{2+}-calmodulin dependent and Ca^{2+}-phospholipid dependent (protein kinase C) protein kinases. Other groups are activated by the cyclic nucleotides cAMP or cGMP. Finally, there are the so-called independent kinases, usually named according to the substrate upon which they act, eg casein-kinases (Rosen and Krebs, 1981).

Protein phosphorylation is well documented in animal systems, up to eighty protein kinases having been described (Hunter, 1987). In contrast, only a few plant proteins are recognised to undergo reversible phosphorylation (see Section 1.3.2). However, the existence of an increasing number of plant protein kinases have been demonstrated more recently using molecular biological techniques. In these studies, oligonucleotide probes corresponding to the highly conserved catalytic regions of mammalian protein kinases, have been used to isolate homologous sequences from plant gene libraries (Lawton *et al.*, 1990). Such studies have indicated the presence of protein kinase C-like genes in *Amaranthus* (Elliott and Brennan, 1990), barley and *Arabodopsis* (Zielinski *et al.*, 1990). Both protein kinase C and cyclic nucleotide dependent protein kinases may be present in *Phaseolus vulgaris* and *Oryzae sativa* (Lawton *et al.*, 1989). Walker and Zhang (1990) reported a gene encoding a novel class of receptor/serine kinase in *Zea mays* , the structure of which resembles the mammalian tyrosine kinases. Despite these advances in identifying novel plant protein kinases, no information is available on substrate specificities or the function of these protein kinases *in vivo*.

Protein kinases are integral components of cellular signal reception and transmission systems in animals. No single pathway of signal transduction in plants has yet been elucidated. It is generally thought that plants lack membrane-associated adenylate cyclase, tyrosine protein-kinases and

diacylglycerol stimulated protein-kinase activity, all of which are involved in signal transduction in animal cells (Harmon, 1990; Yunghans and Morre, 1977). However, several effectors, including light (Morse *et al.*, 1987), auxin (Felle, 1988; Ehlinger *et al.*, 1988), osmotic stress (Srivastava *et al.*, 1989), fungal elicitors (Memon *et al.*, 1989) and touch (Braam and Davis, 1990), have been shown to induce changes in either membrane phospholipids, Ca^{2+} concentrations or protein kinase activity. There is increasing evidence that Ca^{2+} acts as a secondary messenger in plants (Hepler, 1990; Sanders *et al.*, 1990; Melkonian *et al.*, 1990), along with lysophospholipids and fatty acids (Martiny-Baron and Scherer, 1989; Morre, 1990). Scherer *et al.* (1990) described evidence for a signal transduction chain in *Zucchini* hypocotyls. These authors found that auxin stimulated the activation of phospholipase A_2 in isolated tonoplasts and plasma membranes. The resulting lysophospholipids stimulated Ca^{2+} phospholipid dependent protein kinase activity. Although the substrates for this protein kinase are largely unknown, evidence suggests that one of them is a H^+/ATPase (Martiny-Baron and Scherer, 1989).

1.3.2. Plant enzymes regulated by phosphorylation

There are currently eight plant enzymes known to be regulated by reversible phosphorylation. The pyruvate dehydrogenase complex was the first enzyme recognised to be controlled by this covalent modification. The complex is enzymatically inactivated by phosphorylation. This inactivation is enhanced by high ratios of ATP/ADP, acetyl CoA/CoA and NADH/NAD^+ in the mitochondria (Randall *et al.*, 1981). Many processes involved in photosynthesis are also regulated by phosphorylation. It has been demonstrated that the light energy distribution between photosystems I and II is controlled by phosphorylation of the light-harvesting chlorophyll *a/b* complex. The phosphorylation is catalysed by a soluble light-dependent protein kinase (Bennet, 1984). Both the large and small subunits of RuBP carboxylase are susceptible to phosphorylation. A light-independent, Ca^{2+}/phospholipid-stimulated protein kinase was found to phosphorylate the small subunit. The function of this modification is not understood, but is believed to be important in the transport of the subunit across the chloroplast membrane and in its assembly with the large subunit (Soll and Buchanan, 1983; Muto and Shimogawara, 1985). Phosphorylation of the large subunit was demonstrated by Guitton and Mache (1987). The role of this phosphorylation is difficult to interpret as RuBP carboxylase regulation is complex; a protein

activator (Salvucci *et al.*, 1987) and a non-protein inhibitor (carboxyarabinitol 1-phosphate) have also been found (Gutteridge *et al.*, 1987) to regulate RuBP carboxylase activity. Activation is achieved by carbamylation of RuBP carboxylase catalysed by RuBP carboxylase activase. The inhibitor binds to the modified form of the enzyme (Servaites, 1990). Two enzymes involved in sucrose metabolism, namely sucrose phosphate synthase (SPS) and 6-phosphofructo-2-kinase, have been shown to undergo covalent modification (Walker and Huber, 1987; Huber *et al.*, 1989). Light-dark regulation of SPS is due to reversible phosphorylation (inactivation)/dephosphorylation (activation) of the enzyme. SPS also appears to contain phosphorylation sites which are involved in the stimulation of enzyme activity, however these sites are not thought to be involved in the light-dark regulation process (Huber and Huber, 1990).

Other enzymes which are involved in plant secondary metabolism have been shown to be regulated by reversible phosphorylation. These include quinate:NAD^+ 3-oxidoreductase (Ranjeva and Boudet, 1987) and 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which is involved in isoprenoid biosynthesis (Russell *et al.*, 1985).

The enzymes PEP carboxylase and PPDK involved in CAM and C_4 metabolism are also known to be regulated by phosphorylation. PPDK undergoes an unusual ADP-dependent phosphorylation (inactivation) on a threonine residue and a P_i-dependent dephosphorylation (activation) (Burnell and Hatch, 1984; Edwards *et al.*, 1985). A bifunctional protein is thought to catalyse both the phosphorylation and dephosphorylation process (Burnell and Hatch, 1985). Although PPDK is activated during the light, the mechanism of activation/inactivation is not thought to be directly controlled by light. Chollet *et al.* (1989) postulated that stromal metabolites may be key regulatory factors controlling the phosphorylation of PPDK.

The phosphorylation of PEP carboxylase in C_4 and CAM plants induces a change in the allosteric properties of the enzyme (see Section 1.2.3), such that the phosphorylated form of the enzyme has a lower sensitivity to inhibition by malate (Nimmo *et al.*, 1984; Nimmo *et al.*, 1987; Brulfert *et al.*, 1986; Echevarria *et al.*, 1988). PEP carboxylase is phosphorylated exclusively on serine residues *in vivo*, in both C_4 and CAM plants (Nimmo *et al.*, 1984; Nimmo *et al.*, 1987). The phosphorylation of PEP carboxylase in C_4 plants occurs primarily in the light, whereas in CAM it occurs during the night period.

Before the work described in this thesis was started very little was known about the enzymes responsible for the reversible phosphorylation of PEP carboxylase. However, during the course of this study, ATP-dependent soluble protein kinase(s) activities which phosphorylated PEP carboxylase *in vitro* were detected in several C_4 and CAM species; these included *B. fedtschenkoi* (this thesis), maize (Jiao and Chollet, 1989; McNaughton *et al.*, 1991), several *Kalanchoë* species (Kluge *et al.*, 1988) and sorghum (Echevarria *et al.*, 1990). Jiao and Chollet (1989) and McNaughton *et al.* (1991) partially purified this protein kinase from maize. A PEP carboxylase kinase activity was similarly partially purified from *B. fedtschenkoi* as described in Chapter 4. Phosphorylation of PEP carboxylase by the maize protein kinase was reported to reduce the malate sensitivity of the enzyme (Jiao and Chollet, 1989; McNaughton *et al.*, 1991). More recently Jiao and Chollet (1990) demonstrated that serine-15 at the N-terminus was the residue phosphorylated *in vitro* (see Hudspeth and Grula, 1989). This residue of PEP carboxylase was also phosphorylated by mammalian cyclic AMP-dependent protein kinase, and phosphorylation of the holoenzyme induced a change in allosteric properties (Terada *et al.*, 1990). There is currently no evidence for the regulation of the plant PEP carboxylase kinase by various putative effector metabolites or by Ca^{2+}/calmodulin/EGTA combinations (Chollet, 1990; McNaughton *et al.*, 1991; this thesis), apart from one report of a Ca^{2+}-calmodulin dependent activity in sorghum (Echevarria *et al.*, 1988). However, there does appear to be light-dark modulation of PEP carboxylase kinase activity in C_4 species, the activity being greater in highly illuminated leaf tissue (McNaughton *et al.*, 1991; Echevarria *et al.*, 1990).

1.3.3. Protein phosphatases

The phosphorylation process is reversed by hydrolysis of the P_i groups from the enzyme and is catalysed by protein phosphatases. Any system which is controlled by reversible phosphorylation requires both a protein kinase and a protein phosphatase. The balance between these two regulatory enzymes is therefore of paramount importance.

In recent years, a significant insight into plant protein phosphatases has been achieved by comparing them with their mammalian counterparts. Protein phosphatases are certainly one of the most highly conserved enzyme groups throughout evolution (Cohen and Cohen, 1989), the mammalian protein

phosphatases showing very high similarity to the *Drosophila* and yeast enzymes. The mammalian serine/threonine-specific protein phosphatases show broad substrate specificity *in vitro* and their classification is based on their response to specific inhibitors and activators. Four major classes of protein phosphatase catalytic subunit have been identified (reviewed in Cohen, 1989). Type 1 protein phosphatases dephosphorylate the ß-subunit of phosphorylase kinase. They do not require metal ions for activity and are inhibited by thermostable proteins, inhibitor-1 and inhibitor-2 (Cohen *et al.*, 1988b). The type 2 protein phosphatases preferentially dephosphorylate the α-subunit of phosphorylase kinase. The type 2 phosphatases comprise three enzymes (types 2A, 2B and 2C) which can be distinguished by their requirements for cations. Protein phosphatase 2A does not require cations, while type 2B requires Ca^{2+}/calmodulin and type 2C requires Mg^{2+} (Cohen, 1989). The identification and quantification of protein phosphatases have been revolutionised by the discovery of the tumour promotor okadaic acid (Haystead *et al.*, 1989; Cohen *et al.*, 1990). Okadaic acid is a specific inhibitor of type 1 and type 2A protein phosphatases. However, it is a much more potent inhibitor of the type 2A protein phosphatase (which it inhibits at 1nM) than of the type 1 enzyme (which it inhibits at 10-15nM) (Cohen *et al.*, 1989). More recently a cyanobacterial compound (microcystin-LR) and a polyketide isolated from *Streptomyces* (tautomycin) have also been identified as inhibitors of type 1 and type 2A protein phosphatases (MacKintosh *et al.*, 1990; MacKintosh and Klumpp, 1990).

Cohen *et al.* (1989) devised a novel method for identifying and quantifying protein phosphatases in mammalian tissues, employing okadaic acid and the heat stable protein inhibitors. This method was extended to the analysis of protein phosphatase activities in yeast (Cohen *et al.*, 1989) and in higher plants (Mackintosh and Cohen, 1989), and indicated remarkable similarities to the mammalian protein phosphatases. A protein phosphatase activity must be present in *B. fedtschenkoi* in order for a diurnal change in the phosphorylation state of PEP carboxylase to be achieved (Nimmo *et al.*, 1984, 1987). The protein phosphatase activities present in CAM or C_4 plants had not been investigated prior to this study (see Chapters 5 and 6). However, Nimmo *et al.* (1986) showed that PEP carboxylase pre-labelled *in vivo* could be dephosphorylated *in vitro* by alkaline phosphatase, demonstrating the reversibility of the phosphorylation system.

1.4 Objectives

The efficiency of CO_2 assimilation in CAM and C_4 plants is influenced to a large extent by the activity of the primary CO_2 fixing enzyme PEP carboxylase. The general objective of this study was to investigate the regulation of this enzyme in the CAM plant *B. fedtschenkoi.* Detached leaves of *B. fedtschenkoi* display a circadian rhythm of CO_2 fixation, which is directly attributable to changes in flux through PEP carboxylase (Warren and Wilkins, 1961). This rhythm can be easily monitored and readily manipulated and therefore offers an ideal system for studying biological rhythms at a biochemical level. An understanding of the factors controlling PEP carboxylase activity should provide an insight into the nature of the basic oscillator responsible for the circadian rhythm of CO_2 fixation. PEP carboxylase was shown to undergo phosphorylation and dephosphorylation in both C_4 and CAM species (Nimmo *et al.*, 1984, 1987). However the putative protein kinase(s) and protein phosphatse(s) responsible for this covalent modification had not been identified or characterized. The major aim of this work was therefore to identify these two regulatory enzymes. Further studies were undertaken in an attempt to understand the role of the protein kinase and phosphatase in generating the rhythm of CO_2 fixation.

Chapter 2

MATERIALS AND METHODS

2.1. Materials

[γ-^{32}P] ATP (triethylammonium salt, approx. 5000Ci/mmol) and carrier free [^{32}P] orthophosphate were from Amersham International, Bucks, U.K.

Antipain hydrochloride, benzamidine hydrochloride, blue dextran-agarose, Bromophenol Blue, bovine serum albumin (BSA), Coomassie Brilliant Blue G250, cycloheximide, glucose 6-phosphate (monosodium salt), leupeptin (hemisulphate salt), L- and D-malate (disodium salt), M_r marker proteins for SDS/polyacrylamide gel electrophoresis, phenylmethanesulphonyl fluoride (PMSF), puromycin, silver nitrate and sodium azide were obtained from Sigma (London) Chemical Co., Poole Dorset, U.K.

Dithiothreitol (DTT), NADH (disodium salt), phosphoenolpyruvate (monosodium salt), ATP (disodium salt), malate dehydrogenase and Tris were from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K.

Sephadex G-25M, DEAE-cellulose, FPLC Mono Q and Superose 6 pre-packed columns were from Pharmacia, Milton Keynes, Bucks., U.K.

Acrylamide monomer, ammonium persulphate, ethanol, glycine, hydrogen peroxide, 2-mercaptoethanol, N',N' methylenebisacrylamide, octan-1-ol, potassium dihydrogen orthophosphate, sodium dodecyl sulphate (SDS), N,N,N',N' -tetramethylethylenediamine (TEMED) and trichloracetic acid were 'AnalaR' grade materials from BDH Chemicals, Poole, Dorset, U.K.

Hydroxylapatite (Bio-gel HTP) was from Bio-Rad Laboratories (England) Ltd., Bramley, Kent, U.K.

Staphlococcus aureus V8 protease was from Miles Laboratories, East Mosley, Surrey, U.K.

Mammalian protein phosphatases types 1 and 2A , mammalian inhibitor 2, okadaic acid and [^{32}P] phosphorylase *a* were gifts from Dr C. MacKintosh and Prof. P. Cohen of the Department of Biochemistry, University of Dundee, Dundee, Scotland.

All other reagents used were of the highest grade commercially available.

2.2. Plant material

The succulent plant *Bryophyllum (Kalanchoë) fedtschenkoi* Hamet et Perrier was propagated vegetatively from the original stock (Wilkins, 1959, 1960) used in earlier studies. The cuttings were grown in a glasshouse under a 16h photoperiod, maintained throughout the year with mercury-vapour lamps. Four to six month old plants were then transferred to controlled enviroment growth rooms. Two growth rooms were used, both having an 8h photoperiod, one from 0800h-1600h and the other from 1600h-2400h (reverse-phase). The light was provided by white fluorescent lamps and 12 100W tungsten lamps, giving a radient fluence rate of $20W/m^2/s$. The temperature during the illumination period was 28°C and 15°C during the dark period. The definitions of 'day' and 'night' referred to in this thesis signify the periods of illumination and darkness respectively.

Plants were watered every 4 to 5 days. Leaves of similar size and age, usually taken between nodes four to eight, were used from plants that had been under controlled growth conditions for at least two weeks.

2.3. General biochemical methods

2.3.1. pH calibrations were done using a Rusell pH probe. The pH of buffers and solutions were adjusted at the temperature of use , either 5°C or room temperature.

2.3.2. Conductivity measurement was carried out at 5°C using a Radiometer conductivity meter, type CDM 2c (Radiometer, Copenhagen, Denmark).

2.3.3. Glassware and plastics were cleaned in "Haemo-sol" solution (Alfred Cox (Surgical) Ltd., UK), rinsed in distilled water and oven dried.

2.3.4. Chromatographic materials. Sephadex G-25M, DEAE-cellulose hydroxylapatite and blue dextran-agarose were swollen and packed according to the manufacturers instructions. Sephadex G-25 was stored in 0.02% (w/v) sodium azide. Blue dextran-agarose was regenerated by washing in 2 M NaCl followed by 90% (v/v) ethanol and stored in 0.02% sodium azide. Hydroxylapatite and DEAE-cellulose

retained plant pigments and were therefore discarded after one use.

2.3.5. Protein concentrations were estimated using the Bradford (1976) method based on the binding of the dye Coomassie Brilliant Blue to proteins. Bradford's reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G250 in 50 ml of 95% (v/v) ethanol and 100 ml of 85% (w/v) orthophosphoric acid prior to dilution to 1 litre with distilled H_2O. The solution was then filtered before use. BSA was used to construct a calibration curve of absorbance at 595 nm versus protein concentration (0 - 25 µg). Protein samples (5 - 20 µl) were added to 2.5 ml of reagent, mixed, and the absorbance read at 595 nm after 5 min. The concentration of the unknown could then be determined from the standard curve.

2.3.6. Concentration of protein samples was done using Centricon 30 microconcentrators (Amicon Ltd., Stonehouse, Glos., UK) according to the manufacturer's instructions.

2.3.7. Spectrophotometric assays were carried out in semi-micro quartz cuvettes or plastic disposable cuvettes (1 cm path, 1 ml). A Cecil CE272 linear ultraviolet spectrophotometer was used to measure PEP carboxylase activity. Enzyme assays, apparent K_i determinations and protein concentration determinations were carried out on a Philips PU8720 UV/VIS scanning spectrophotometer.

2.3.8. Centrifugation was carried out in a Beckman model J2-21 centrifuge using a JA20 rotor. Small volume samples (1 - 10 ml) were centrifuged in a Beckman microfuge model E.

2.3.9. Micropipetting (5 µl - 1 ml) was done using adjustable Finnpipettes (Labsystems Oy, Pulttitie 9 - 11, 00810 Helsinki 81, Finland) or Gilson pipetmen (Gilson Medical Electronics, 72 rue Gambetta, 954 00 Villiers-le-Bel, France). Volumes less than 5 µl were dispensed using a microsyringe (Scientific Glass Engineering Ply Ltd., North Melbourne, Australia 3051).

2.3.10. Scanning laser densitometer (Shimadzu Model CS-9000) The laser densitometer used in some experiments was only available for use during a trial period in the Department.

2.4. Methods developed for the purification of enzymes

2.4.1. Buffers

Buffer A (extraction): 100 mM Tris/HCl, pH 8.0, containing 2 mM EDTA, 10 mM malate, 1 mM

DTT, 1 mM benzamidine and 2% (w/v) poly (ethylene glycol) 20000.

Buffer B: 50 mM Tris/HCl, pH 7.5, containing 1 mM benzamidine, 1 mM DTT and 5% (v/v) glycerol.

Buffer C: 50 mM Tris/HCl, pH 7.8, containing 1 mM benzamidine, 1 mM DTT and 20% (v/v) glycerol.

Buffer D: 20 mM Tris/HCl, pH 7.5, containing 0.1 mM EGTA, 0.1 mM benzamidine and 10% (v/v) glycerol.

Buffer A was derived from the extraction buffer employed by Nimmo *et al.* (1986) for the purification of PEP carboxylase from *B. fedtschenkoi*. Buffers B and C were used by Jiao and Chollet (1989) during the partial purification of maize leaf PEP carboxylase kinase. Buffer D was used by Cohen *et al.* (1988) for the purification of mammalian protein phosphatases.

All purification steps were carried out at 5°C except Superose 6 and Mono Q chromatogrphy which was done at room temperature.

Benzamidine and DTT were stored as 1 M solutions at -20°C and added to the buffers just before use.

2.4.2. Preparation of leaf extracts

Leaf extracts were made by cutting up leaves with scissors and homogenising them in 1 ml of Buffer A per g FW of tissue. A few drops of octan-1-ol, 0.5% (w/w) acid-washed sand and 5% (w/w) sodium bicarbonate was added prior to homogenisation for 30 s at low speed in a Waring blender. Homogenates were filtered through two layers of muslin and the pH adjusted to 7.5 if necessary. Extracts were centrifuged for 15 min at 15000 g.

2.4.3. Purification of PEP carboxylase

(i) Extraction. *B. fedtschenkoi* leaves (30 g) were taken from plants between 09.00 and 11.00 h in the growth rooms (Section 2.2). The leaves were extracted and centrifuged as described in Section 2.4.2.

(ii) Sephadex G-25M chromatography. The supernatant was desalted on a 15 cm x 4.5 cm (200 ml) bed volume Sephadex G-25 M column pre-equilibrated in 100 mM Tris/HCl, pH 7.5, containing 2 mM EDTA, 10 mM malate, 1 mM DTT and 1 mM benzamidine. The flow rate was 5 ml/min and 5 min fractions were collected.

(iii) Hydroxylapatite chromatography. A 10 ml bed volume of hydroxylapatite was pre-equilibrated in the desalting buffer. The desalted extract was mixed with the hydroxylapatite and swirled gently on ice for 5 min until all the PEP carboxylase activity had bound to the matrix. The suspension was then poured back into the column and washed with 30 ml of desalting buffer. Protein was eluted in 100 mM Tris/HCl, pH 7.5 containing 40 mM potassium phosphate, 0.1 mM EDTA, 1 mM benzamidine, 1 mM DTT and 10mM malate until the A_{280} fell to zero. PEP carboxylase was then eluted by increasing the potassium phosphate concentration in the buffer to 150 mM. The flow rate was 2 ml/min and 1 min fractions were collected.

(iii) Sephadex G-25M chromatography. The active fractions from the hydroxylapatite column were pooled and desalted on a 55 cm x 2.2 cm (200 ml) column of Sephadex G-25M, pre-equilibrated in 50 mM Tris/HCl, pH 7.5 containing 0.1 mM EDTA, 1 mM DTT and 1 mM benzamidine. The flow rate was 7 ml/min and 0.5 min fractions were collected.

(iv) DEAE-cellulose chromatography. The desalted PEP carboxylase pool was loaded onto a 1.2 cm x 1 cm (1 ml) DEAE-cellulose column which was equilibrated in the 50 mM Tris/HCl desalting buffer. The column was washed in this buffer until the A_{280} was zero. PEP carboxylase was then eluted in the same buffer containing 100 mM NaCl. The flow rate was 0.5 ml/min and 2 min fractions were collected. The pool was concentrated to 0.5 ml in a Centricon 30 microconcentrator (Section 2.3.6).

(v) Superose 6 chromatography. The concentrated PEP carboxylase pool was chromatographed on a 30 cm x 1.0 cm Pharmacia (FPLC) Superose 6 column, in 50 mM 1, 3-bis [Tris hydroxymethyl) methylamino] propane/HCl, pH 7.5, containing 50 mM KCl, 0.1 mM EDTA, 1 mM DTT and 1 mM benzamidine. The flow rate was 0.3 ml/min and 0.5 min fractions were collected. The active fractions were pooled and dialysed overnight into 50 mM 1, 3-bis [Tris (hydroxymethyl)

methylamino] propane/HCl, pH 7.5, containing 0.1 mM EDTA, 1 mM DTT, 1 mM benzamidine and 50% (v/v) glycerol. Purified PEP carboxylase was stored at -20°C.

2.4.4. Partial purification of PEP carboxylase kinase

(i) Extraction. Leaves (60 g) taken from plants in the middle of the dark period were extracted in Buffer A as described in Section 2.4.2.

(ii) Ammonium sulphate fractionation. The supernatant was brought to 50% saturation with ammonium sulphate, stirred for 30 min on ice, then centrifuged for 15 min at 150000 g. The precipitate was resuspended in 4 ml of Buffer B.

(iii) Sephadex G-25M chromatography. The dissolved pellet was desalted into Buffer B on a 45 cm x 1.2 cm (50 ml) Sephadex G-25 M column. The flow rate was 2.5 ml/min and 1 min fractions were collected. The peak green fractions were pooled.

(iv) Blue dextran-agarose chromatography. The desalted pool was loaded onto a 4.5 cm x 2.7 cm (25 ml) column of blue dextran-agarose equilibrated in Buffer B. The column was washed in the same buffer until the A_{280} fell to zero, then protein was eluted in Buffer B containing 0.5 M NaCl. The flow rate was 0.4 ml/min and 5 min fractions were collected. The A_{280} peak fractions were pooled and desalted on a 55 cm x 2.2 cm (200 ml) Sephadex G-25 m column into Buffer B.

(v) Mono Q chromatography. The desalted pool was chromatographed on a Mono Q column (0.5 cm x 5.0cm) connected to a Pharmacia FPLC system. A linear 10 ml gradient of 0 - 400 mM NaCl in Buffer B was applied at a flow rate of 1 ml/min and 1 min fractions were collected. The fractions were dialysed individually overnight into Buffer C. The dialysed fractions were assayed for protein kinase activity as described in Section 2.5.2 (ii). Active fractions were pooled and concentrated to approx. 1 ml. The protein kinase was stored at -20°C in 0.2 ml aliquots.

2.4.5. Partial purification of PEP carboxylase phosphatase

(i) Extraction. Leaves (60 g) were removed from *B. fedtschenkoi* plants during the light period between 0900 and 1000h (Section 2.2). Extracts were prepared as described in Section 2.4.2.

(ii) Ammonium sulphate precipitation. The supernatant was brought to 75% saturation with ammonium sulphate, stirred for 30 min on ice and centrifuged for 15 min at 15000 g. The precipitate was resuspended in 8 ml of Buffer D.

(iii) Ethanol precipitation. 10 vols of 95% ethanol containing 1 mM PMSF was added to the resuspended protein fraction at room temperature, mixed and centrifuged immediately at 15000 g for 10 min. The precipitate was resuspended in 4 ml of Buffer D at 4°C then recentrifuged for 10 min. The supernatant was collected and the precipitate re-extracted with 4 ml of Buffer D.

(iv) Sephadex G-25M chromatography. The pooled supernatants from extraction of the pellet were desalted on a 45 cm x 1.2 cm (50 ml) Sephadex G25-M column into Buffer D. The active phosphatase fractions were pooled (Section 2.5.2(ii)).

(v) Mono Q chromatography. The desalted protein phosphatase pool was chromatographed on a Pharmacia (FPLC) Mono Q (0.5 cm x 5.0 cm) column equilibrated in Buffer D. Protein was eluted in a 25 ml linear gradient of 0 - 400 mM NaCl in Buffer D at 1 ml/min and 1 min fractions were collected. The peak protein phosphatase fractions were pooled (Section 2.5.3 (i)), concentrated fourfold (approx.) and stored at 4°C.

2.5. Assay procedures

2.5.1. PEP carboxylase activity

The standard spectrophotometric assay mixture contained, in 1 ml, 50 mM Tris/HCl, pH 7.8, 5 mM $MgCl_2$, 2 mM PEP, 0.2 mM NADH, 10 mM $NaHCO_3^-$, 5 units of MDH and the enzyme sample. The decrease in A_{340} at 25°C caused by the oxidation of NADH by the coupling enzyme MDH was proportional to the PEP carboxylase concentration.

The malate sensitivity of PEP carboxylase was determined using an appropriate range of L-malate concentrations in the standard assay mixture. The concentration of malate required for 50% inhibition of enzyme activity (apparent K_i) was estimated from a plot of rate versus malate concentration.

One unit of enzyme activity is the amount required to catalyse the formation of 1 µmol of product per min.

2.5.2. PEP carboxylase kinase activity

(i) Leaf extracts. Extracts were prepared as described in Section 2.4.2. Small samples (1 ml) were desalted on a Sephadex G-25M 12 cm x 1 cm (10 ml) column equilibrated in 50 mM Tris/HCl, pH 7.8, containing 1 mM benzamidine, 1 mM DTT and 5% (v/v) glycerol.

Desalted extract containing 0.002 units of PEP carboxylase activity (unless otherwise stated) was incubated in 50 mM Tris/HCl, pH 7.8, containing 1 mM benzamidine, 10 µg antipain/ml, 10 µg leupeptin/ml, 5 nM okadaic acid, 5 mM $MgCl_2$, 0.01 mM [γ-^{32}P] ATP (1µCi) and 0.03 units of PEP carboxylase purified from illuminated tissue, in a total volume of 25 µl. Incubations were carried out for 10 min at 30°C.

(ii) Partially purified 'PEP carboxylase kinase activity. Assays were based on the method of Jiao and Chollet (1989). Protein kinase (17 µl), which had been dialysed into Buffer C (see Section 2.4.1), was incubated with 0.03 units of purified 'day' form PEP carboxylase or 7.5 µg of glycogen synthase in Buffer C, containing 10 µg antipain/ml, 10 µg leupeptin/ml, 5 mM $MgCl_2$, and 0.1 mM [γ-^{32}P] ATP (1 µCi), in a total volume of 25 µl at 30°C. Large scale incubations (x 10) were carried out in order to assess the time-course of phosphsorylation of PEP carboxylase. In such experiments 35 µl aliquots were removed from the incubation at intervals; 10 µl of this was used to determine the apparent K_i for malate (Section 2.5.1) and the degree of phosphorylation of PEP carboxylase was determined in the remaining 25 µl. Control assays contained buffer C in place of protein kinase.

Protein kinase assays were terminated by boiling for 2 min with 5 µl of x5 SDS sample buffer and analysed by SDS/polyacrylamide gel electrophoresis and autoradiography (Section 2.6). Alternatively, 1.0 ml of 10% (w/v) TCA was added to stop the reaction and the $^{32}P_i$ incorporation into protein measured as described in Section 2.9.4.

(iii) PEP carboxylase kinase activity in mixtures of 'day' and 'night' extracts. Leaves were extracted and desalted as described in Section 2.5.2(i). Samples (concentrations given in relevant text) were preincubated in Buffer C containing 5nM okadaic acid, 10µg antipain/ml, 10µg

leupeptin/ml and 1mM benzamidine, for 10 min at 30°C in a total volume of 20µl. Purified 'day' form PEP carboxylase (0.03 units) was then added with 0.01 mM [γ-32P] ATP (1µCi) and 5mM $MgCl_2$ (30µl total volume), and incubated for 10 min at 30°C. Buffer C was added in place of PEP carboxylase in the control assays. The reactions were terminated by the addition of SDS sample buffer and analysed by SDS/polyacrylamide gel electrophoresis.

2.5.3. Protein phosphatase activity

Protein phosphatase activity was quantified by measuring the release of $^{32}P_i$ from ^{32}P-labelled phosphorylase *a* or casein, by the method of Cohen *et al.* (1988a). The dephosphorylation of these substrates was limited to ≤30% to ensure linearity, hence samples to be assayed were diluted appropriately.

One unit of phosphatase activity (U) catalyses the dephosphorylation of 1 µmol of ^{32}P-labelled substrate per min.

^{32}P-phosphorylase *a* stock was obtained in crystalline form (gift from Dr. C. MacKintosh and Prof. P. Cohen, Dundee University) . This was resuspended in 50 mM Tris/HCl, pH 7.0, containing 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 15 mM caffeine, at a concentration of 3 mg (30 nmol)/ml.

^{32}P-casein was prepared by the method of McGowan and Cohen (1988b). Casein (10 mg) was incubated with 2 mU of cyclic AMP-dependent protein kinase for 8 h at 30°C in 50 mM Tris/HCl, pH 7.0, containing 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 10 mM magnesium acetate and 0.2 mM [γ-^{32}P] ATP (90 µCi). The reaction was terminated by the addition of 0.1 ml of 100 mM EDTA, 100 mM sodium pyrophosphate, pH 7.0, and then desalted on a Sephadex G-50 superfine 15 cm x 1.5 cm column equilibrated in 50 mM Tris/HCl, pH 7.5, containing 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 5% (v/v) glycerol to separate the ATP from the ^{32}P-casein. The incorporation of phosphate was approx. 4 nmol/mg casein. ^{32}P-casein was diluted in the desalting buffer to a concentration of 3 nmol $^{32}P_i$ /ml and stored at 4°C.

(i) Measurement of protein phosphatase activity using ^{32}P-labelled substrates. Sample (10 µl, diluted in 50 mM Tris/HCl, pH 7.0, containing 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 1 mg/ml BSA) was preincubated for 10 min at 30°C with 10 µl of the dilution buffer containing 0.03% Brij-35 in place of the BSA. The reaction was initiated by the addition of either 10 µM phosphorylase *a*, 4 µM casein or 0.5 µM ^{32}P-labelled PEP carboxylase (see Section 2.9.1) to a total volume of 30 µl (larger scale incubations were carried out to monitor the time course of dephosphorylation of PEP carboxlase). The reaction was terminated by the addition of 0.2 ml of 10% (w/v) TCA. Samples were kept on ice for 2 min followed by centrifugation for 5 min at 12000 g. A 0.2 ml sample of the supernatant was added to 1 ml of Ecoscint and the $^{32}P_i$ released determined by scintillation counting. In some experiments the dephosphorylation of PEP carboxlase was analysed by SDS/polyacrylamide gel electrophoresis and autoradiography.

(ii) Dephosphorylation of PEP carboxylase The dephosphorylation of PEP carboxlase purified from dark leaf tissue by either mammalian or plant protein phosphatases was determined by measuring the change in apparent K_i for malate of the enzyme. Incubations were carried out at 30°C and contained 3 mU/ml of type 2A protein phosphatase activity (unless otherwise stated), 1 U/ml purified PEP carboxlase in 50 mM Tris/HCl, pH 7.0, containing 0.1 mM EGTA, 0.1% (v/v) 2-mercoptoethanol and 0.03% Brij-35. Aliquots (10 µl) were removed at intervals and the apparent K_i for malate determined (Section 2.5.1). Control assays were carried out containing no protein phosphatase.

(iii) Determination of type 1 and type 2A protein phosphatase activities. This analysis was based on studies by Mackintosh and Cohen (1989) . Type 1 and type 2A protein phosphatase activities were distinguished by pre-incubating 10 µl of sample with either (i) 1 µM inhibitor-2, (ii) 1nM okadaic acid, (iii) 1 µM inhibitor-2 and 1 nM okadaic acid, or (iv) buffer (50 mM Tris/HCl, pH 7.0, containing 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 0.03% Brij-35), in a volume of 20 µl, for 10 min at 30°C. Phosphorylase *a* (10 µM) was then added to give a final volume of 30 µl and incubated for a further 10 min. Protein phosphatase activity was determined by TCA precipitation method as described in part (i) of this section. Type 1 protein phosphatase activity was measured as both the activity sensitive to inhibitor-2 and the activity insensitive to 1 nM okadaic acid.

These two values were averaged. Type 2A protein phosphatase activity was taken as the average of the activity sensitive to 1 nM okadaic acid and the activity insensitive to inhibitor-2.

2.6. Polyacrylamide gel electrophoresis techniques

2.6.1. SDS/polyacrylamide gel electrophoresis (discontinuous system)

Gel electrophoresis was carried out by the method of Laemmli (1970). Gels contained an 8% or 15% polyacrylamide separating gel and a 3% stacking gel. Samples were denatured by addition of an equal volume of 50mM Tris/HCl, pH 6.8, containing 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue and 1% 2-mercaptoethanol (sample buffer), and boiling for 2 min. A 45mA current was applied until the tracking dye had reached the end of the separating gel.

2.6.2. Staining SDS/polyacrylamide gels

Gels were routinely stained for protein in 0.1% Coomassie Brilliant Blue G250, 50% (v/v) methanol, 10% (v/v) acetic acid for 30 min at 45°C and destained in several changes of 10% (v/v) methanol, 10% (v/v) acetic acid at 45°C.

A more sensitive protein staining technique was obtained by using the method of Wray *et al.* (1981). Gels were soaked for 1 or 2 days in 50% methanol. The staining solution was prepared by adding solution A (0.8 g $AgNO_3$ in 4 ml H_2O) to solution B (1.4 ml 14.8 M NH_4OH and 21 ml 0.36% (w/v) NaOH) dropwise while stirring vigorously. This mixture was made up to 100 ml with distilled water. The gel was then shaken gently for 10 min in the staining solution and rinsed for 1 h with 6 changes of distilled water. To develop the stain the gel was immersed in a solution comprising 2.5 ml 1% (w/v) citric acid and 0.25 ml of 38% (v/v) formaldehyde in 500 ml distilled water. Once the bands had appeared the staining was stopped by removing the gel to distilled water.

2.6.3. Drying and autoradiography of gels

Slab gels were dried onto Whatman 3MM chromatography paper using a Biorad Laboratories Gel Drier model 1125 connected to an Aquavac Junior multi-purpose vacuum unit (Uniscience Ltd., London).

Dried gels were autoradiographed using Fuji RX X-ray film with an intensifying screen at -70°C for a period between 1 to 5 days. The X-ray film was developed using a Kodac X-OMAT Processor Model ME-3.

2.6.4. Gel slicing and counting

In order to quantify the phosphorylation of PEP carboxylase, the protein band was excised from the polyacrylamide gel. The gel segments were placed in scintillation vials and incubated with 0.3ml hydrogen peroxide (30%(w/v)) for 24h at 37°C. Ecoscint (2ml) was then added to the samples and they were counted for 10 min in a Beckman LS8100 liquid scintillation counter.

2.7. Treatment of *B. fedtschenkoi* leaves with protein synthesis inhibitors

Cycloheximide was solubilized in 1ml of ethanol per 100ml of distilled water to a concentration of 5.0mM. Serial dilutions of this solution were made in the range 5 $x10^{-4}$ M to 5 $x10^{-7}$M. A 1mM stock solution of puromycin was prepared in distilled water and serial dilutions between 1 $x10^{-3}$M and 1 $x10^{-5}$M were made. Leaves were detached from *B. fedtschenkoi* plants and placed with their petioles in solutions of cycloheximide or puromycin. Control leaves were allowed to take up either distilled water or a 0.1% ethanol solution. For analysis of the effects of the protein synthesis inhibitors on the CO_2 rhythm, the leaves were detached at the end of the illumination period (Section 2.2) and immediately transferred to controlled enviromental conditions as described in Section 2.8. Those leaves in which the PEP carboxylase kinase activity was to be determined were detached at the onset of the illumination period and kept under the normal growth conditions (Section 2.2). The leaves were then extracted between 0900h-1100h (middle of the dark period) the following day (section 2.5.2 (i)), and the apparent K_i for malate and the protein kinase activity determined as described in Sections 2.5.1 and 2.5.2 (i).

2.8. Measurement of the CO_2 output from detached leaves of *B. fedtschenkoi.*

Leaves were detached from plants, weighed and placed in air-tight chambers, with their petioles resting in the solution to be analysed (Section 2.7). A single leaf was put into each chamber. The leaf

chambers were surrounded by a water jacket to maintain the temperature at 15°C (± 0.05°C). A stream of CO_2-free air was passed over the leaves at a rate of 1.55l/h and the CO_2 content of the emergent gas was determined using an infra-red gas analyser (ADC 225 mark 3, Analytical Development Company Ltd., Hoddeson, Herts, U.K.). The system was controlled by a BBC micro-computer (model B, Commscot, Glasgow, U.K.) and the data was printed out as μg CO_2/h/g fresh weight of tissue on a Radioshack TRS-80 colorgraphic printer (Tandy corp., Glasgow, U.K.). The leaves were maintained in continuous darkness for several days. A 6-8h illumination treatment was given after 3-4 days, provided by white fluorescent tubes at an average photon fluence rate (400-700nm) of 15μmol/m^2/s, in order to assess leaf viability.

2.9. Miscellaneous techniques

2.9.1. *In vivo* labelling with [^{32}P] orthophosphate

Leaves were labelled by the method of Nimmo *et al.* (1987). Detached leaves were allowed to take up 1.0mCi of carrier-free $^{32}P_i$ through the transpiration stream. Treatment was started at the onset of the illumination period, 1600h in the reverse-phase growth room (Section 2.2). They were then extracted during the middle of the following 'night' period between 0900h and 1100h, as described in Section 2.4.2. The PEP carboxylase was immunoprecipitated (Section 2.9.2) or purified by the method described in Section 2.4.2. For the purification, four leaves were each treated with 1.0mCi of $^{32}P_i$. The leaves were extracted and pooled with extract from unlabelled leaves (22g approx.).

2.9.2. Immunoprecipitation of PEP carboxylase

PEP carboxylase antiserum was prepared as described by Nimmo *et al.* (1986). Plant extract containing 0.06 units of PEP carboxylase was mixed with 30μl of antiserum and left on ice for 30 min. Phosphate buffered saline (0.85g NaCl, 0.128g Na_2HPO_4, 0.156g NaH_2PO_4 in 100ml of water) was added to 1ml. The samples were then centrifuged at 11600g for 2min, the supernatent (which contained no PEP carboxylase activity) was discarded and the pellet washed with 1ml of phosphate buffered saline. The

pellet was resuspended in 30µl of SDS sample buffer (Section 2.6.1), boiled for 2 min and analysed by SDS/polyacrylamide gel electrophoresis (Section 2.6). Immunoprecipitation of purified PEP carboxylase following *in vitro* phosphorylation was achieved by adding 15µl of antiserum/0.03 units of enzyme.

2.9.3. Peptide mapping

Buffers: (I) 125mM Tris/HCl, pH 6.8 containing 1mM DTT and 0.1% SDS

(II) 125mM Tris/HCl, pH 6.8 containing 1mM DTT, 0.1%SDS, 0.01% Bromophenol Blue and 20% glycerol

(III) 125mM Tris/HCl, pH 6.8 containing 1mM DTT, 0.1% SDS, 0.01% Bromophenol Blue and 10% glycerol

PEP carboxylase was phosphorylated *in vitro* (Section 2.5.2(ii)) or *in vivo* (Section 2.9.1) and immunoprecipitated as described in Section 2.9.2. The enzyme (0.03 units) was then subjected to SDS/polyacrylamide gel electrophoresis to separate the PEP carboxylase. The PEP carboxylase band was excised from the gel following staining with Coomassie Brilliant Blue. In some experiments the 112kDa and the 123kDa subunit bands were excised separately. The gel slices were washed for 30 min in buffer (I) at room temperature. Limited proteolysis and peptide mapping were carried out by the method of Cleveland *et al.* (1977). The gel chips were inserted into alternate tracks on a 3% SDS/polyacrylamide stacking gel and 10µl of buffer (II) was layered on top. This was then overlaid with 10µl of *Staphylococcous aureus* V8 protease (2.5µg/ml) in buffer (III). A current (45mA) was applied until the tracking dye had reached the end of the stacking gel. The current was then turned off for 30min to allow limited proteolysis of PEP carboxylase to occur. The current was switched back on and the peptides were separated on a 15% polyacrylamide gel. The peptides were visualised by staining with silver nitrate , dried and autoradiographed as described in Section 2.6.

2.9.4. Trichloracetic acid (TCA) precipitation

The incorporation of $^{32}P_i$ into purified PEP carboxylase was determined by TCA precipitation,

essentially by the method of Walsh *et al.* (1971). Aliquots of 25µl were removed from the protein kinase incubation assay mixture (Section 2.5.2(i)) and precipitated by the addition of 10µl of 100mg/ml BSA and 1ml of ice-cold 10% TCA. Samples were kept on ice for 10min and then centrifuged at 11600g. The pellets were dissolved in 0.5ml of 0.1M NaOH and precipitated by 0.5ml of 10% TCA as before. Following centrifugation the pellets were solubolized in 100µl of formic acid and 2ml of Ecoscint was added. Samples were counted for 10min in a Beckmann liquid scintillation counter.

2.9.5. ATP determination

In order to determine the stoichiometry of phosphorylation of PEP carboxylase, the concentration of the ATP used in the incubation mixture was determined biochemically by the method of Trautschold *et al.* (1983). The ATP concentration was determined by measuring the reduction of $NADP^+$ at 340nm in 1ml reaction mixtures containing 50mM Tris/HCl, pH 7.5, 20mM $NADP^+$, 0.1mM $MgCl_2$, 0.5M glucose. The assay was initiated by the addition of 1µl of hexokinase (10mg/ml) and 1µl of glucose 6-phosphate dehydrogenase (5mg/ml). The activity of [γ-^{32}P] ATP was determined by liquid scintillation counting.

Chapter 3

PURIFICATION OF PEP CARBOXYLASE FROM *B. FEDTSCHENKOI*

3.1. Introduction

The purification of PEP carboxylase was essential in order to achieve the objectives of this project. Homogenous enzyme was required as a substrate for studies on the protein kinase/phosphatase. The method of purification used in this study was based on a method developed by Nimmo *et al.* (1986), who purified phosphorylated and dephosphorylated forms of PEP carboxylase from *B. fedtschenkoi.* Some minor adjustments were made to the procedure, largely in order to maintain the enzyme's sensitivity to malate (see Section 1.2.3).

3.2. Results

The phosphorylated and dephosphorylated forms of PEP carboxylase were purified to homogeniety from dark or illuminated leaves as described in Section 2.4.3. The purification of both forms of the enzyme is summarized in Tables 3.1 (a), (b). The apparent K_i for malate was monitored at each stage in the purification (see Section 2.5.1). The dephosphorylated 'day' form of the enzyume is almost ten-fold more sensitive to inhibition by malate (apparent K_i = 0.4 mM) than the phosphorylated 'night' form (apparent K_i = 3.0 mM). An initial desalting step was included in the modified purification procedure because this served to maintain the malate sensitivity of the enzyme, particularly during the purification of the dephosphorylated form of PEP carboxylase. In addition, the desalted extract bound more readily to the hydroxylapatite matrix, than undesalted extract. Use of Superose 6 chromatography immediately after ion exchange chromatography on DEAE-cellulose, instead of dialysing overnight as was done in the original procedure, proved more satisfactory in maintaining malate sensitivity. A rapid purification of PEP carboxylase by this method yielded homogenous enzyme (see Figure 3.1), with no apparent loss in malate sensitivity (Table 3.1 a, b). The activity and apparent K_i for malate of the enzyme remained stable for several weeks if stored at -20°C.

Figure 3.1a. **Purification of PEP carboxylase from 'day' leaves of *B. fedtschenkoi***

Step	Total Volume (ml)	Total protein (mg)	Enzyme activity (Units)	Specific activity (Units/mg)	Purification (fold)	Yield (%)	Apparent K_i for malate (mM)
Homogenate	32	60	52	0.9	1.0	100	0.4
10 000g supernatent	30	40	55	1.4	1.5	106	0.4
Sephadex G25 pool	35	37	47	1.3	1.4	90	0.5
Hydroxyl-apatite pool	17	5.4	23	4.2	4.6	44	0.4
Sephadex G25 pool	20	2.1	20	9.5	10.5	38	0.4
DEAE-cellulose pool	7.0	1.6	17	10.6	11.7	32	0.4
Superose 6 pool	1.5	0.4	5	12.5	13.8	10	0.4

Figure 3.1b. **Purification of PEP carboxylase from 'night' leaves of *B. fedtschenkoi***

Step	Total Volume (ml)	Total protein (mg)	Enzyme activity (Units)	Specific activity (Units/mg)	Purification (fold)	Yield (%)	Apparent K_i for malate (mM)
Homogenate	45	72	108	1.5	1.5	100	3.0
10 000g supernatent	30	48	72	1.5	1.0	66	3.0
Sephadex G25 pool	30	33	68	2.1	1.4	63	3.0
Hydroxyl-apatite pool	17	4.2	27	6.4	4.3	25	3.0
Sephadex G25 pool	20	3.2	26	8.1	5.4	24	2.8
DEAE-cellulose pool	4.0	1.7	19	11.2	7.5	17	2.8
Superose 6 pool	1.8	0.6	10	16.6	11.1	9	2.8

FIGURE 3.1. Purification of PEP carboxylase from darkened leaves of *B. fedtschenkoi.* Denatured samples were run on an 8% SDS/polyacrylamide gel and stained with Coomassie Brilliant Blue. Approx. 0.03 units of PEP carboxylase was loaded in each track. Track A, crude extract; track B, desalted extract; track C, hydroxylapatite pool; track D, Sephadex G-25M pool; track E, DEAE-cellulose pool; track F, Superose 6 pool. The mobilities of the following M_r markers are indicated: 1, myosin (205kDa); 2, β-galactosidase (125kDa); 3, phosphorylase b (100kDa); 4, bovine serum albumin (66kDa); 5, ovalbumin (45kDa); 6, carbonic anhydrase (29kDa).

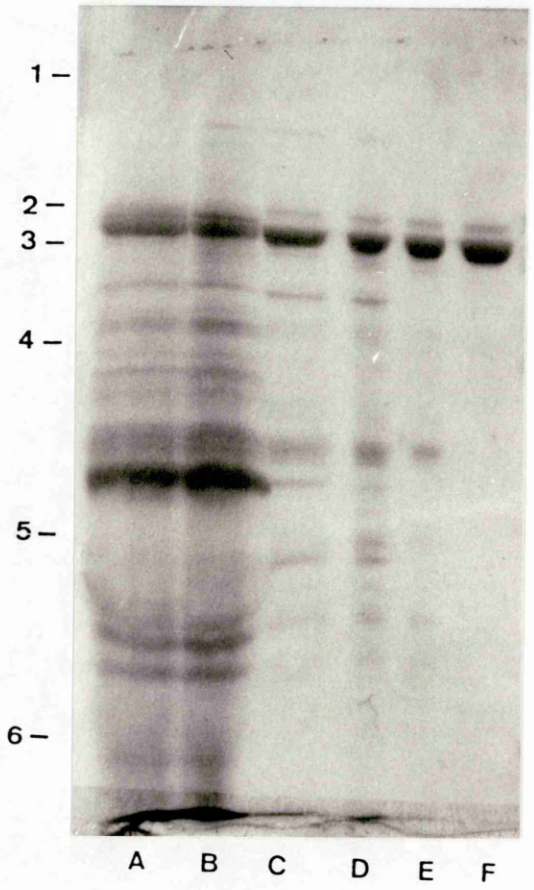
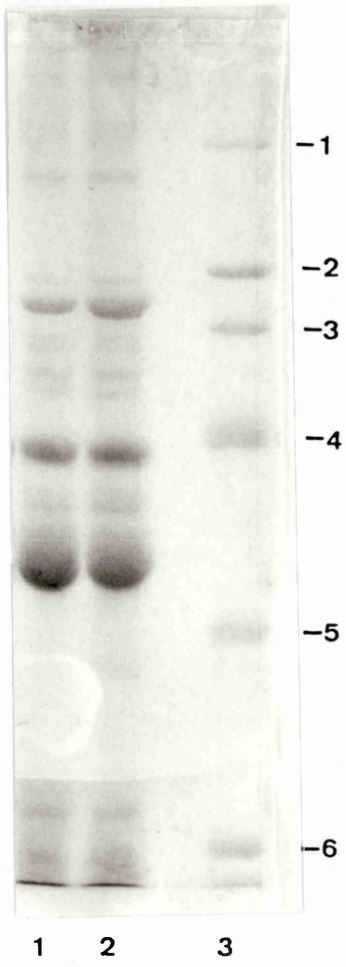

1
2
3
4
5
6
A
B
C
D
E
F

FIGURE 3.2. **Immumoprecipitation of PEP carboxylase from darkened and illuminated leaves of *B. fedtschenkoi*.** Extracts were prepared (Section 2.4.2) and desalted into 100mM Tris/HCl, pH 7.5 containing 10mM malate, 2mM EDTA, 1mM DTT and 1mM benzamidine. PEP carboxylase (0.03units) was immunoprecipitated as described in section 2.9.2. Samples were separated on an 8% SDS/polyacrylamide gel and stained with Coomassie Brilliant Blue. Track 1, 'day' extract; track 2, 'night' extract; track 3, M_r as follows: 1, myosin (205kDa); 2, β-galactosidase (125kDa); 3, phosphorylase b (100kDa); 4, bovine serum albumin (66kDa); 5, ovalbumin (45kDa); 6, carbonic anhydrase (29kDa). The arrow indicates the position of PEP carboxylase.


-1
-2
-3
-4
-5
-6
1
2
3

The apparent K_i for malate of PEP carboxylase purified from illuminated leaves varied between 0.3 to 0.8 mM in different experiments, whereas enzyme purified from darkened tissue had apparent K_i values in the range 2.5 to 3.5 mM. These values are similar to those found in desalted leaf extracts prepared from illuminated or darkened plants, which were assayed immediately after extraction (Nimmo *et al.*, 1984; Chapter 6). Studies by Nimmo *et al.* (1986) indicated that a loss in malate sensitivity of PEP carboxlase was at least partly due to a proteolysis (see Introduction page 21). Hence, the apparent K_i values of the purified enzyme forms suggest that they are non-proteolysed proteins.

Purified PEP carboxylase contains subunits of M_r 123kDa and 112kDa in the ratio 1:10 (approx) as illustrated in Figure 3.1. These two subunits co-purify and show very similar 'Cleveland mapping' patterns (Nimmo *et al.*, 1986, also Chapter 4). Both subunits were found in rapidly prepared and denatured extracts (Nimmo *et al.*, 1986). In this work, similar PEP carboxylase subunits were observed in immunoprecipitated extracts (Figure 3.2), which indicates that the purified form of the enzyme is the same as that found in intact tissue. Two subunit molecular weights of PEP carboxylase have been found in other CAM species (discussed in Section 1.2.2).

3.3. Discussion

The purification of PEP carboxylase by this modified method yields homogenous, unproteolysed enzyme. The minor modifications made in the procedure greatly enhance the stability of the enzyme with respect to its allosteric properties, during the purification and on storage. Both the phosphorylated and dephosphorylated forms of PEP carboxylase were purified to a specific activity of approx. 15U/mg protein. Most importantly, the purified enzyme retained the kinetic properties found in freshly prepared crude leaf extracts. Hence *in vitro* studies using PEP carboxylase as a substrate should reflect the *in vivo* regulation of the enzyme.

Chapter 4

THE PHOSPHORYLATION OF PEP CARBOXYLASE

4.1. Introduction

PEP carboxylase is one of an increasing number of higher plant enzymes that have been shown to be regulated by reversible phosphorylation (see Section 1.3.2). The existence of phosphorylated and dephosphorylated forms of the enzyme which exhibit different kinetic properties has been demonstrated in several CAM species (Nimmo *et al.*, 1986; Brulfert *et al.*, 1988). These preliminary observations incite further characterization of the protein kinase and protein phosphatase that are responsible for carrying out phosphorylation and dephosphorylation of PEP carboxylase.

Four main criteria have been established by Nimmo and Cohen (1977) regarding the physiological relevance of any protein phosphorylation *in vitro*. These were put forward to demonstrate that a cyclic AMP-induced effect was mediated by phosphorylation of a protein. Such criteria can be modified to apply to any phosphorylation system as follows;

1. The rate of phosphorylation of the native protein should be adequate to account for the speed at which the alteration in the function of the protein occurs *in vivo*.
2. The altered function brought about by the phosphorylation should be reversible *in vitro*, catalysed by a protein kinase and protein phosphatase.
3. The reversible change in function of the protein should occur *in vivo* in response to a stimulus.
4. The phosphorylation of the protein *in vivo* should occur at the same site that is phosphorylated by the purified protein kinase *in vitro*.

Taking these criteria into consideration a reconstituted assay system was employed to study the phosphorylation of PEP carboxylase. The main objectives in this part of the study were to investigate the changes in kinetic properties of the enzyme in relation to its phosphorylation state and to determine the stoichiometry of phosphorylation. A comparison of the *in vitro* and *in vivo* phosphorylation sites was also undertaken. The reversibility of the system is discussed in Chapter 5.

4.2. Results

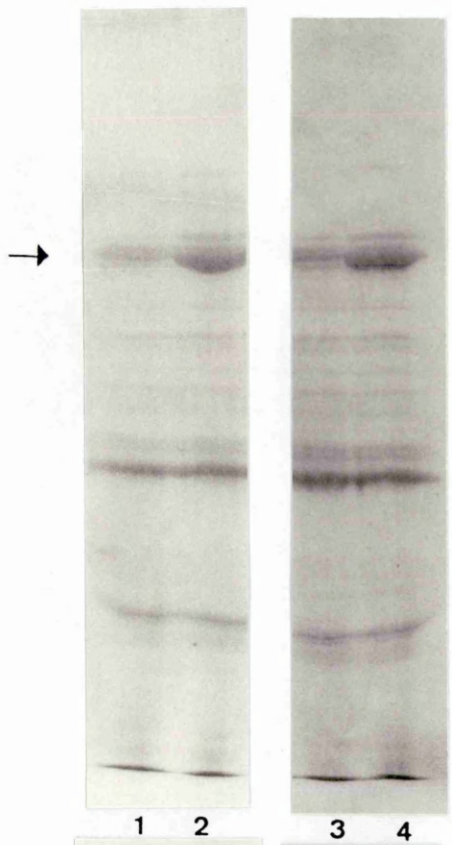
4.2.1. Partial purification of a protein kinase which can phosphorylate PEP carboxylase.

PEP carboxylase purified from illuminated leaves of *B. fedtschenkoi* was used as the substrate for the study of protein kinase activity. This malate-sensitive form (apparent K_i =0.3 mM) was shown by Nimmo *et al.* (1986) to be largely unphosphorylated *in vivo*. PEP carboxylase in leaf tissue taken from plants during the dark period was shown by these authors to be phosphorylated *in vivo* and to be ten-fold less sensitive to malate inhibition (apparent $K_{i=}$ 3.0 mM); so these leaves were used as a source of PEP carboxylase kinase in the present studies.

Experiments showed that desalted extracts prepared from leaves in the middle of the 16 h dark period contained a soluble protein kinase capable of phosphorylating PEP carboxylase. No such activity was found in leaf extracts prepared during the illumination period (Figure 4.1). The presence of protein kinase activity in leaves extracted at other times will be discussed in Chapter 6. For the purpose of purifying the protein kinase, extracts were routinely made 8 to 10 hours after the end of the illumination period (see Section 2.2). The protein kinase was purified by the method described in Section 2.4.4, which was based on a procedure developed by Jiao and Chollet (1989) for the purification of a PEP carboxylase kinase in maize. The first step involved carrying out a 0 - 50% ammonium sulphate fractionation. The resuspended protein contained kinase activity and PEP carboxylase activity; the main objective was, therefore, to separate these two activities. A blue dextran-agarose column proved excellent in achieving this aim; the protein kinase activity bound to the matrix, while the PEP carboxylase activity eluted in the wash. The protein kinase was subsequently eluted in 0.5 M NaCl in buffer C (Section 2.4.1) as shown in Figure 4.2. Chromatography on Mono Q was used to purify the protein kinase further and also to concentrate the protein pool. The protein was eluted over a 10 ml 0 - 0.4M NaCl gradient and the fractions were dialysed individually overnight into buffer C. Each fraction was assayed for PEP carboxylase kinase and glycogen synthase kinase activity as described in Section 2.5.2 (ii) (Figure 4.3). The active fractions were pooled and then concentrated four-fold. Activity remained stable for several weeks at -20°C.

FIGURE 4.1. PEP carboxylase kinase activity in extracts from darkened and illuminated leaves of *B. fedtschenkoi.* Leaves were extracted and desalted as described in Section 2.5.2(i). The protein kinase assays contained approx. 10μg of desalted extract incubated either with or without purified PEP carboxylase (0.03units), in the presence of 4μCi/nmol ATP as described in the methods Section 2.5.2(i). The samples were loaded onto 8% SDS/polyacrylamide gels (a) and autoradiographed (b). Tracks 1 and 2, 'night' extracts incubated in the absence and presence of purified exogenous PEP carboxylase. Tracks 3 and 4, 'day' extracts incubated in the absence and presence of purified exogenous PEP carboxylase. The arrow indicates the position of the PEP carboxylase on the gel.

(a)



(b)

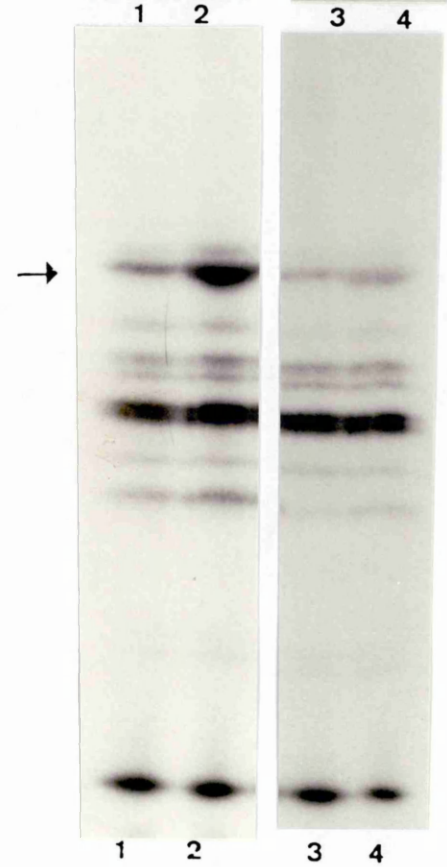


FIGURE 4.2 Blue dextran-agarose chromatography of PEP carboxylase kinase. Enzyme from the 0-50% ammoniun sulphate fraction which had been desalted was loaded onto a 4.5 cm x 2.7cm (25ml) blue dextran-agarose column equlibrated in buffer B (Section 2.4.1) . The protein kinase activity was eluted from the column by washing with buffer B containing 0.5M NaCl. The arrow indicates the point where the buffer was changed. The flow rate was 0.2ml/min and 5min fractions were collected. The peak A_{280} fractions from the NaCl elution were pooled.

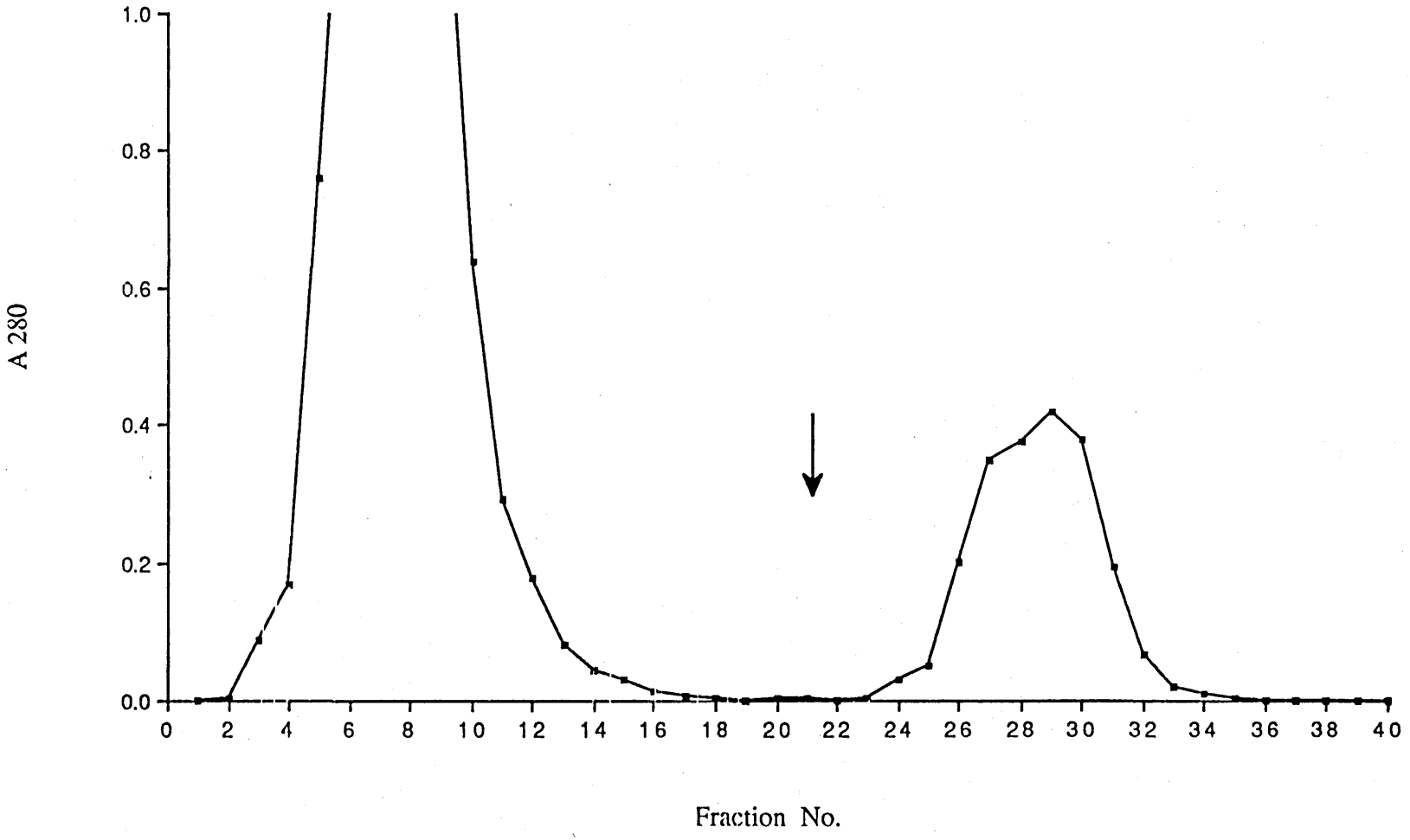
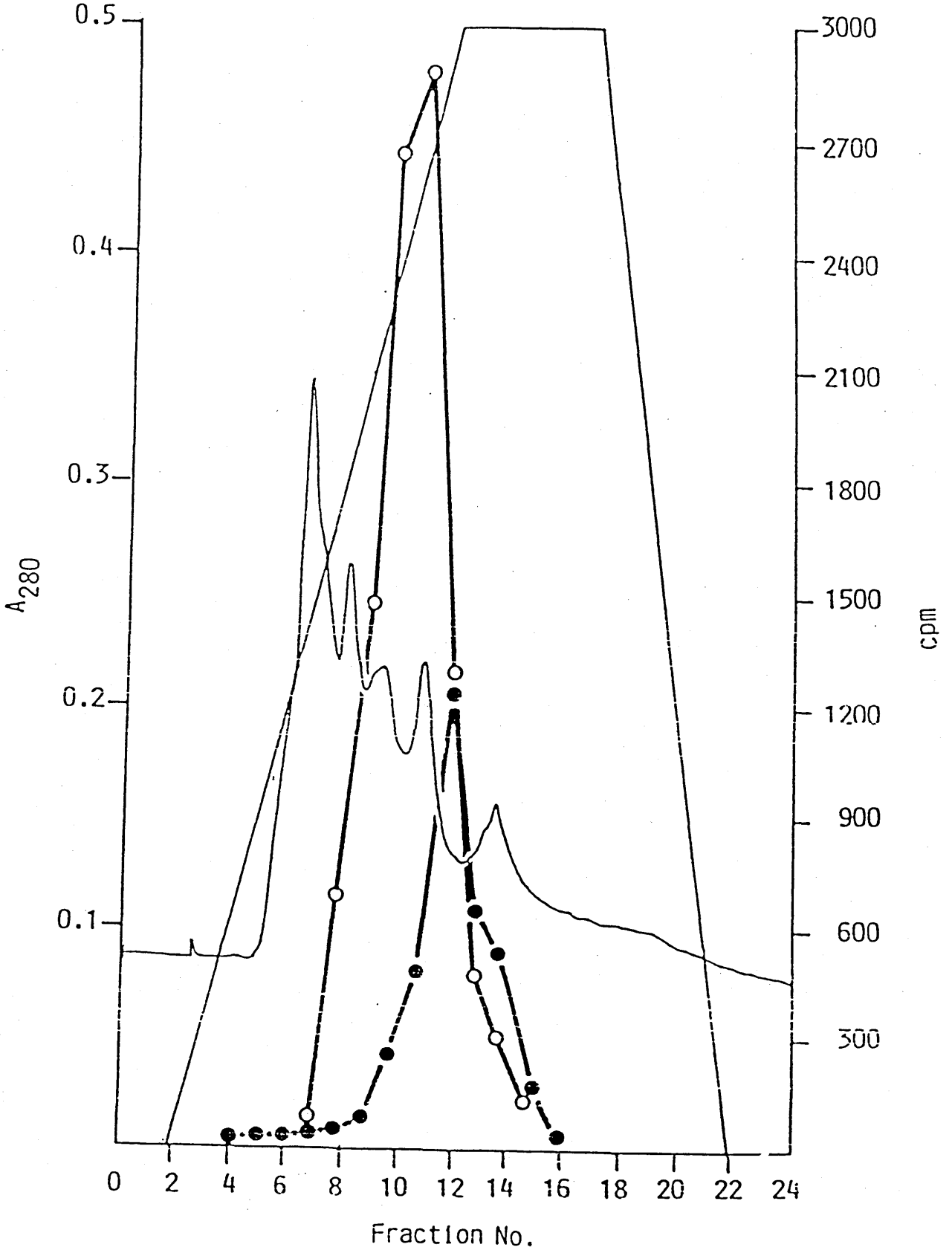

A 280
1.0
0.8
0.6
0.4
0.2
0.0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Fraction No.

FIGURE 4.3. Mono Q chromatography of PEP carboxylase kinase. The protein pool from the blue dextran-agarose column was desalted on a 55 cm x 2.2cm (200 ml) Sephadex G-25M column and loaded onto a Mono Q column equlibrated in buffer B (Section 2.4.1). The protein kinase activity was eluted from the column with a linear gradient of 0-0.4M NaCl over a 10min gradient as indiated by the diagonal line on the figure. The flow rate was 1ml/min and 1min fractions were collected. The PEP carboxylase kinase (●) and glycogen synthase kinase (○) activities were determined as described in Section 2.5.2(ii). Incubations were carried out for 10min and protein kinase activity analysed by the TCA precipitation method. The fractions showing PEP carboxylase kinase activity (10-15) were pooled.


0.5
0.4
0.3
0.2
0.1
A_{280}
3000
2700
2400
2100
1800
1500
1200
900
600
300
cpm
0 2 4 6 8 10 12 14 16 18 20 22 24
Fraction No.

The protein kinase activity was not quantified at each stage of the purification nor were yields determined because of the presence of endogenous PEP carboxylase in the initial stages of the purification and also because of the presence of proteases in the preparation. Proteolysis of the substrate PEP carboxylase was evident on prolonged incubation with the partially purified protein kinase. The addition of several protease inhibitors to the incubation assay (Section 2.5.2) proved beneficial only after the protein kinase was purified on Mono Q. The PEP carboxylase kinase activity obtained at each stage of the purification is shown in Figure 4.4. The final kinase preparation contains a number of proteins but no further purification was attempted. The partially purified protein kinase pool was used for all further studies.

4.2.2. Studies on the kinetic properties of PEP carboxylase on phosphorylation.

The degree of phosphorylation of PEP carboxylase by the *B. fedtschenkoi* protein kinase was investigated by carrying out time courses of phosphorylation using [γ-^{32}P] ATP as described in Section 2.5.2(ii). Figure 4.5 shows one such time course where the phosphorylation appears to be complete after 30 - 60 min. The stoichiometry of phosphorylation from several experiments was found to be in the range 0.7 to 0.9 mol P_i incorporated per mol of PEP carboxylase subunit. These values were calculated from scintillation counting of gel fragments or by TCA precipitation of the phosphorylated enzyme. The incorporation of P_i into the 112kDa and 123kDa subunits was determined (Figure 4.6). The values obtained from a single experiment show that the phosphorylation of the two subunits occurs at the same relative rate.

The change in the malate sensitivity of PEP carboxylase was studied in relation to the phosphorylation state of the enzyme. In the experiment shown in Figure 4.7, the apparent K_i for malate increased from 0.48 mM to 2.7 mM with a maximum stoichiometry of 0.82 mol P_i per mol subunit after 60 min incubation. The change in malate sensitivity appeared to be directly proportional to the degree of phosphorylation over a 100 min incubation period tested (Figure 4.8).

No apparent increase in PEP carboxylase activity (V_{max}) was found on phosphorylation of the enzyme. Studies by Jiao and Chollet (1989) on PEP carboxylase in maize showed an ATP and protein

FIGURE 4.4. Purification of PEP carboxylase kinase activity from darkened leaves of *B. fedtschenkoi.* Denatured samples were run on an 8% SDS/polyacrylamide gel stained with Coomassie Brilliant Blue (a) and autoradiographed (b). Samples were incubated in the presence or absence of PEP carboxylase (0.03units) purified from 'day' leaves using 0.4μCi/nmol ATP (Section 2.5.2(ii)). Tracks 1 and 2, desalted 0-50% ammonium sulphate fraction (5μl) incubated in the absence and presence of exogenous PEP carboxylase; tracks 3 and 4, blue dextran-agarose pool (17μl) incubated in the absence and presence of exogenous PEP carboxylase; tracks 5 and 6, Mono Q pool (17μl) incubated in the absence and presence of exogenous PEP carboxylase.
The arrow indicates the position of PEP carboxylase

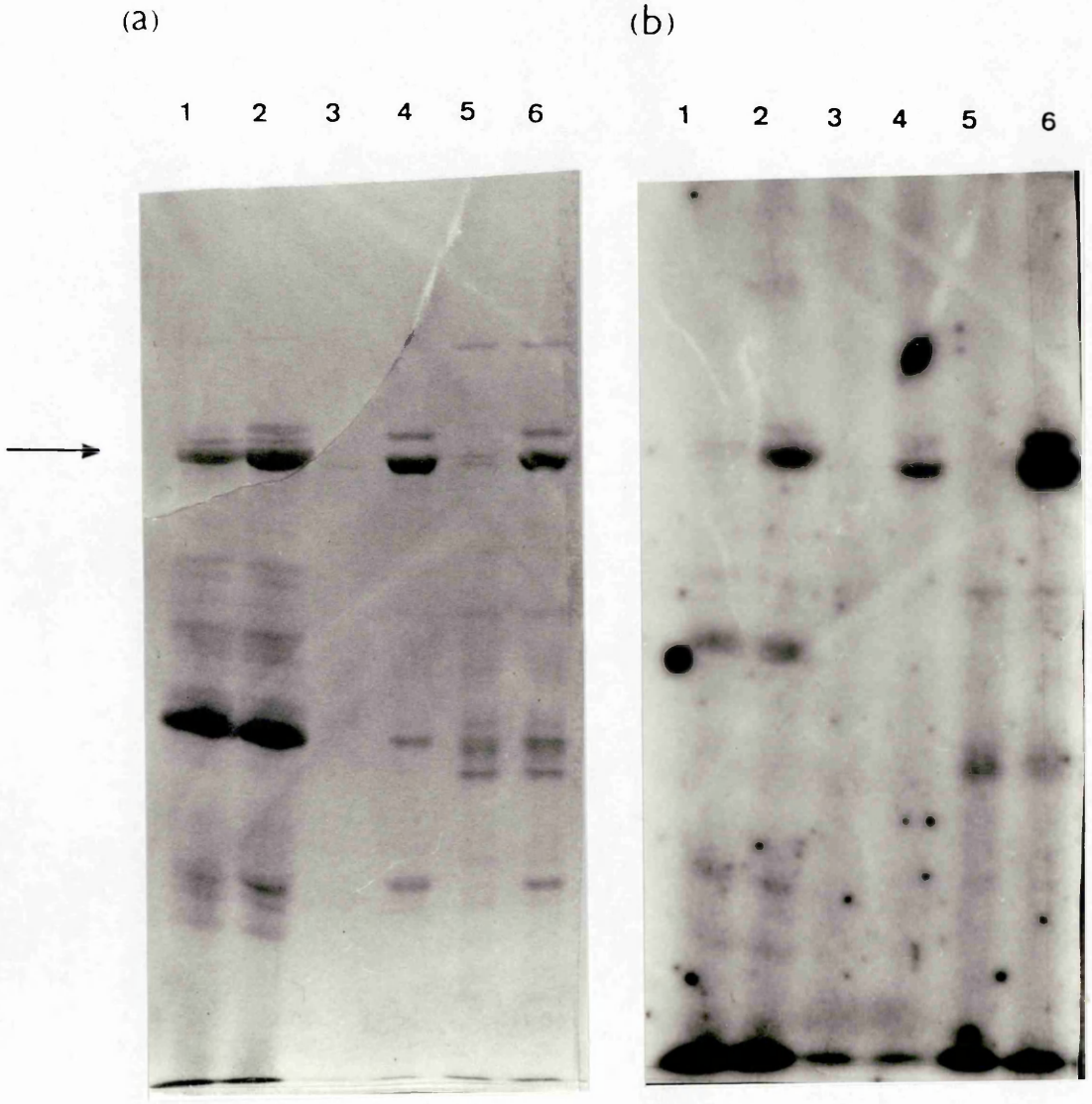

(a)
(b)
1 2 3 4 5 6
1 2 3 4 5 6

FIGURE 4.5. **Time course of phosphorylation of purified PEP carboxylase on incubation with partially purified protein kinase.** Incubations were carried out as described in Section 2.5.2(ii). Samples were denatured and run on an 8% SDS/polyacrylamide gel and stained with Coomassie Brilliant Blue (not shown) and autoradiographed. The phosphorylation was monitored over a 180min period as follows:

Track	Time
1	0min
2	10min
3	20min
4	30min
5	60min
6	90min
7	180min

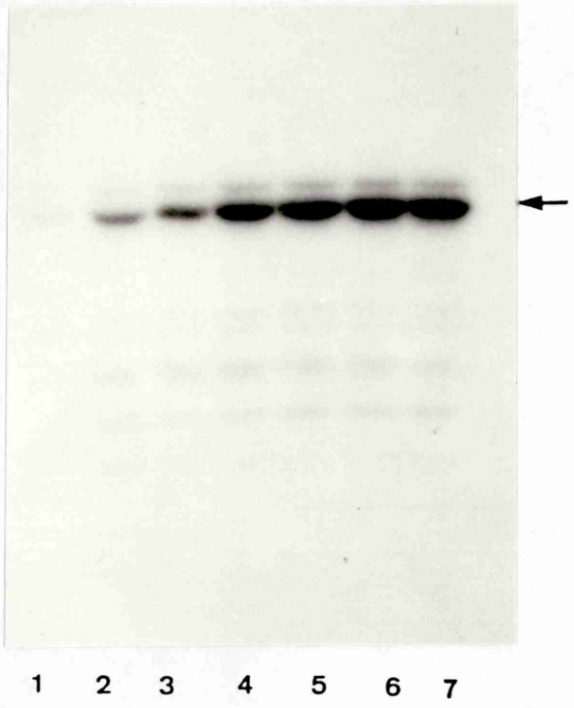

1 2 3 4 5 6 7

FIGURE 4.6. **Time course of phosphorylation of the 112kDa and 123 kDa subunits of purified PEP carboxylase on incubation with partially purified protein kinase.** Incubations were carried out as described in Section 2.5.2 (ii). Phosphorylation was determined from an autoradiograph of an SDS/polyacrylamide gel using a "flying spot " laser densitometer to calculate the intensity of phosphorylation in both the 112kDa and 123kDa subunits of PEP carboxylase. The relative phosphorylation is given as a percentage of the phosphorylation after 40 min incubation.

112kDa subunit (—●—)

123kDa subunit (—○—)

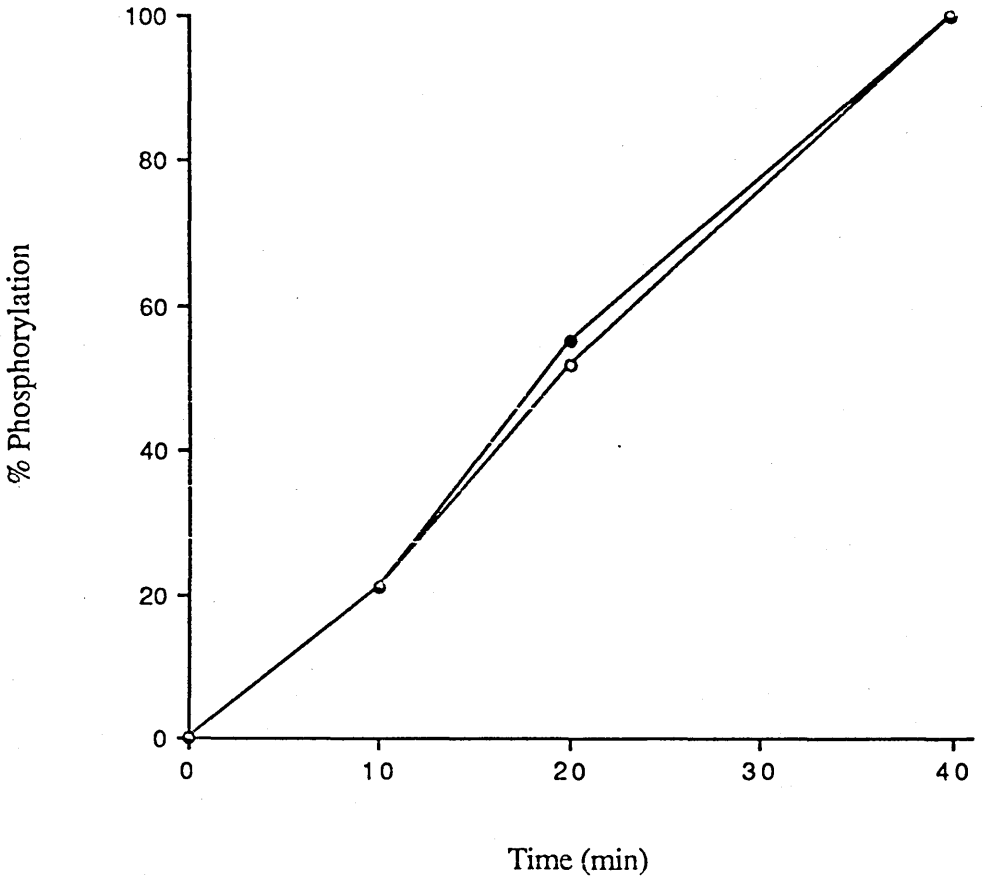
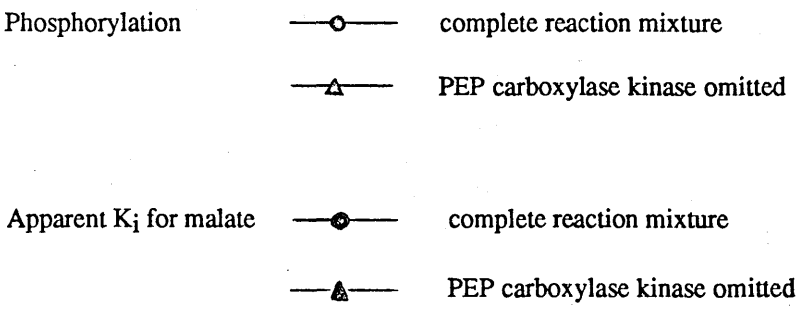

% Phosphorylation
100
80
60
40
20
0
0
10
20
30
40
Time (min)

FIGURE 4.7. **Time course of phosphorylation and change in the apparent K_i for malate of purified PEP carboxylase on incubation with partially purified kinase from *B. fedtschenkoi*.** Incubations were carried out as described in Section 2.5.2 (ii). The apparent K_i for malate and the stoichiometry of phosphorylation were determined in samples removed from the incubation mixture. The incorporation of phosphate into PEP carboxylase was measured by the TCA precipitation method (Section 2.9.4).



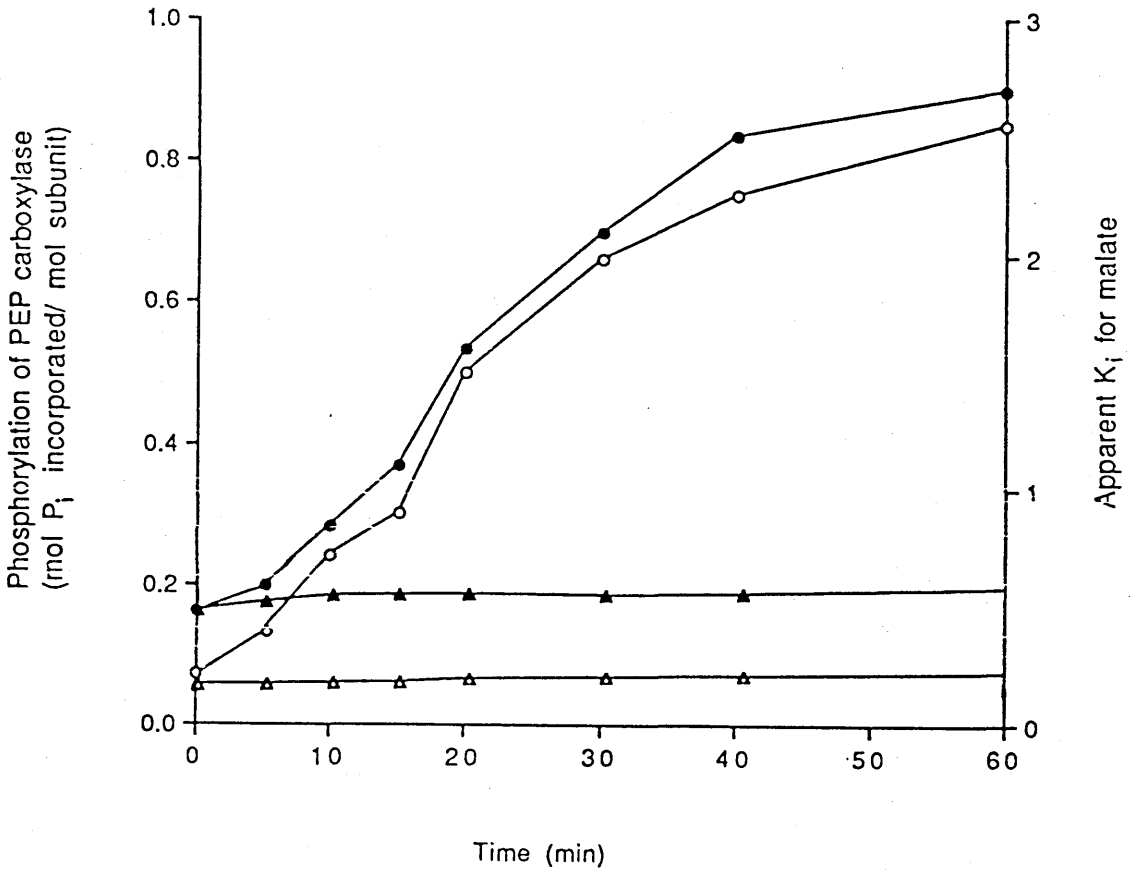
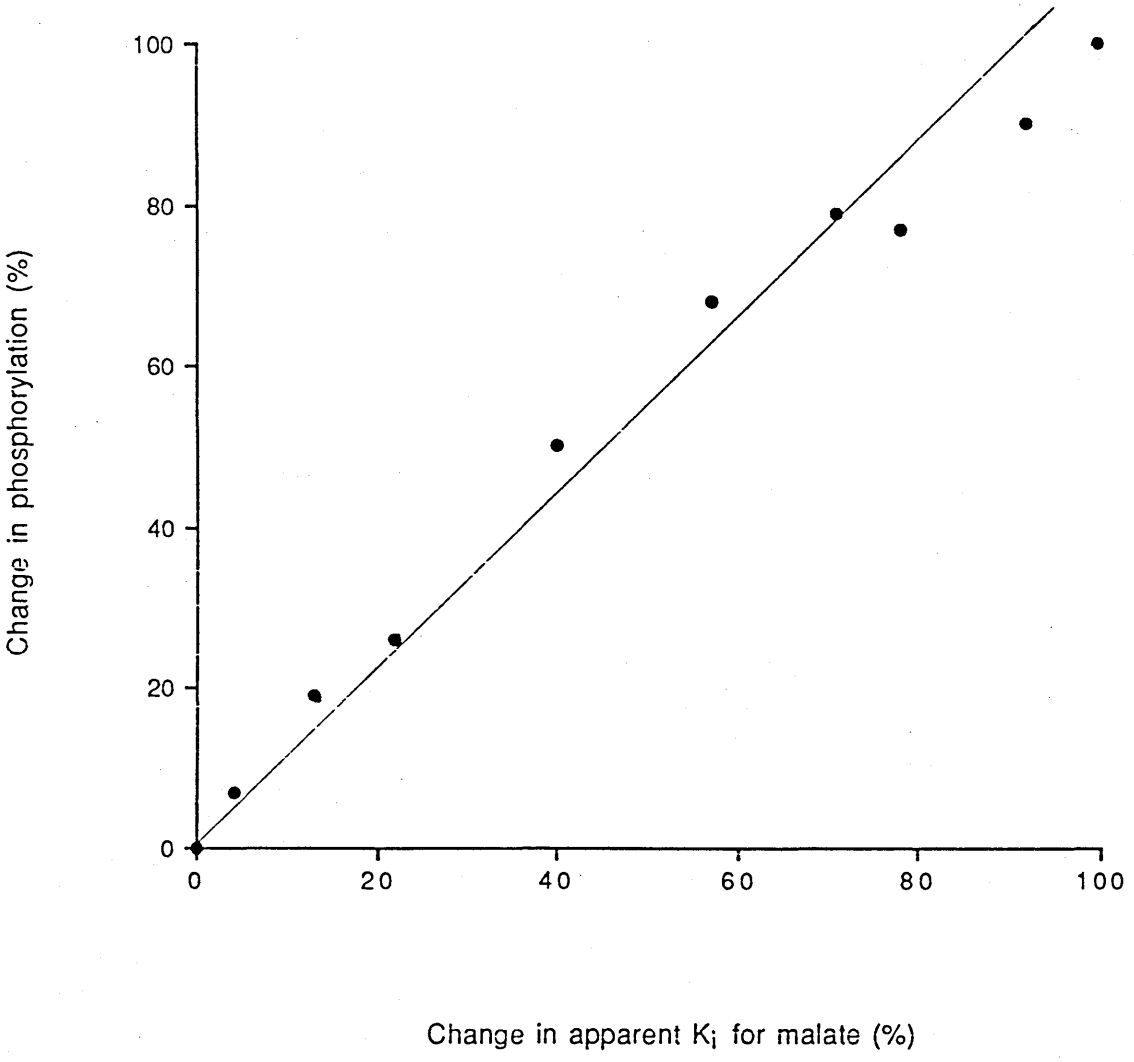

Phosphorylation of PEP carboxylase
(mol P_i incorporated/ mol subunit)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Time (min)
3
2
1
0
Apparent K_i for malate

FIGURE 4.8. **Relationship between the change in the malate sensitivity and the change in phosphorylation state of PEP carboxylase.** PEP carboxylase was incubated with the partially purified protein kinase as described in Section 2.5.2 (ii). The stoichiometry of phosphorylation and the apparent K_i for malate were determined at intervals over a 100 min time course and each point represents a seperate time of sampling. The change in phosphorylation state of PEP carboxylase was calculated as a percentage of the overall change observed. The apparent K_i for malate was calculated as a percentage of the overall change in K_i.


Change in phosphorylation (%)
100
80
60
40
20
0
0
20
40
60
80
100
Change in apparent K_i for malate (%)

kinase-dependent activation of the enzyme, which correlated with a change in its sensitivity to malate. However, the specific activity of PEP carboxylase in *B. fedtschenkoi* crude extracts does not change over the diurnal cycle (Nimmo *et al.*, 1984 and Section 6.2), also the specific activity of the enzyme purified from darkened or illuminated leaf tissue was the same (see Chapter 3, Nimmo *et al.*, 1986).

4.2.3. The effects of various metabolites on the phosphorylation of PEP carboxylase

The role of various possible effectors on the *in vitro* phosphorylation of PEP carboxylase was investigated (Table 4.1). Although Ca^{2+}-calmodulin plays an important role in the regulation of numerous protein kinases (Rosen and Krebs, 1980), Ca^{2+} and EGTA had no effect on the *B. fedtschenkoi* protein kinase activity. A range of free Ca^{2+} concentrations (1 - 500μM) using Ca^{2+}/EGTA buffers also showed no effects on the phosphorylation (not shown). Okadaic acid and sodium fluoride were investigated as inhibitors of putative endogenous protein phosphatases, which would otherwise compete with protein kinase activity. However, okadaic acid did not increase the phosphorylation whereas fluoride had an inhibitory effect. PEP showed slight inhibition, but both L-malate and glucose 6-phosphate showed a significant inhibition of the phosphorylation. This inhibition may be due to the interaction of the metabolites with the PEP carboxylase or with the kinase or both.

In an attempt to assess whether the malate had a direct effect on the protein kinase, studies using glycogen synthase as a substrate were carried out. The glycogen synthase substrate exhibited "autophosphorylation", presumably because of the presence of an endogenous kinase. Therefore, control incubations were done which contained no added protein kinase. The control assay and the protein kinase assay were done in the presence and absence of 10 mM L-malate (Table 4.2). The results from two separate experiments showed that the malate inhibited the "autophosphorylation" of the glycogen synthase. However, the malate actually increased the phosphorylation brought about by the exogenous protein kinase. Interpretation of these data is difficult. It is possible that malate has two separate effects. It may activate the protein kinase but interact with PEP carboxylase so that the overall effect is reduction of PEP carboxylase kinase activity. The effects of a range of concentrations of L-malate on the PEP

TABLE 4.1. The effect of various compounds on the phosphorylation of PEP carboxylase by the partially purified *B. fedtschenkoi* kinase. The degree of phosphorylation is calculated as a percentage of that obtained with the complete reaction mixture as described in Section 2.5.2 (ii). Means for four separate experiments are listed ± standard deviation.

Addition to/ omission from the assay mixture	Relative phosphorylation (%)	
None (control)	100	
No kinase	0	
L-malate (5mM)	30.2 ± 11.0	(4)
Glucose 6-phosphate (10mM)	15.2 ± 15.4	(4)
$CaCl_2$ (1mM)	96.5 ± 5.1	(4)
EGTA (5mM)	95.5 ± 7.1	(4)
Okadaic acid (1μM)	87.2 ± 13.9	(4)
NaF (5mM)	50.7 ± 18.9	(3)
PEP (5mM)	61.3 ± 13.6	(3)

Table 4.2. **Effect of L-malate on the phosphorylation of glycogen synthase by partially purified *B. fedtschenkoi* protein kinase.** Incubations were carried out in the absence or presence of 10mM L-malate for 15min as described in Section 2.5.2 (ii). The phosphorylation of glycogen synthase is given in counts per minute as determined by the TCA precipitation method (Section 2.9.4). The experiment was carried out twice using two different *B. fedtschenkoi* protein kinase preparations.

Additions to glycogen synthase incubation mixture	Counts per minute	
	Experiment 1	Experiment 2
No additions	25,362	20,323
10mM L-malate	5,927	5,932
Protein kinase	28,369	22,103
Protein kinase with 10mM L-malate	15,830	14,951

carboxylase kinase activity was also investigated. Figure 4.9 illustrates the effect of this metabolite in inhibiting the phosphorylation. It is apparent that only 0.5 mM L-malate was required to obtain 60% inhibition whereas in all other experiments carried out 5.0 mM L-malate gave 60% inhibition. The reason for this almost ten-fold shift in inhibition in this particular experiment is unknown.

The effect of D-malate on the phosphorylation of PEP carboxylase was also studied. D-malate (5.0 mM, which was shown to contain 2% L-malate) was found to inhibit the protein kinase activity, albeit to a lesser extent (30% inhibition). The inhibition by malate is therefore not entirely stereospecific.

4.2.4. Comparison of the *in vitro* and *in vivo* labelled phosphorylation sites of PEP carboxylase.

'Cleveland mapping' was used to compare the sites in PEP carboxylase that were phosphorylated *in vitro* and *in vivo*. The *in vivo* phosophorylated form of the enzyme was obtained by immunoprecipitation of extracts from darkened leaves, which had taken up a solution of $^{32}P_i$ as described in Section 2.9.1. The *in vitro* phosphorylated enzyme was prepared by incubating purified PEP carboxylase with partially purified protein kinase for 10 min as described in Section 2.5.2. All samples were separated on SDS/polyacrylamide gels. V8 protease was used to partially digest the phosphorylated enzyme (Section 2.9.3). The ^{32}P-labelled peptides were visualized by autoradiography (Figures 4.10, 4.11). The results show that the peptide(s) phosphorylated *in vitro* is the same as that found *in vivo* (Figure 4.11) In addition, the maps for the 112kDa and 123kDa subunits of PEP carboxylase appear to be identical and the two subunits seem to contain the same phosphorylation site(s) (Figure 4.10).

4.3. Discussion

The results described in this section clearly demonstrate the presence of a soluble protein kinase in *B. fedtschenkoi* leaves that can phosphorylate PEP carboxylase purified from illuminated tissue. Studies on other CAM species by Kluge *et al.* (1988) showed the presence of a PEP carboxylase kinase activity in extracts but no further purification of this enzyme has been reported. PEP carboxylase kinase activity

FIGURE 4.9. The effect of L-malate on the phosphorylation of the 112kDa and 123kDa subunits of PEP carboxylase. PEP carboxylase was incubated with the partially purified plant protein kinase as described in Section 2.5.2 (ii). A range of L-malate concentrations (0.0, 0.5, 1.0, 2.0, 5.0 mM) were added to the incubations . The reactions were terminated after 20 min and the samples run on an SDS/polyacrylamide gel. A "flying spot" laser densitometer was used to estimate the intensity of phosphorylation on an autoradiograph. The relative phosphorylation of the 112kDa and 123kDa subunits is shown as a percentage of the control incubation which contained no L-malate.

123kDa subunit (—●—)

112kDa subunit (—○—)

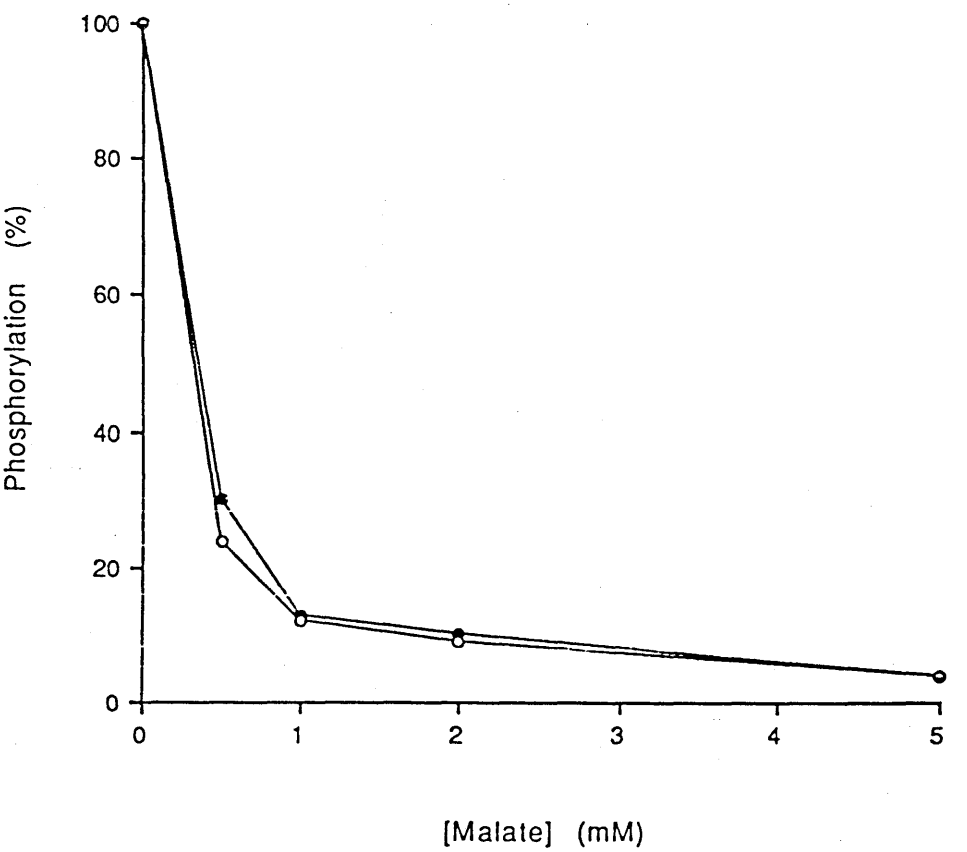
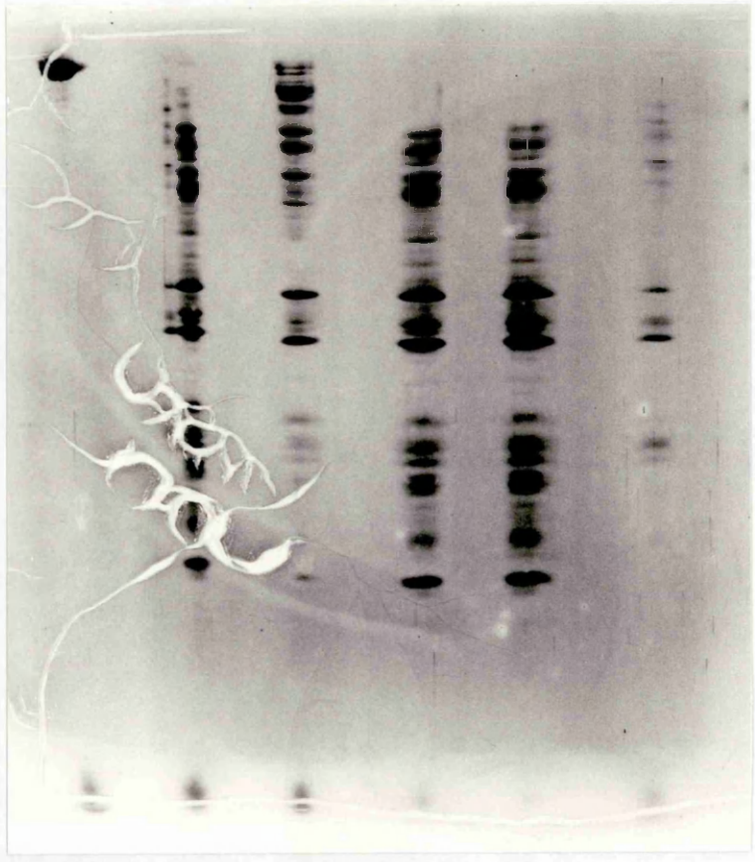

100
80
60
40
20
0
Phosphorylation (%)
0
1
2
3
4
5
[Malate] (mM)

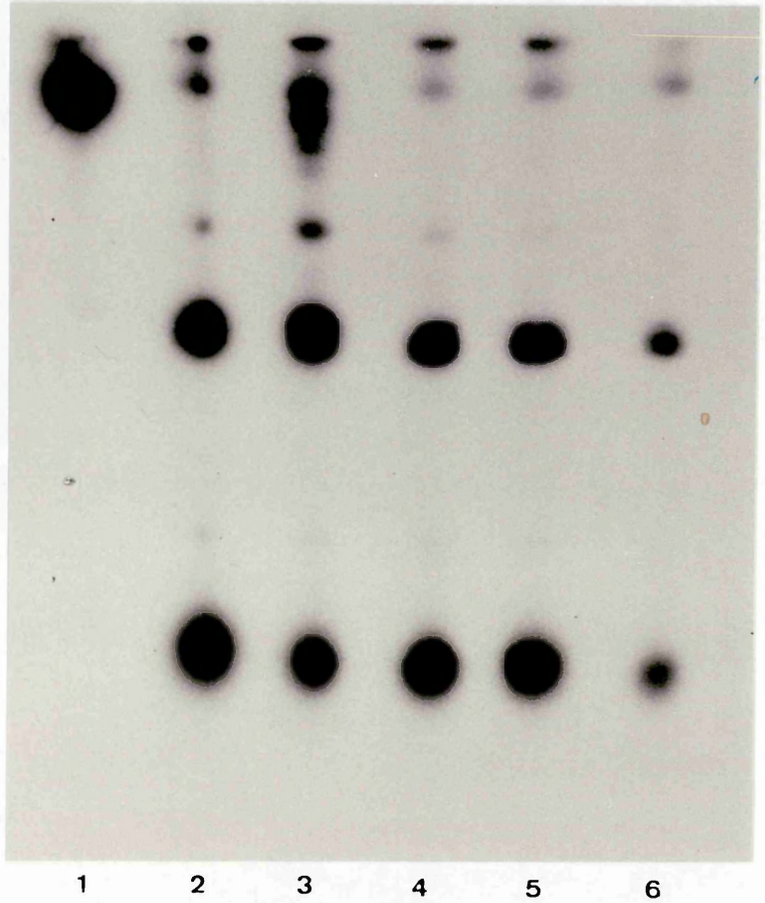
FIGURE 4.10. Peptide mapping of phosphorylated PEP carboxylase. PEP carboxylase was phosphorylated *in vitro* using the partially purified protein kinase (Section 2.5.2(ii)). The 112kDa and the 123kDa subunits (0.03 units total) of the enzyme were digested for 30 min with V8 protease (25ng) as described in Section 2.9.3. The peptides were separated on a 15% SDS/polyacrylamide gel (a) stained with $AgNO_3$ (Section 2.6.2) and autoradiographed (b). Track 1, undigested 112kDa and 123kDa subunits ; track 2, 112kDa and 123kDa subunits digested with V8 protease; track 3 112kDa and 123kDa subunits digested with V8 protease; tracks 4 and 5, 112kDa subunit (0.03 units) digested with V8 protease; tracks 6, 123kDa subunit (0.06units) digested with V8 protease.

(a)



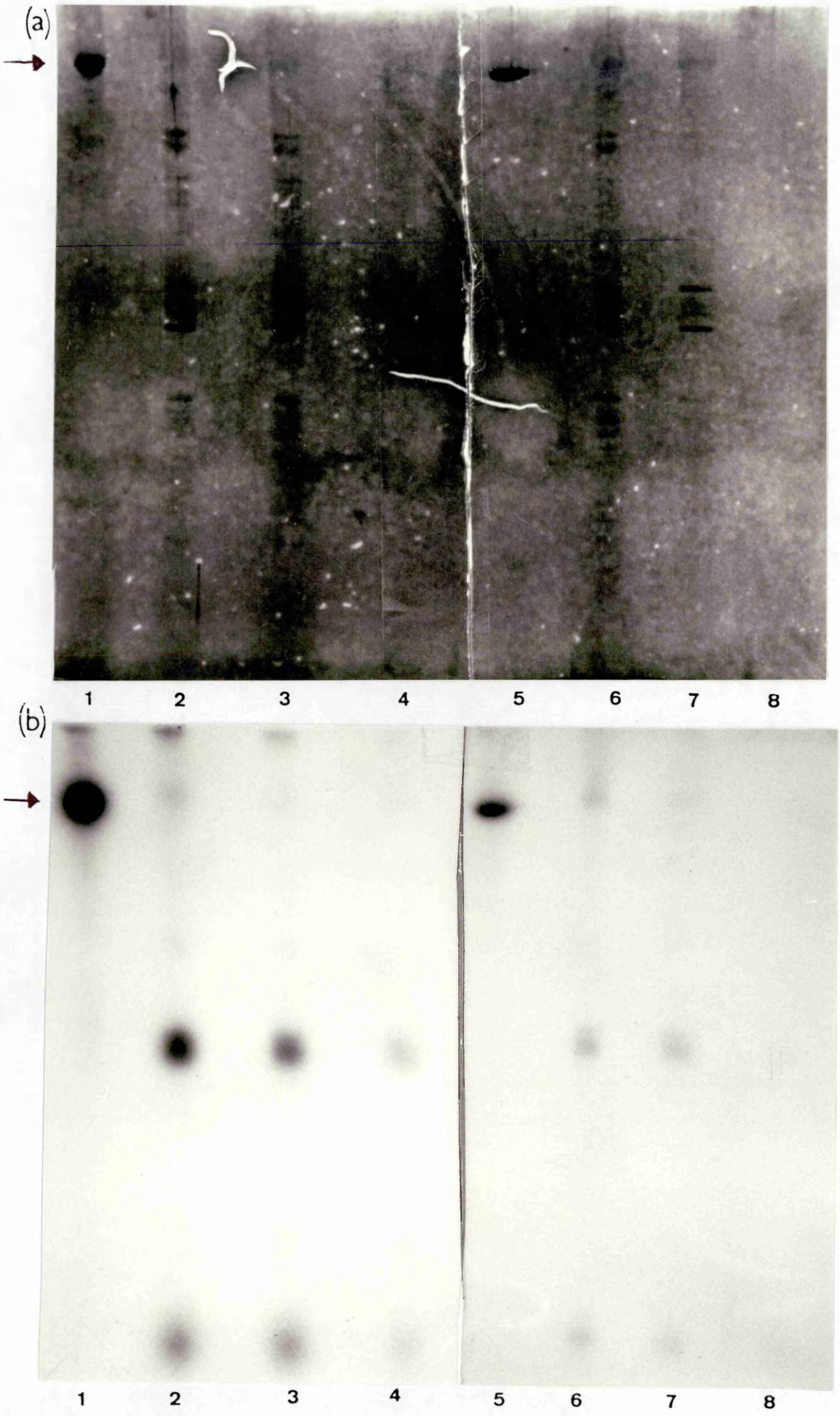
1 2 3 4 5 6

(b)



1 2 3 4 5 6

FIGURE 4.11. Comparison of *in vivo* and *in vitro* phosphorylation sites of PEP carboxylase. PEP carboxylase was either phosphorylated *in vitro* (Section 2.5.2 (ii)) or phosphorylated *in vivo* and isolated by immunoprecipitation (Section 2.9.1). The 112 kDa and 123 kDa subunits (0.03 units total) of the enzyme were digested for 30 min with V8 protease (25ng) as described in Section 2.9.3. The peptides were separated on a 15% SDS/polyacrylamide gel (a) stained with $AgNO_3$ and autoradiographed (b). Tracks 1,2,3,4 show PEP carboxylase which was phosphorylated *in vitro*. Tracks 5,6,7,8 show PEP carboxylase which was phosphorylated *in vivo*. Tracks 1 and 5, undigested enzyme; tracks 2 and 6, both the 112kDa and the 123kDa subunits digested with V8 protease; tracks 3 and 7, 112 kDa subunit digested with V8 protease; tracks 4 and 8, 123 kDa subunit (0.06 units) digested with V8 protease. The arrow indicates the position of undigested PEP carboxylase.


(a)
1
2
3
4
5
6
7
8
(b)
1
2
3
4
5
6
7
8

has also been observed in C_4 species; sorghum (Echevarria *et al.*, 1988) and partially purified from maize (Jiao and Chollet, 1989; McNaughton *et al.*, 1991). The partially purified protein kinase from *B. fedtschenkoi* phosphorylates PEP carboxylase at the same site(s) that is labelled *in vivo* and causes a change in the allosteric properties of the enzyme. This suggests that the phosphorylation is of physiological significance as discussed in the Introduction of this Chapter. PEP carboxylase can be phosphorylated relatively quickly (30-60 min) to a maximum stoichiometry of 0.9 mol P_i per mol subunit, although lower stoichiometries were also obtained. Studies on the phosphorylation of PEP carboxylase in the C_4 plant maize by McNaughton *et al.* (1991) showed a maximum stoichiometry of 0.76 mol of P_i per mol subunit. It has been suggested by these authors that a low stoichiometry may reflect a partial proteolysis of the substrate or a partial phosphorylation of the substrate prior to the protein kinase catalysed reaction.

The time course of phosphorylation of PEP carboxylase and the change in its malate sensitivity indicate that the increase in apparent K_i of the enzyme must be caused by its phosphorylation. The overall change in the K_i value obtained *in vitro* (almost ten-fold) correlates with the change in K_i observed between day and night leaves (0.3mM and 3.0mM). The net K_i change therefore suggests that the phosphorylation state of the enzyme increases from 0% to approximately 100% *in vivo*.

Control of the phosphorylation state of PEP carboxylase may be mediated through changes in the activity of the protein kinase. In many plant systems, light is one of the signals controlling intracellular metabolism. Studies by several workers have shown that high illumination increases the PEP carboxylase activity in C_4 plants (Huber *et al.*, 1986; Nimmo *et al.*, 1987; Chollet *et al.*, 1989). This activation is now thought to occur by light-modulation of the protein kinase which phosphorylates the enzyme, although the mode of action is as yet unknown (McNaughton *et al.*, 1991). Studies by Samaras *et al.* (1988) on C_4 plants have suggested that phosphorylation of PEP carboxylase is activated by a Calvin cycle product that diffuses from bundle-sheath to mesophyll tissue. PEP carboxylase activity in CAM plants is not directly affected by light but is controlled by an endogenous circadian rhythm. It is possible that the concentration of specific metabolites in the cytoplasm act as an oscillator of this

biological clock. Attempts to identify metabolites that might regulate *B. fedtschenkoi* PEP carboxylase kinase gave negative results (Table 4.1). However, further work is described on the regulation of PEP carboxylase kinase activity in Chapter 6.

Chapter 5

THE DEPHOSPHORYLATION OF PEP CARBOXYLASE

5.1. Introduction

At the time of this study very few papers had been published on the protein phosphatase activity in higher plants. The presence of a histone phosphatase in soybean (Lin *et al.*, 1980) and in wheatgerm (Polya *et al.*, 1988) had been reported and an activity associated with a phosphoprotein has been observed in pea thylakoids (Bennet, 1988). Studies by MacKintosh and Cohen (1989) illustrated the similarity of the protein phosphatase activities found in *Brassica napus* to those found in mammalian tissues. These authors identified type 1 and type 2A plant protein phosphatase activities (see Section 1.3.3). One of the aims of the present work was therefore to identify the type(s) of protein phosphatases present in *B. fedtschenkoi*, with a view to investigating the dephosphorylation of PEP carboxylase. The availability of purified mammalian phosphatases and protein phosphatase inhibitors greatly assisted the identification of the putative protein phosphatase responsible for the dephosphorylation of PEP carboxylase *in vivo*.

5.2. Results

5.2.1. Dephosphorylation of PEP carboxlase by mammalian protein phosphatases

Preliminary studies with mammalian protein phosphatases showed that incubation of type 2A protein phosphatase (30mU/ml) with PEP carboxylase purified from darkened leaf tissue (K_i = 2.5 mM) caused an increase in the malate sensitivity of the enzyme (to K_i approx. 0.9 mM), whereas type 1 protein phosphatase (3 or 30 mU/ml) had no effect on the apparent K_i (Table 5.1). In addition, PEP carboxylase purified from dark leaves which had been pre-labelled with $^{32}P_i$ (as described in Section 2.9.1) was readily dephosphorylated by the type 2A protein phosphatase (Figure 5.1). In order to correlate this dephosphorylation with the observed increase in malate sensitivity, the time dependence of dephosphorylation of PEP carboxylase labelled *in vivo* was quantified by TCA precipitation and scintillation counting of the supernatants. Figure 5.2 represents one such time course where

Table 5.1. **The effect of mammalian protein phosphatases type 1 and type 2A on the malate sentivity of PEP carboxylase purified from darkened leaves of *B. fedtschenkoi*.** PEP carboxylase was incubated with either 30mU/ml of type 1 or type 2A protein phosphatase (see Section 2.5.3 (ii)) purified from rabbit skeletal muscle . The apparent K_i for malate of PEP carboxylase was determined at 2h intervals.

	Apparent K_i for malate (mM)		
Time (min)	Type 1 protein phosphatase (30mU/ml)	Type 2A protein phosphatase (30mU/ml)	No protein phosphatase added
0	2.4	2.5	2.5
120	2.5	1.1	2.4
240	2.4	0.9	2.5
360	2.4	0.9	2.5

FIGURE 5.1. Dephosphorylation of PEP carboxylase by mammalian protein phosphatase type 2A. PEP carboxylase was phosphorylated *in vivo* using $^{32}P_i$ (Section 2.9.1) and purified as described in Section 2.4.3. The labelled enzyme (0.03 units) was incubated with 3mU of mammalian type 2A protein phosphatase/ml (Section 2.5.3(i)) and the dephosphorylation monitored over a 90 min period. Samples were denatured and separated on an 8% SDS/polyacrylamide gel (a) stained with Coomassie Brillant Blue and autoradiographed (b). Track 1, 0 min; track 2, 10 min; track 3, 20 min; track 4, 30 min; track 5, 60 min; track 6, 90 min. The arrow indicates the position of PEP carboxylase.

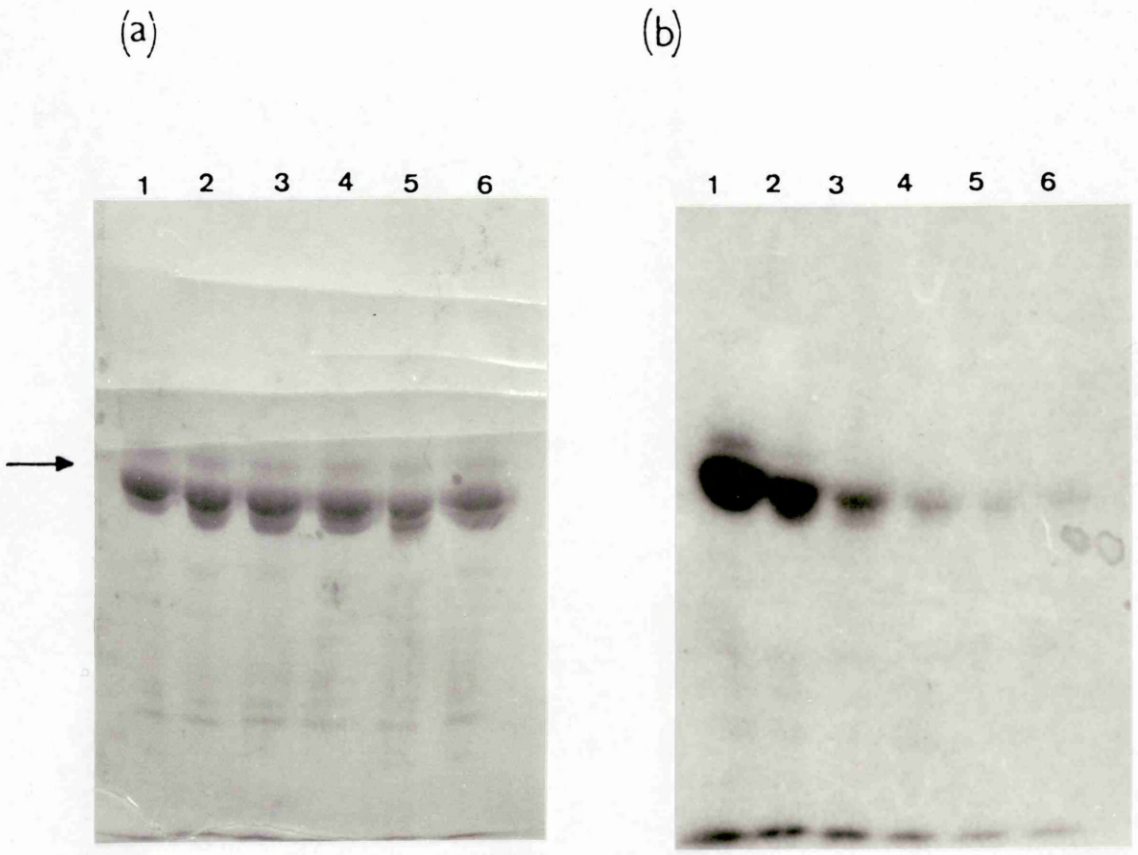
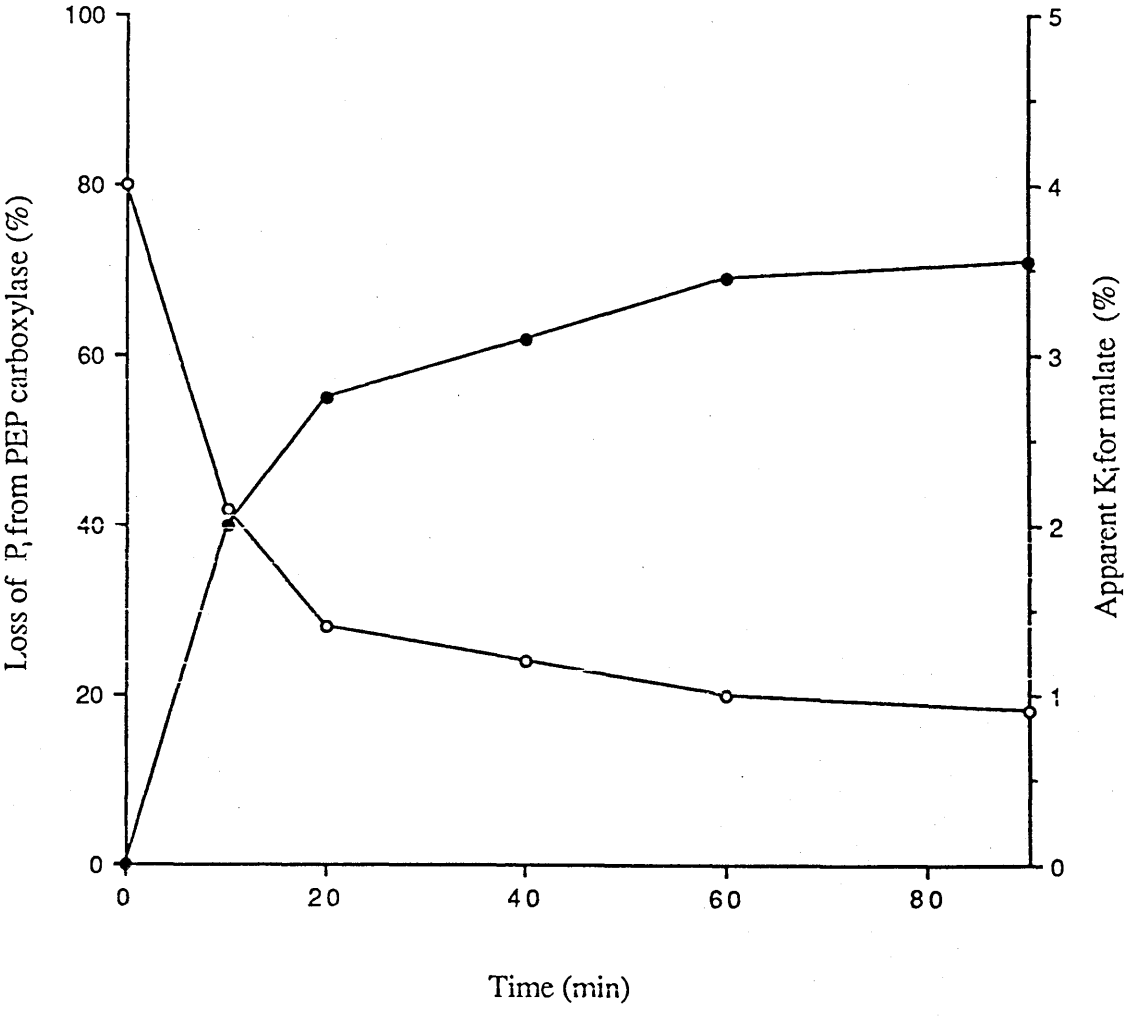

(a)
(b)
1 2 3 4 5 6
1 2 3 4 5 6

FIGURE 5.2. **The effect of mammalian protein phosphatase type 2A on the dephosphorylation and change in apparent K_i for malate of purified PEP carboxylase.** *In vivo* $^{32}P_i$-labelled PEP carboxylase (0.03units) was incubated with protein phosphatase type 2A (3mU/ml) at 25°C as described in Section 2.5.3 (i). Samples were removed at intervals and the loss of $^{32}P_i$ from the PEP carboxylase was determined by TCA precipitation as described in the methods (Section 2.9.4). The apparent K_i for malate was also determined at the same times.

Loss of ^{32}P (—●—)

Apparent K_i (—○—)


100
80
60
40
20
0
0
20
40
60
80
5
4
3
2
1
0
Loss of P_i from PEP carboxylase (%)
Apparent K_i for malate (%)
Time (min)

dephosphorylation seemed complete after 60 min; the apparent K_i for malate decreased from 4.0 to 0.9 mM. Similar results were obtained in three separate experiments. The dephosphorylated form of PEP carboxylase *in vivo* has a K_i of approximately 0.3 mM. The reason for the higher apparent K_i for malate of the enzyme that was dephosphorylated *in vitro* was presumably due to partial proteolysis of the original phosphorylated substrate.

5.2.2. Protein phosphatase activity in *B. fedtschenkoi*

The activity of protein phosphatases in *B. fedtschenkoi* leaf extracts was investigated by measuring their activities towards phosphorylase *a* (purified from rabbit muscle and ^{32}P-labelled by phosphorylase kinase) or casein (^{32}P-labelled by cyclic AMP-dependent kinase). Phosphorylase *a* is a good substrate for type 1 and type 2A protein phosphatases, whereas casein is only a substrate for the type 2A enzyme (Cohen *et al.*, 1988a). Desalted leaf extracts showed a protein phosphatase specific activity of 0.8 (± 0.2) mU/mg (n = 3) with phosphorylase *a* and 0.24 (± 0.03) mU/mg with casein (n = 4).

Initial studies of PEP carboxylase phosphatase were done using desalted 'day' extracts on the assumption that they would contain the activity responsible for the dephosphorylation of PEP carboxylase. Dephosphorylation was monitored by measuring a decrease in the apparent K_i for malate. However, no significant change was evident after 60 min incubation and on prolonged incubation the apparent K_i actually increased from 3.0 mM to 5.0 mM indicating that proteolysis of the PEP carboxylase had occurred. Some protein phosphatases are stimulated by metal ions or polyamines (Ballou and Fischer, 1986), but the addition of Mg^{2+} (20 mM), Mn^{2+} (5 mM), Ca^{2+} (5 mM) or spermidine (1 mM) had no effect on the dephosphorylation of PEP carboxylase by desalted extracts. The endogenous phosphorylase *a* and casein phosphatase activities found in *B. fedtschenkoi* were therefore partially purified using a modification of the procedure for the isolation of the catalytic subunits of protein phosphatases from rabbit skeletal muscle (Cohen *et al.*, 1988) as described in Section 2.4.5. The phosphorylase phosphatase and casein phosphatase activities were determined at each stage of the purification (Table 5.2). An increase in the activity was observed after treatment of the ammonium

Table 5.2. Partial purifiaction of protein phosphatases from leaves of *B. fedtschenkoi* taken during the day period. Protein phosphatase was purified as described in Section 2.4.5. Phosphorylase phosphatase and casein phosphatase activities were determined at each stage of the purification (see Section 2.5.3 (i)). The final Mono Q pool was assayed for type 1 and type 2A protein phosphatase activity using okadaic acid and inhibitor-2 to differentiate between the two types of activity (see Section 2.5.3(iii)), as discussed in the text.

Purification step	Volume (ml)	Phosphorylase phosphatase activity (mU/ml)	Casein phosphatase activity (mU/ml)
Extract	60.0	0.88	0.22
0-75% $(NH_4)_2SO_4$	8.0	3.71	0.68
Desalted ethanol precipitate	10.0	6.10	1.25
Mono Q pool concentrated	0.7	71.00	6.00

sulphate fraction with ethanol at room temperature. This is attributed to the dissociation of catalytic subunits from regulatory subunits of the phosphatases (Cohen, 1989). These free catalytic subunits were then chromatographed on a Mono Q column using a 25 min linear gradient from 100 mM to 400 mM-NaCl in buffer D (see Section 2.4.1) at 1 ml/min. Individual fractions were assayed for phosphorylase phosphatase activity (Section 2.5.3) (Figure 5.3). Active fractions were pooled and stored at 4°C. This three-step purification does not separate type 1 and type 2A protein phosphatases. These two protein phosphatases were distinguished and quantified as described in Section 2.5.3 (iii). The 71 mU phosphorylase phosphatase activity/ml measured in the Mono Q pool (Table 5.2) contained 54 mU type 1 activity/ml and 16 mU type 2A activity/ml. The casein phosphatase activity was almost exclusively type 2A. Unsuccessful attempts were made to separate these two activities by chromatography on heparin-sepharose which, like poly (L-lysine)-sepharose was found by Cohen *et al.* (1988) to bind type 1 but not type 2A protein phosphatases.

The Mono Q protein phosphatase mixture was used to study the dephosphorylation of PEP carboxylase. Incubations contained 3 mU of type 2A protein phosphatase activity/ml (quantified using phosphorylase *a* as a substrate) and 0.5 µM PEP carboxylase purified from leaves taken during the dark period (apparent K_i = 3.0 mM). Under these conditions a rapid decrease in the apparent K_i for malate from 2.7 to 0.6 mM occurred within 30 min incubation, after which it steadily increased again to 4.0 mM (Figure 5.4). This increase was prevented by 1 mM L-malate, which probably acts by protecting the dephosphorylated form of PEP carboxylase from proteolysis (Nimmo *et al.*, 1986). This explanation was confirmed by the observation that the addition of a mixture of protease inhibitors (5 µg antipain/ml, 5 µg leupeptin/ml, 10 µg chymostatin/ml, 1.0mM benzamidine) also prevented an increase in the K_i (Figure 5.4). Further studies on the effects of malate showed that 10 µM L-malate was sufficient to maintain the low K_i for malate over 150 min incubation. However, concentrations of L-malate greater than 2.0 mM had an inhibitory effect on the plant phosphatase activity. The dephosphorylation of PEP carboxylase by plant phosphatase in the presence of either 1 mM or 5 mM L-malate is illustrated in Figure 5.5. High concentrations of L-malate (5 mM) also inhibited phosphatase activity when measured with casein (not shown). Similar effects were found with D-malate in that at moderate concentrations

Figure 5.3. Mono Q chromatography of protein phosphatase(s) from *B. fedtschenkoi.* The desalted ethanol precipitate was loaded onto a Mono Q column equilibrated in buffer D. A 25ml linear gradient (indicated by the diagonal line) of 100-400mM NaCl in buffer D was applied. The flow rate was 1ml/min and 1min fractions were collected. The casein phosphatase activity was determined as described in Section 2.5.3(i).

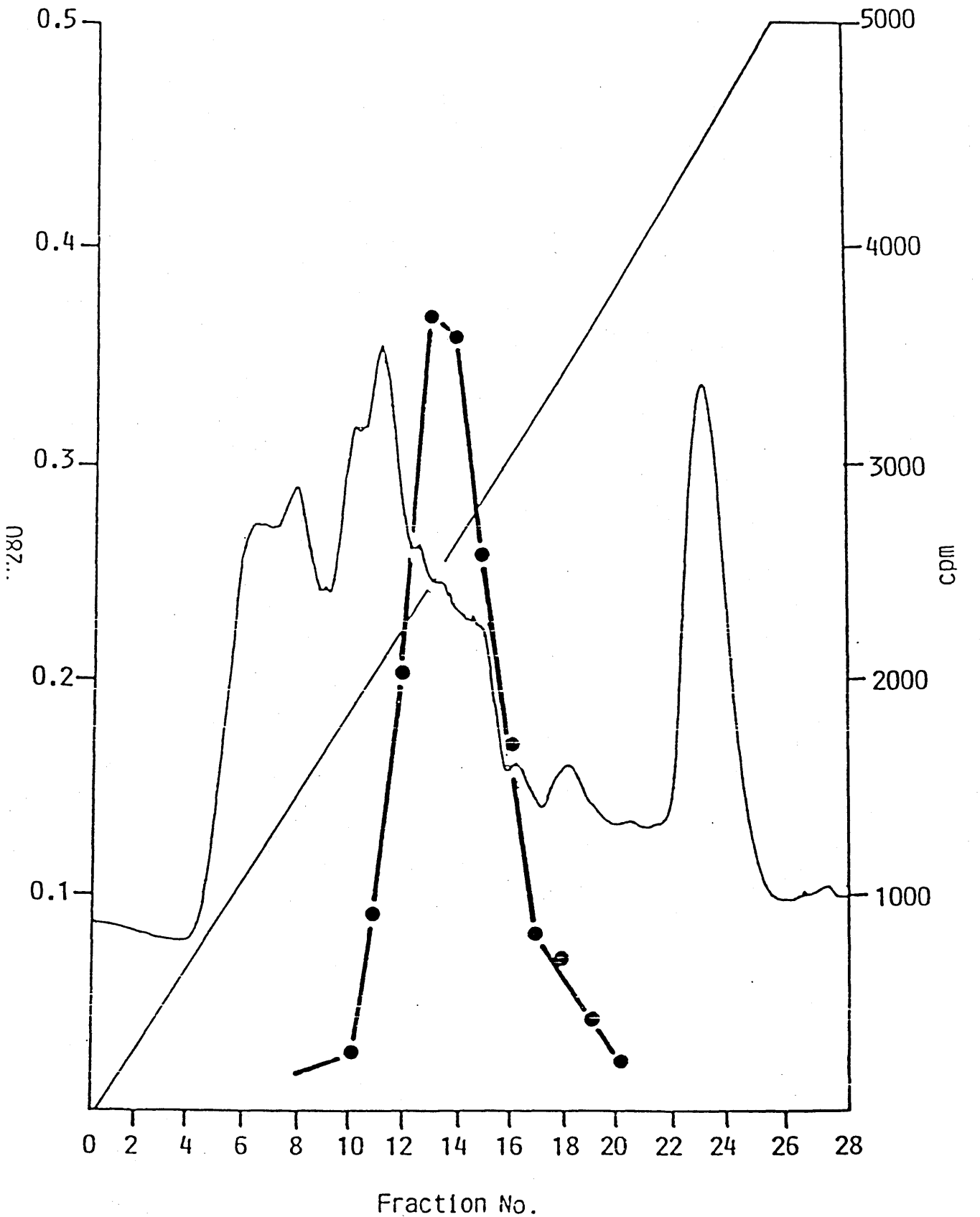

0.5
0.4
0.3
0.2
0.1
OD280
5000
4000
3000
2000
1000
cpm
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Fraction No.

FIGURE 5.4. The effect of partially purified *B. fedtschenkoi* protein phosphatase on the malate sensitivity of purified PEP carboxylase. PEP carboxylase was incubated with the plant phosphatase (equivalent to 3mU/ml of type 2A activity) in the presence (o) or absence (•) of 1mM L-malate, or in the presence of protease inhibitors (1mM benzamidine, 10μg antipain/ml, 10μg leupeptin/ml) (△) , as described in Section 2.5.3(ii). The apparent K_i for malate was determined over the time course.

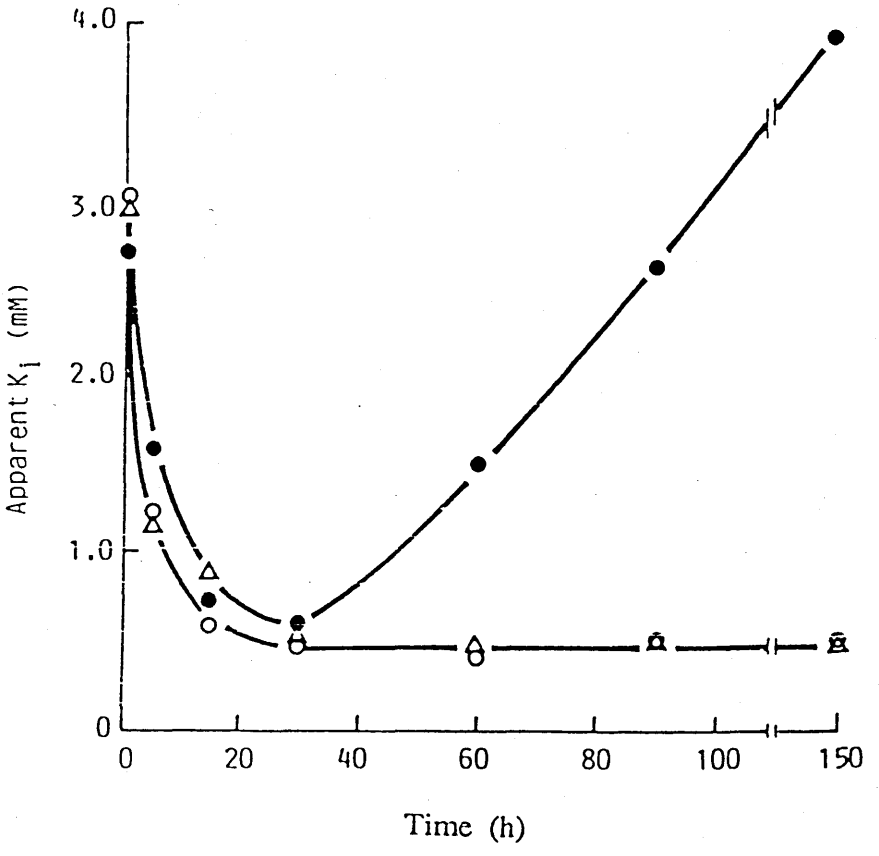
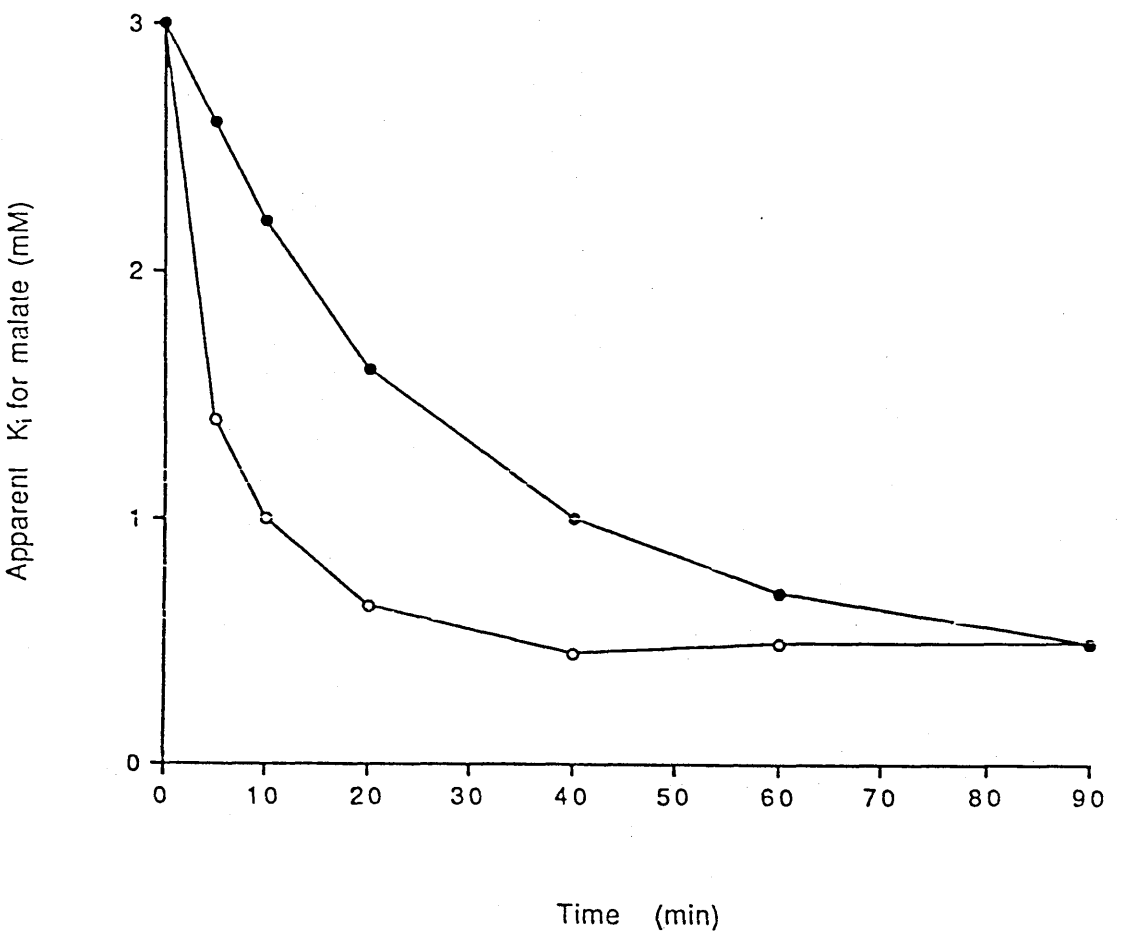

4.0
3.0
2.0
1.0
0
Apparent K_i (mM)
0
20
40
60
80
100
150
Time (h)

FIGURE 5.5. **The effect of L-malate on the dephosphorylation of PEP carboxylase by plant protein phosphatases.** Incubations contained 3mU of type 2A plant protein phosphatase activity /ml, 0.5μM purified PEP carboxylase and either 1mM (—○—) or 5mM (—●—) L-malate in buffer D (Section 2.5.3).


Apparent K_i for malate (mM)
3
2
1
0
0
10
20
30
40
50
60
70
80
90
Time (min)

(1 mM) it stabilized the K_i of dephosphorylated PEP carboxylase, although lower concentrations (100 μM, 10 μM) were less effective. At higher concentrations (5 mM) D-malate inhibited the casein phosphatase activity but its effects on dephosphorylation of PEP carboxylase were not studied.

In order to ascertain whether *B. fedtschenkoi* type 1 or type 2A protein phosphatase activity catalysed the dephosphorylation of PEP carboxylase, the effects of inhibitor-2 and okadaic acid were investigated (see Section 6.3.3). The dephosphorylation of PEP carboxylase (0.5 μM) by 3 mU of type 2A mammalian or plant protein phosphatase/ml (determined using phosphorylase *a* as a substrate), was assessed in the presence of a range of okadaic acid concentrations. The apparent K_i for malate was determined initially and after 60 min incubation (Table 5.3). Between 10 and 25 nM okadaic acid was required to inhibit both the plant and mammalian protein phosphatase activity. Addition of 1 μM inhibitor-2 did not inhibit the dephosphorylation of PEP carboxylase by the plant protein phosphatase preparation. The apparent K_i for malate decreased from 3.3. mM to 0.8 mM over 60 min in one such experiment. This suggests that it is the type 2A activity and not the type 1 activity that is responsible for the dephosphorylation of PEP carboxylase.

5.3. Discussion

This work shows the existence of two types of protein phosphatase activity in *B. fedtschenkoi*, namely type 1 and type 2A. Similar types of activities were found in maize by McNaughton *et al.* (1991) and in oilseed rape by MacKintosh and Cohen (1989). The physiological roles of these plant phosphatases is largely unknown. However, this work suggests that PEP carboxylase is a physiological substrate for the type 2A protein phosphatase found in *B. fedtschenkoi*. PEP carboxylase may be just one of many substrates dephosphorylated by protein phosphatase, type 2A, since protein phosphatases exhibit broad substrate specificities. This is illustrated by the fact that the plant protein phosphatase can dephosphorylate mammalian phosphorylase *a* and ^{32}P-labelled casein.

When phosphorylase *a* was used as a substrate for *B. fedtschenkoi* protein phosphatases, any activity remaining after the addition of 1 M inhibitor-2 could be removed with 1 nM okadaic acid. This implies that there are no other metal-ion independent protein phosphatases present apart from type 1 and

Table 5.3. **Inhibition by okadaic acid on the effect of mammalian and plant protein phosphatase activity on the malate sensitivity of purified PEP carboxylase.** The incubations contained 3mU of type 2A plant or mammalian protein phosphatase activity /ml, as determined using phosphorylase *a* as a substrate (see Section 2.5.3). A range of okadaic acid concentrations (0 to 25nM) was added to the incubations and the apparent K_i for malate of PEP carboxylase determined initially and after 60 min.

[Okadaic acid]	K_i value after incubation with plant protein phosphatase		K_i value after incubation with mammalian protein phosphatase	
(nM)	0 min	60 min	0 min	60 min
0	3.4	0.7	3.3	0.8
5	3.3	0.7	N.D	N.D
10	3.3	0.7	3.0	1.0
25	3.5	3.4	3.0	3.0

type 2A.

No attempts were made in this study to optimize the incubation conditions used for measuring dephosphorylation. Results from a single experiment did show that a twenty-fold lower concentration of plant type 2A protein phosphatase (0.13 mU/ml) activity could also decrease the apparent K_i within 30 to 60 min incubation.

The effects of L- and D- malate on the dephosphorylation of PEP carboxylase suggest that malate stabilizes the low K_i form of the enzyme. It is difficult to postulate the mechanism by which it does this, since it appears to be a non-stereospecific effect. However, the D-malate stock was found to be contaminated by 2% L-malate which may explain why 1 mM D-malate (containing 20 μM L-malate) prevented the increase in apparent K_i. The inhibition of both phosphorylase *a* and PEP carboxylase phosphatase activity by higher concentrations of L- and D- malate indicate that it is not a substrate-specific effect. It is unlikely that the malate interacts directly with the protein phosphatase since only the catalytic subunits of these are present in the incubation. Mammalian phosphatases type 1 and type 2A are controlled by regulatory subunits associated with the catalytic subunit *in vivo* (Cohen, 1989). If malate was a significant effector of plant phosphatase activity, then it would most likely interact with similar regulatory subunits. Therefore, in order to study the regulation of the plant protein phosphatases, it will be necessary to purify them in their holoenzyme form.

Chapter 6

THE REGULATION OF PROTEIN KINASE AND PROTEIN PHOSPHATASE ACTIVITIES IN *B.FEDTSCHENKOI*

6.1. Introduction

The diurnal CAM cycle is partly maintained through the periodic control of PEP carboxylase activity. This requires the regulation of the activities of PEP carboxylase kinase and phosphatase. The initial aim of this work was therefore to investigate the activities of these two regulatory enzymes over the diurnal cycle, in order to see how their activities might be integrated *in vivo* . At the time of this study there were no reports on the mechanism of regulation of the phosphorylation of PEP carboxylase in CAM plants.

Bollig and Wilkins (1979) showed that cycloheximide, an inhibitor of protein synthesis (Baliga *et al.*,1969) abolished the circadian rhythm of CO_2 metabolism in detached leaves of *B. fedtschenkoi* (see Section 1.1.3). These authors suggested that cycloheximide maintained the presence of an inhibitor of PEP carboxylase in the cytoplasm. Their interpretation was based on the idea that cycloheximide disrupted membrane permeability , an effect that was reported by McMahon (1975) . Since the covalent modification of PEP carboxylase had not been discovered at the time of their work, Bollig and Wilkins (1979) postulated that malate leaked across the tonoplast membrane thereby inhibiting PEP carboxylase. In view of the fact that cycloheximide was reported to interfere with cellular processes such as respiration, amino acid uptake and ion transport (McMahon, 1975), it is possible that the interpretation of Bollig and Wilkins is at least in part correct. However the possible role of cycloheximide in inhibiting protein synthesis *per se* cannot be ruled out. In the present studies two chemically diverse protein synthesis inhibitors, namely puromycin and cycloheximide, were used in order to investigate the latter possibility. The effects of these inhibitors on the rhythm of CO_2 fixation and on the activities of PEP carboxylase kinase and phosphatase were investigated, with a view to understanding how the rhythm might be generated.

6.2. Results

6.2.1. Measurement of protein kinase and protein phosphatase activities over the diurnal cycle

The results described in this section were obtained from *B. fedtschenkoi* leaf extracts, made at various intervals over the normal 8h 'day' 16h 'night' cycle (Section 2.2) The extracts were desalted as described in Section 2.5.2 (i) and the enzyme assays carried out within one hour of extraction, by the methods described in Section 2.5, with the assistance of Dr H.G. Nimmo. Results (Table 6.1) show that there is no significant change in the type 2A protein phosphatase activity in leaf extracts over the diurnal cycle. PEP carboxylase kinase activities were determined in this experiment and in another experiment in which extracts prepared between 18 and 24h were examined (Figures 6.1a and 6.1b). In contrast to type 2A protein phosphatase, there is a diurnal variation in the PEP carboxylase kinase activity. The PEP carboxylase kinase activity is present only in the middle of the 'night' period, at which time the PEP carboxylase has a high K_i value for malate (Table 6.1). The changes in the apparent K_i for malate occur during the dark period as was observed by Nimmo *et al.* (1984). PEP carboxylase kinase activity appears 4-5h after the onset of darkness, and its activity subsequently diminishes 2-3h before the beginning of the 'day' period. These rhythmic changes in the protein kinase activity could account for the periodic control of PEP carboxylase activity.

6.2.2. The effects of inhibitors of protein synthesis on CO_2 metabolism in *B. fedtschenkoi*

The observation that protein kinase activity appears and disappears over the diurnal cycle, and the inhibtory effect of cycloheximide on the rhythm of CO_2 fixation (Bollig and Wilkins, 1979), suggests that protein synthesis may play a role in regulating the CAM pathway. In an attempt to assess this possibility, the effects of treating detached *B. fedtschenkoi* leaves with either cycloheximide or puromycin were investigated. Initially, the effects of a range of concentrations of inhibitor on the rhythm of CO_2 fixation were studied. Leaves were detached at the onset of the 8h illumination period and put into

TABLE 6.1. Diurnal variations in the activity of protein phosphatase type 2A and in the activity and malate sensitivity of PEP carboxylase, in desalted extracts of leaves of *B. fedtschenkoi*. The plants were maintained under normal short day growth conditions; 'day' was from 0800 to1600h (see Section 2.2). Extracts were prepared from pairs of leaves using 3ml of Buffer A /g FW of tissue and desalted into buffer C (Section 2.4.1). Extracts were assayed immediately after preparation for PEP carboxylase and protein phosphatase (PP) activities (Section 2.5.3). The PEP carboxylase protein kinase activities were also determined as shown in Figures 6.1a and 6.1b. The protein phosphatase type 2A activity was determined using ^{32}P-casein as a substrate as described in Section 2.5.3(i).

Time (h)	[Protein] (mg/ml)	Protein phosphatase type 2A activity (pmol/min/ml)	PEP carboxylase activity (U/ml)	Apparent K_i for malate (mM)	Specific activity of type 2A PP (pmol/min/mg)	Ratio type 2A PP/PEP carboxylase
0200	0.52	110.0	0.32	2.60	212	344
0400	0.15	35.1	0.13	2.70	234	270
0500	0.26	70.6	0.23	2.50	272	307
0600	0.18	37.9	0.11	1.90	211	345
0800	0.15	27.3	0.10	0.33	182	273
1100	0.11	22.7	0.12	0.32	206	189
1600	0.14	40.5	0.12	0.42	289	338
1800	0.19	50.0	0.13	0.55	263	370
2000	0.19	37.8	0.15	0.75	199	252
2100	0.13	25.1	0.10	1.30	193	251
2200	0.10	29.8	0.09	2.30	298	331
2400	0.17	51.4	0.14	3.00	302	367

FIGURE 6.1a. **Diurnal variation in PEP carboxylase kinase activity in *B. fedtschenkoi* leaves.** Extracts were prepared from pairs of leaves using 3 ml of buffer A /g FW of tissue and desalted into buffer C (Section 2.4.1) The PEP carboxylase kinase activity in freshly prepared extracts was determined by incubating in the presence or absence of purified exogenous 'day' form PEP carboxylase (0.03 units) for 10 min using 4 μCi/nmol ATP as described in Section 2.5.2 (i). The samples were denatured and separated on 8% SDS/polyacrylamide gels (a) and autoradiographed (see Figure 6.1b). Tracks 1,3,5,7,9,11,13,15,17,19,21, no exogenous PEP carboxylase added. Tracks 2,4,6,8,10,12,14,16,18,20,22, exogenous PEP carboxylase added. Tracks 1 and 2, 1100h extract; tracks 3 and 4, 1800h extract; tracks 5 and 6, 2000h extract; tracks 7 and 8, 2100h extract; tracks 9 and 10, 2200h extract; tracks 11 and 12, 2400h extract; tracks 13 and 14, 0200h extract; tracks 15 and 16, 0400h extract; tracks 17 and 18, 0500h extract; tracks 19 and 20, 0600h extract; tracks 21 and 22, 0800h extract. The arrow indicates the position of PEP carboxylase.

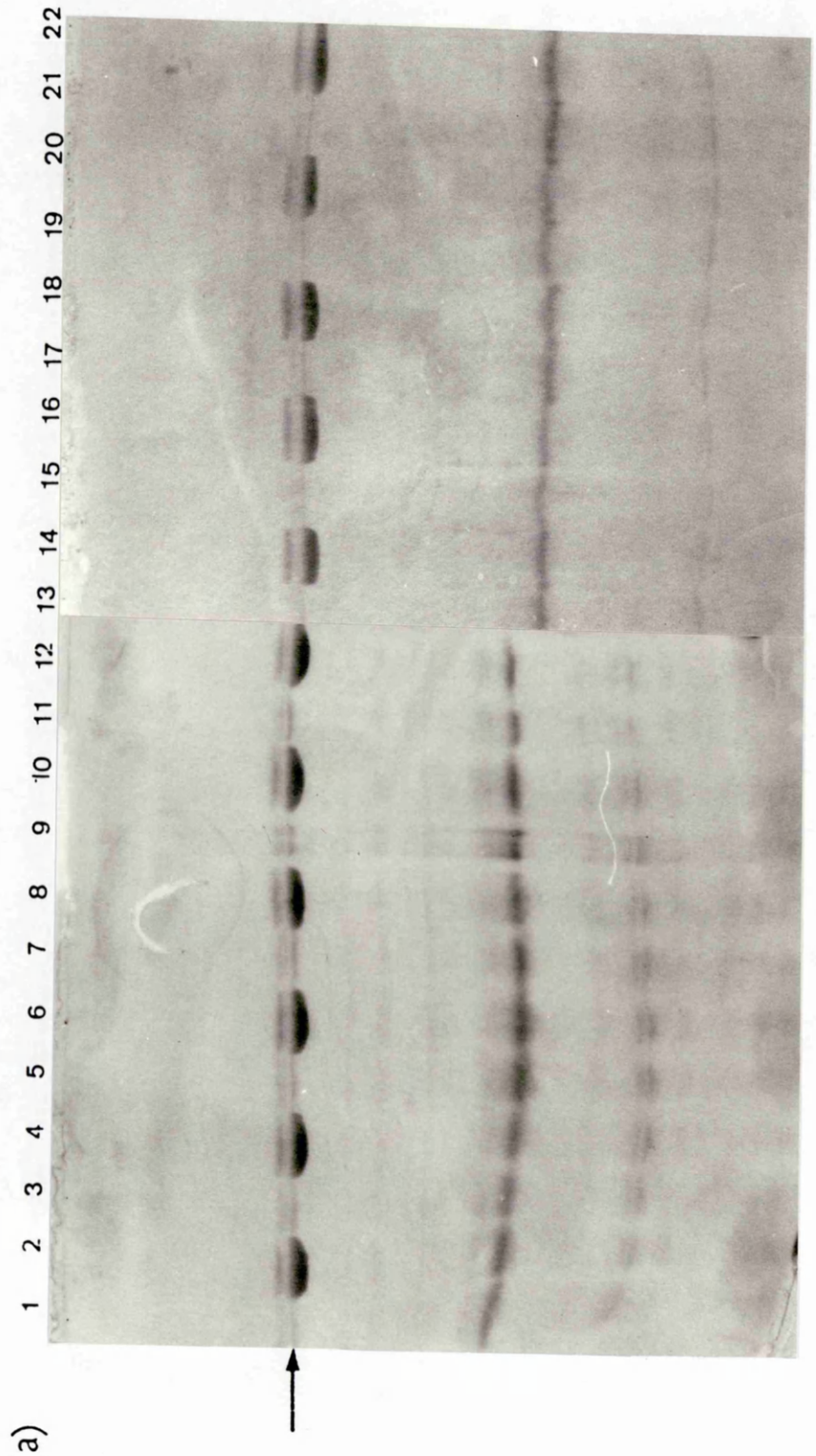
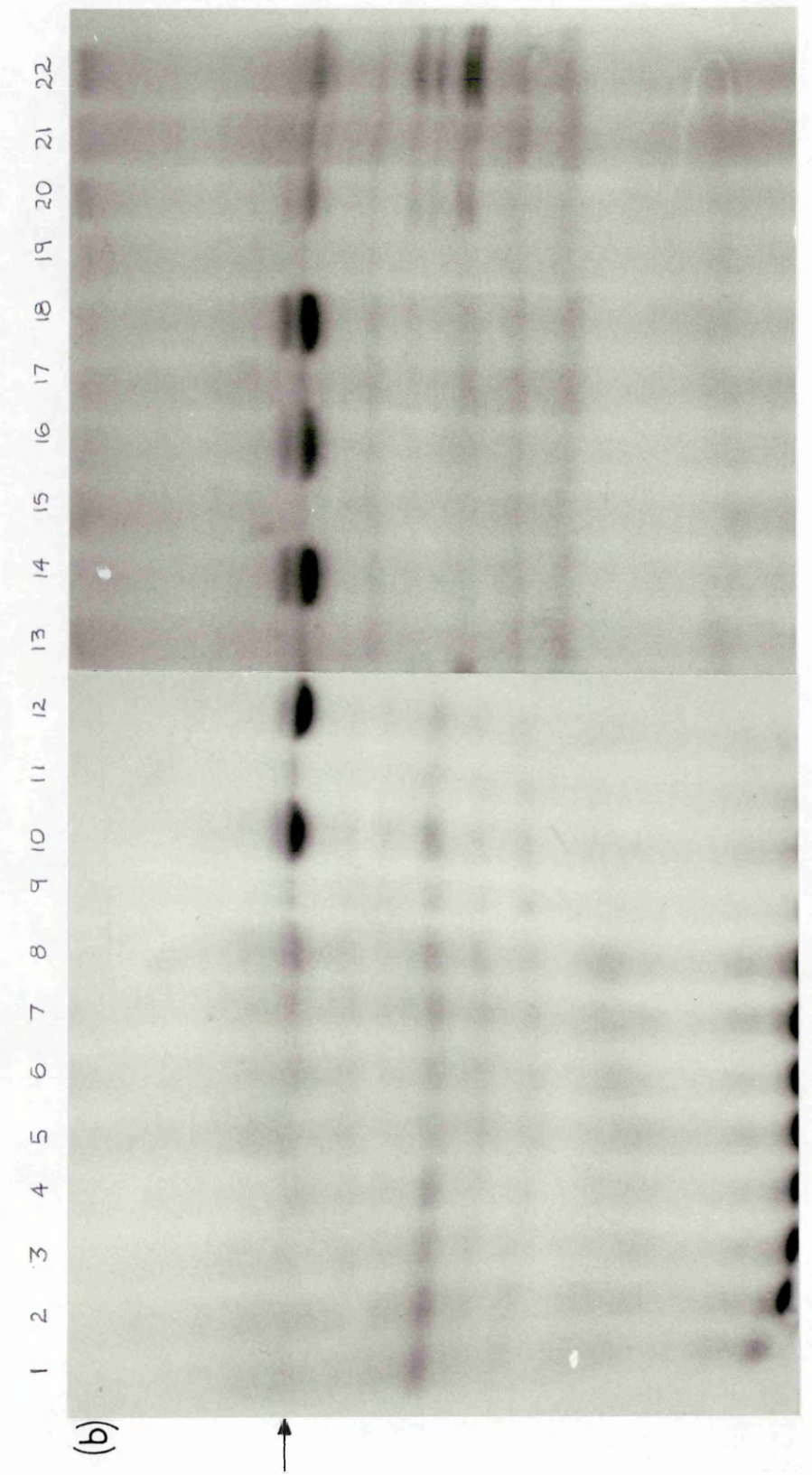

(a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

FIGURE 6.1b. **Diurnal variation in PEP carboxylase kinase activity in *B. fedtschenkoi* leaves.** Autoradiographs from the corresponding SDS/polyacrylamide gels shown in Figure 6.1a. Tracks 1,3,5,7,9,11,13,15,17,19,21, no exogenous PEP carboxylase added. Tracks 2,4,6,8,10,12,14,16,18,20,22, exogenous PEP carboxylase added. Tracks 1 and 2, 1100h extract; tracks 3 and 4, 1800h extract; tracks 5 and 6, 2000h extract; tracks 7 and 8, 2100h extract; tracks 9 and 10, 2200h extract; tracks 11 and 12, 2400h extract; tracks 13 and 14, 0200h extract; tracks 15 and 16, 0400h extract; tracks 17 and 18, 0500h extract; tracks 19 and 20 0600h extract; tracks 21 and 22, 0800h extract. The arrow indicates the position of PEP carboxylase on the SDS/polyacrylamide gels.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
(b)

enviromentally controlled chambers, with their petioles immersed in a solution of either puromycin or cycloheximide (Section 2.7) . The leaves were kept in continuous darkness in a stream of CO_2-free air at 15°C. The CO_2 output was monitored over several days by an infra-red gas analyser (Section 2.8) as illustrated in Figures 6.2 and 6.3. The control leaves which were not treated with protein synthesis inhibitor showed a circadian rhythm of CO_2 output which persisted for several days. The troughs in CO_2 output correspond with CO_2 fixation or periods of PEP carboxylase activity (Nimmo *et al.*,1987). There was a progressive inhibition of the rhythm of CO_2 fixation with increasing concentration of cycloheximide 5 $x10^{-6}$M to 5 $x10^{-4}$M (Figure 6.2), as was observed by Boillg and Wilkins (1979). Leaves treated with puromycin in the range 1 $x10^{-5}$M to 1 $x10^{-3}$M behaved in a similar manner (Figure 6.3). In order to assess the 'viability' of the leaves after three days in the presence of protein synthesis inhibitors, they were given a 5-6h light treatment (as indicated in Figures 6.2 and 6.3). The control leaves and those treated with lower concentrations of inhibitor showed a 'spike' of CO_2 output on illumination. This is presumably due to decarboxylation of malate by malic enzyme, which suggests that these leaves had accumulated significant amounts of malate. However, those leaves treated with higher concentrations of inhibitor showed a trough of CO_2 output on illumination. This is presumably due to fixation of CO_2 by RuBP carboxylase and indicates that the leaves were still capable of carrying out photosynthesis in spite of their treatment with the protein synthesis inhibitors.

6.2.3. The effects of puromycin and cycloheximide on the regulation of PEP carboxylase

The effects of puromycin and cycloheximide on the protein kinase activity in *B. fedtschenkoi* leaves was studied. Detached leaves were allowed to take up a solution of either 5 $x10^{-4}$M cycloheximide or 1 $x10^{-3}$ M puromycin through their petioles. Treatment was started at the begining of the 'day' period and the leaves extracted in the middle of the following 'night' period (Secton 2.7). The PEP carboxylase activity and the apparent K_i for malate were determined in freshly prepared desalted extracts. The results showed that there was no effect on the PEP carboxylase activity, but the apparent K_i for malate of the

Figure 6.2. **Effect of cycloheximide on the circadian rhythm of CO_2 output in detached leaves of *B. fedtschenkoi*.** Detached leaves of *B. fedtschenkoi* were placed with their petioles in a solution of cycloheximide (Section 2.7), in air-tight chambers. The leaves were held in continuous darkness and CO_2-free air at 15°C for several days and the CO_2 output was monitored as described in Section 2.8. The arrow indicates the onset of a 6h illumination treatment. The following concentrations of cycloheximide were analysed: a, control (1% ethanol replaced cycloheximide); b, 5 x10^{-6}M cycloheximide; c, 5 x10^{-5}M cycloheximide; d, 5 x10^{-4}M cycloheximide.

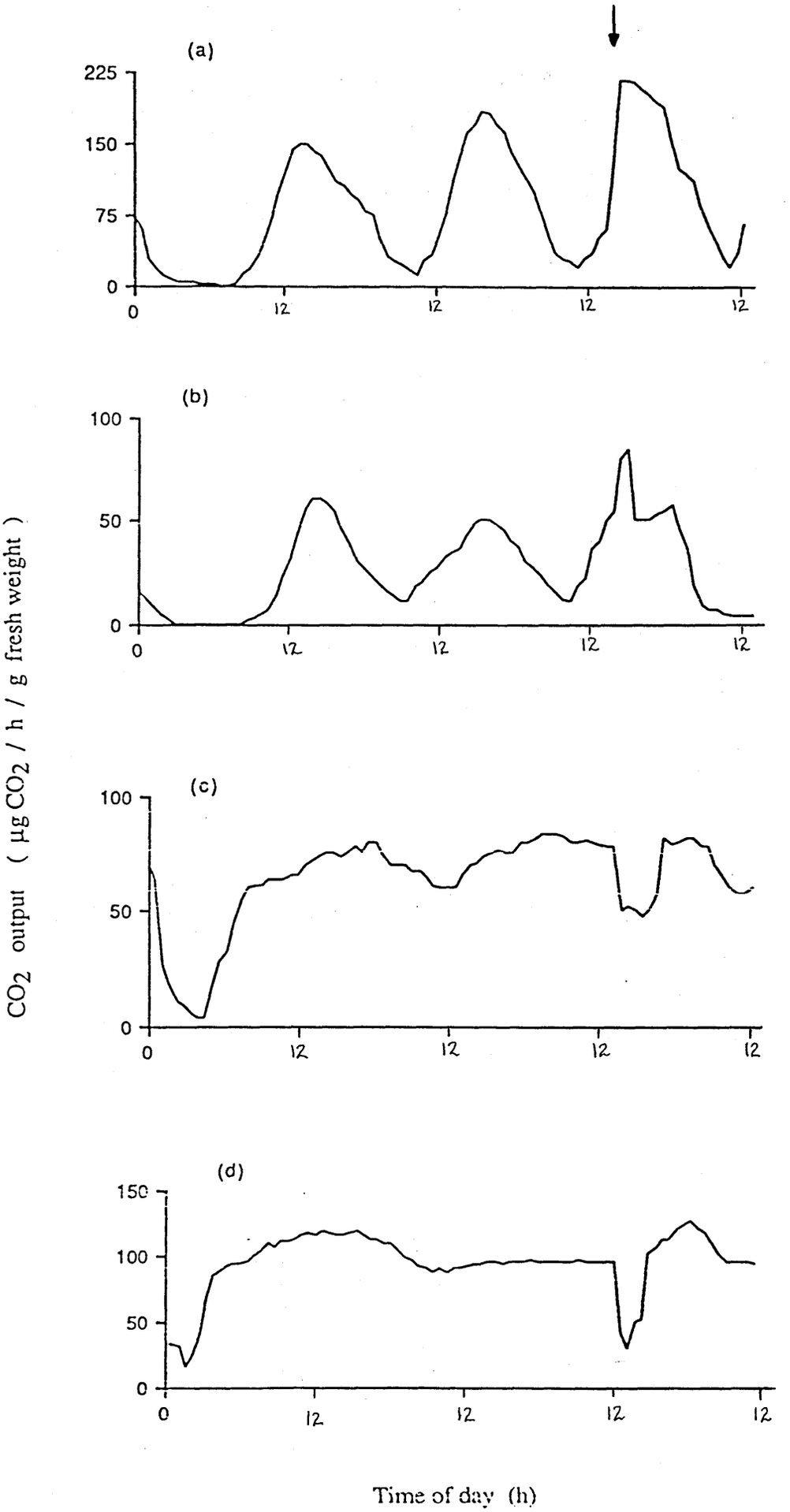
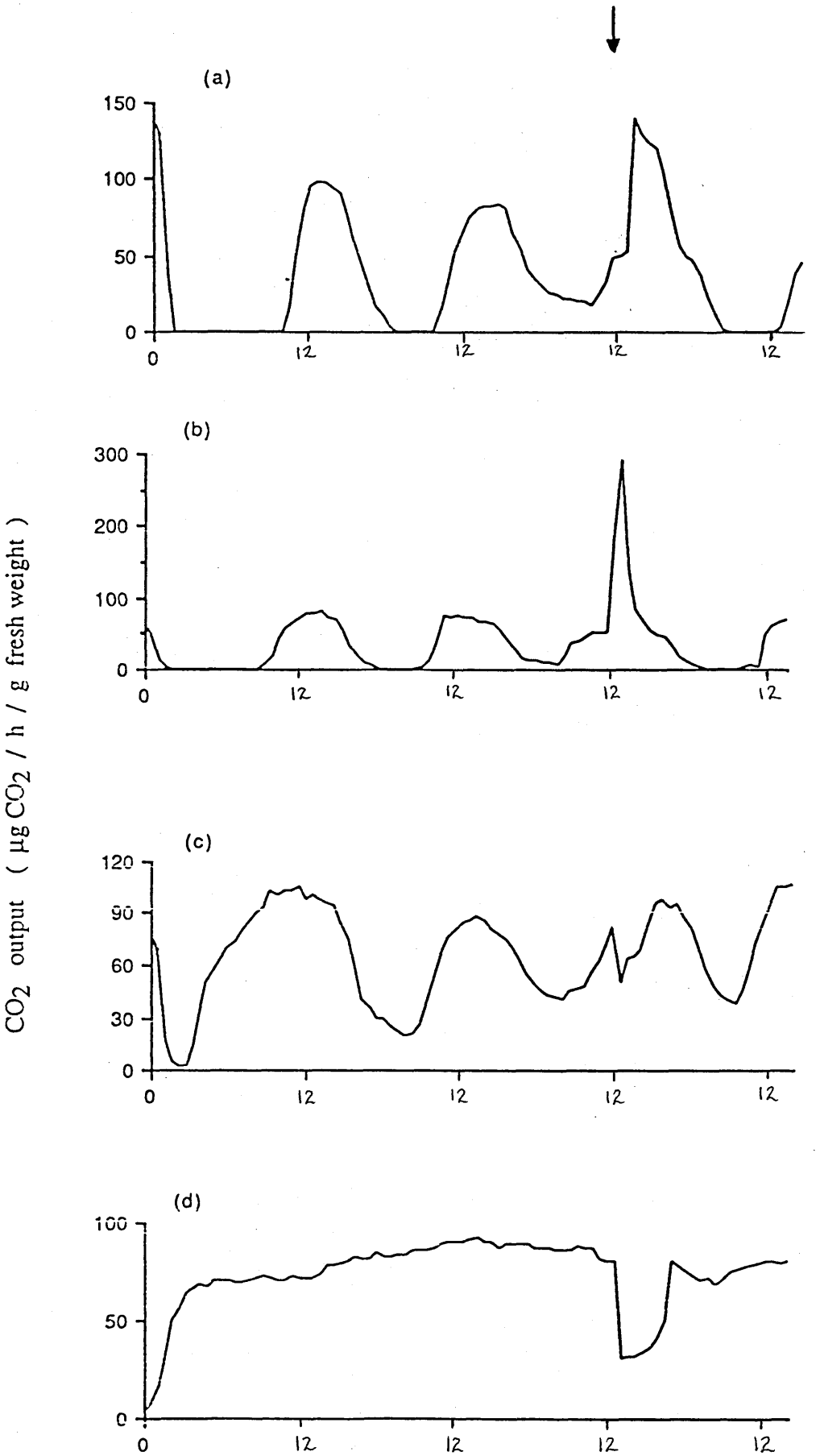

(a)
225
150
75
0
0
12
12
12
12
(b)
100
50
0
0
12
12
12
12
(c)
100
50
0
0
12
12
12
12
(d)
150
100
50
0
0
12
12
12
12
CO_2 output (µg CO_2 / h / g fresh weight)
Time of day (h)

Figure 6.3. **Effect of puromycin on the circadian rhythm of CO_2 output in detached leaves of *B. fedtschenkoi*.** Detached leaves of *B. fedtschenkoi* were placed with their petioles in a solution of puromycin (Section 2.7), in air-tight chambers. The leaves were held in continuous darkness and CO_2-free air at 15°C for several days and the CO_2 output was monitored as described in Section 2.8. The arrow indicates the onset of a 6h illumination treatment. The following concentrations of puromycin were analysed: a, control (water replaced puromycin); b, $1x10^{-5}M$; c, $1 \times 10^{-4}M$; d, $1 x10^{-3}M$.


(a)
150
100
50
0
0
12
12
12
12
(b)
300
200
100
0
0
12
12
12
12
(c)
120
90
60
30
0
0
12
12
12
12
(d)
100
50
0
0
12
12
12
12
CO_2 output (μg CO_2 / h / g fresh weight)
Time of day (h)

enzyme was lower in the leaves pretreated with cycloheximide and puromycin. The results from one such experiment are shown in Table 6.2. The apparent K_i was 0.4mM and suggests that the endogenous PEP carboxylase was in the dephosphorylated form. Further experiments were therefore carried out to determine the PEP carboxylase kinase activity in the pretreated leaves.

Desalted extracts were incubated with [^{32}P] ATP in the presence or absence of exogenous purified PEP carboxylase (Section 2.5.2(i)). Analysis of protein kinase activity by SDS/polyacrylamide gel electrophoresis and autoradiography (see Figure 6.4) showed that PEP carboxylase kinase was absent from both puromycin and cycloheximide pretreated leaves. Hence protein synthesis inhibitors prevent the appearence of the protein kinase in 'night' leaves. If *de novo* synthesis of the enzyme is the key regulatory mechanism, then there should be no protein kinase present during the 'day' period. The results in Section 6.2.1 show this to be the case in desalted extracts . In attempts to show the absence of the enzyme from leaves in which the endogenous PEP carboxylase is in the dephosphorylated form, the procedure for the partial purification of the protein kinase was carried out with 'day' leaves and with leaves pretreated with 1 x10^{-3}M puromycin. Some protein kinase activity appeared during the purifications, mainly after the blue dextran-agarose step (Section 2.4.5), as shown in Figures 6.5 and 6.6. The phosphorylation of PEP carboxylase by these protein kinase preparations was low. A tenfold higher specific activity of [^{32}P] ATP was used in the incubations in comparison with incubations carried out with protein kinase purified from 'night' leaves (see Section 4.2.1). Although the stoichiometry of phosphorylation was not determined, these experiments illustrate the presence of the protein kinase in leaves where the PEP carboxylase exhibits a low K_i for malate. These results suggest that the protein kinase may be activated during the dark period or on purification. It is also possible that the PEP carboxylase kinase cannot be detected in desalted 'day' extracts due to interference by other factors which complicate the assay.

6.2.4. Inhibitors and activators of protein kinase activity

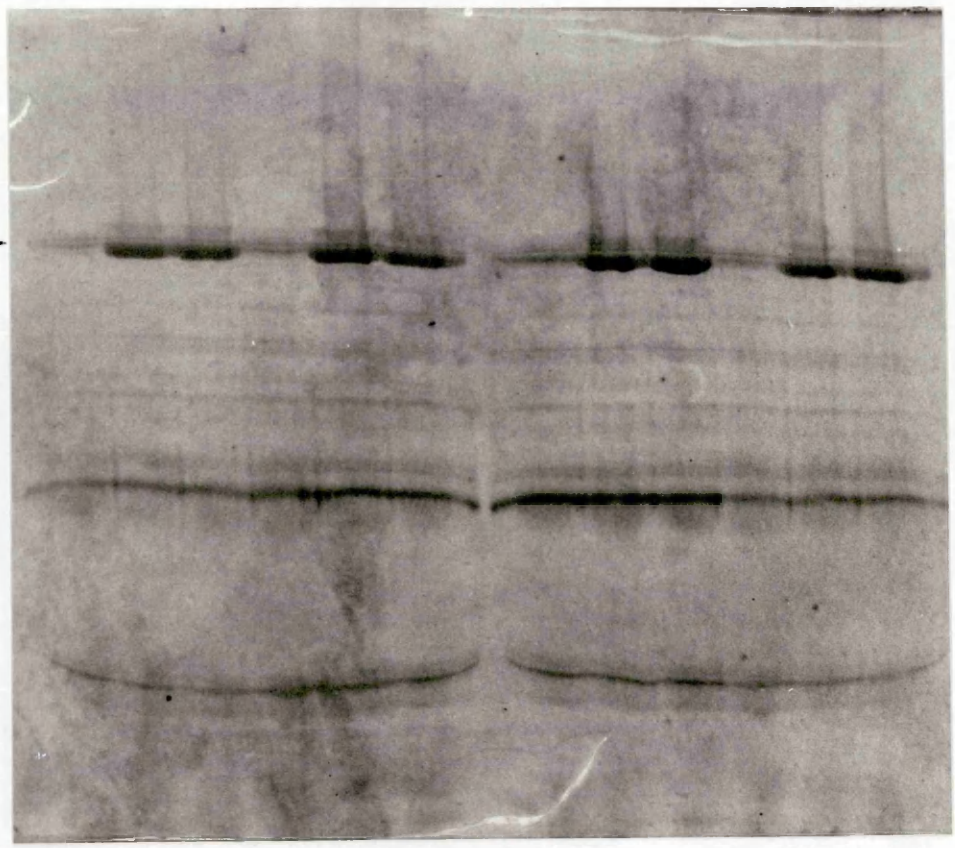
PEP carboxylase kinase may be regulated by activators or inhibitors present in *B. fedtschenkoi* leaves. In an attempt to find evidence for such effectors, desalted extracts of 'day' and 'night' leaves

Table 6.2. **Apparent K_i for malate of PEP carboxylase from *B. fedtschenkoi* leaves pretreated with either cycloheximide or puromycin.** Detached leaves were treated with $1x10^{-3}$ M puromycin or $5x10^{-4}$ M cycloheximide during the 'day' period as described in Section 2.7. Extracts were prepared the following 'night' and desalted into buffer C (Section 2.4.1). The malate sensitivity of the PEP carboxylase was determined. Control leaves were pretreated with either water or ethanol/water (see Section 2.7).

Leaf pretreatment	[Protein] (mg/ml)	PEP carboxylase activity (U/ml)	Apparent K_i for malate (mM)
H_2O	0.45	0.44	3.00
$1x10^{-3}$M puromycin	0.44	0.45	0.38
Ethanol/H_2O (1/1000) dilution	0.56	0.64	3.50
$5x10^{-4}$M cycloheximide	0.40	0.43	0.40

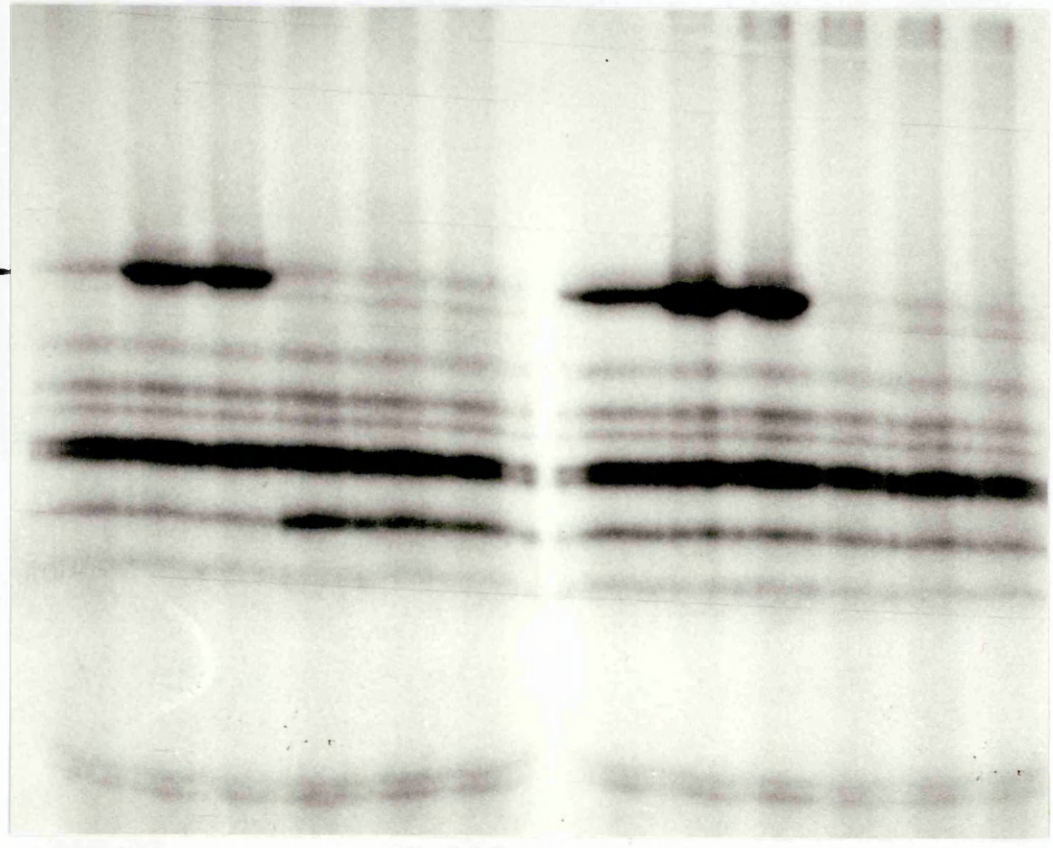
FIGURE 6.4. **Effect of cycloheximide and puromycin on PEP carboxylase kinase activity in *B. fedtschenkoi* leaves.** Detached leaves were allowed to take up solutions of puromycin (1×10^{-3} M) or cycloheximide (5×10^{-4} M) through the transpiration stream as described in Section 2.7. The control leaves were treated with either water or ethanol/water (1/1000). The leaves were extracted during the 'night' period, desalted into buffer C and the PEP carboxylase kinase activity determined by the method described in Section 2.5.2(i). Incubations were carried out in the absence (tracks 1,4,7,10) or presence (tracks 2,3,5,6,8,9,11,12) of purified exogenous 'day form' PEP carboxylase. Denatured samples were separated on an 8% SDS/polyacrylamide gel (a) and autoradiographed (b). Duplicate incubations are shown in tracks 2 and 3; 5 and 6; 8 and 9; 11 and 12. Tracks 1,2,3, control (ethanol/water) leaf extracts; tracks 4,5,6, cycloheximide pretreated leaf extracts; tracks 7,8,9 control (water) leaf extracts; tracks 10,11,12, puromycin pretreated leaf extracts.

(a)



1 2 3 4 5 6 7 8 9 10 11 12

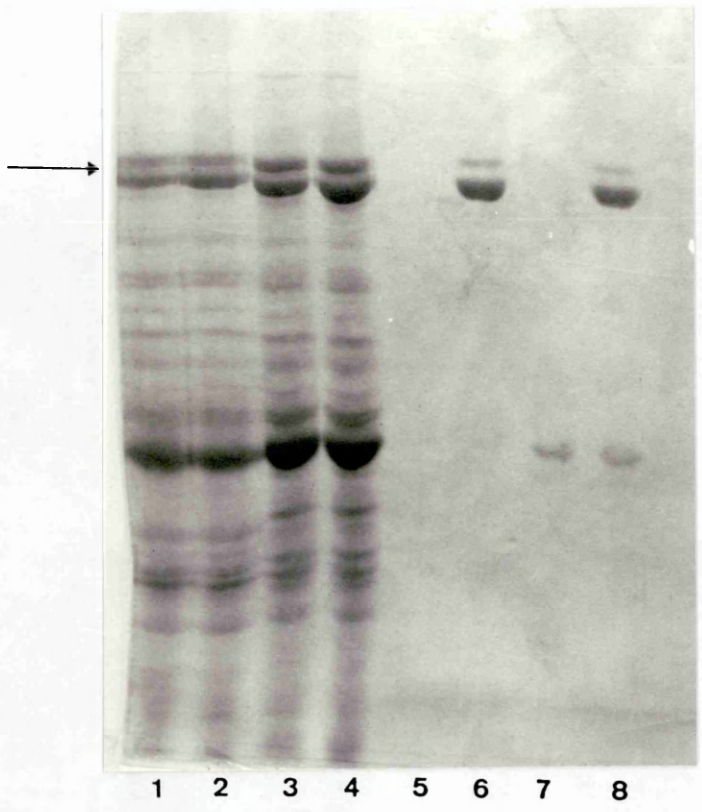
(b)



1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 6.5. Partial purification of PEP carboxylase kinase from 'day' leaves of *B. fedtschenkoi*. PEP carboxylase kinase was partially purified by the method described in Section 2.4.4. Samples from each stage in the purification (17µl or 5µl of the ammonium sulphate fraction) were analysed for protein kinase activity. The incubations were carried out in the absence or presence of purified 'day form' PEP carboxylase using 4µCi/nmol ATP for 30 min as described in Section 2.5.2 (ii). The samples were denatured and separated on an 8% SDS/polyacrylamide gel stained with Coomassie Brilliant Blue (a) and autoradiographed (b). Tracks 1,3,5,7, no exogenous PEP carboxylase added. Tracks 2,4,6,8, exogenous PEP carboxylase added. Tracks 1 and 2, desalted 'day' extract; tracks 3 and 4, desalted 0-50% ammonium sulphate fraction; tracks 5 and 6, desalted blue dextran-agarose pool; tracks 7 and 8, Mono Q pool. The arrow indicates the position of PEP carboxylase.

(a)



(b)

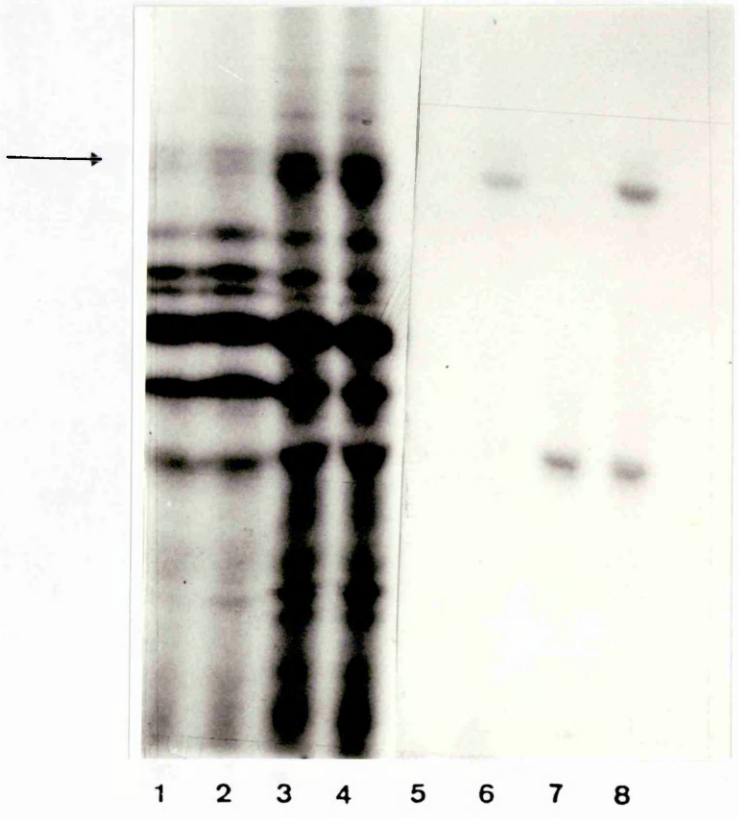
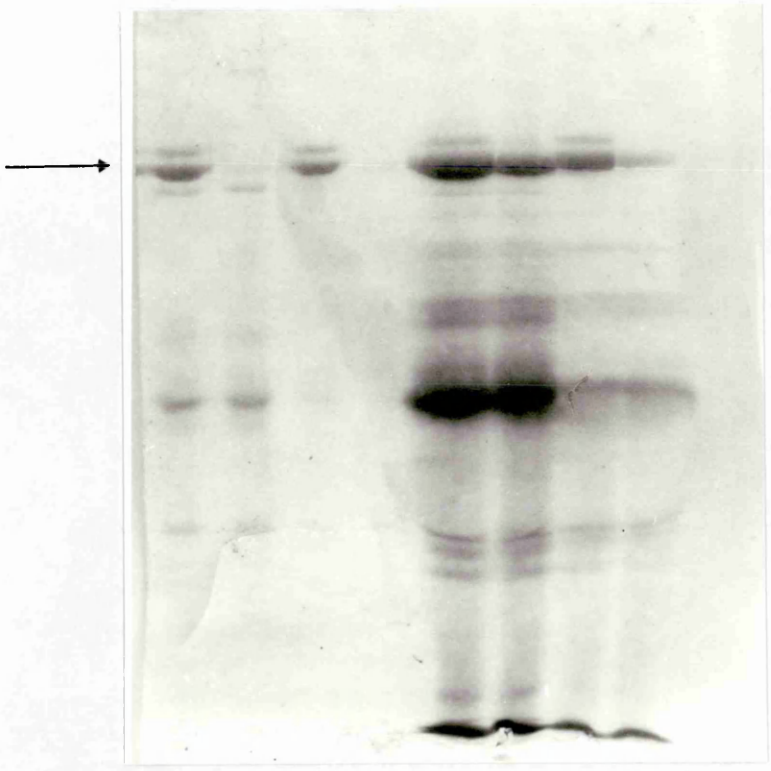
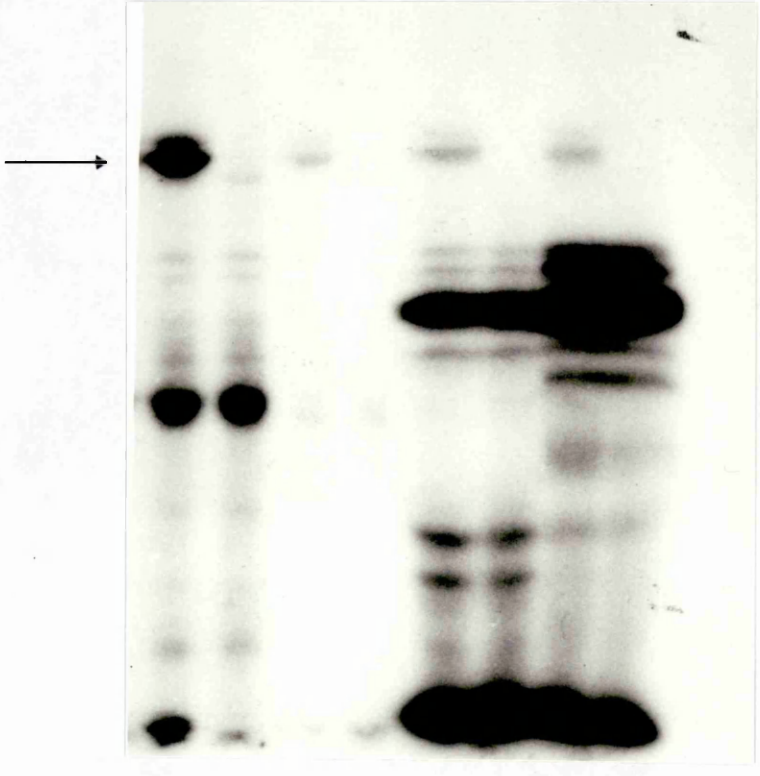


FIGURE 6.6. **Partial purification of PEP carboxylase kinase from 'night' leaves of *B. fedtschenkoi* pretreated with puromycin.** Detached leaves (25g) were allowed to take up a solution of puromycin (1 x10^{-3}M) through the transpiration stream (Section 2.7) . The leaves were extracted and the PEP carboxylase kinase purification procedure was carried out as described in Section 2.4.4. Samples from each stage of the purification (17µl or 5µl of the ammonium sulphate fraction) were analysed for PEP carboxylase kinase activity . The samples were incubated in the absence or presence of purified 'day form' PEP carboxylase with 4µCi/nmol ATP for 30 min as described in Section 2.5.2 (ii). Denatured samples were separated on an 8% SDS/polyacrylamide gel (a) and autoradiographed (b). Tracks 1,3,5,7, no exogenous PEP carboxylase added. Tracks 2,4,6,8, exogenous PEP carboxylase added. Tracks 1 and 2, desalted 'night' extract; tracks 3 and 4, desalted 0-50% ammonium sulphate fraction; tracks 5 and 6, desalted blue dextran-agarose pool; tracks 7 and 8, Mono Q pool. The arrow indicates the position of PEP carboxylase.

(a) 8 7 6 5 4 3 2 1



(b) 8 7 6 5 4 3 2 1



were mixed and the protein kinase activity in the mixtures determined (see Section 2.5.2 (iii)). Initial incubations contained 0.02mg of desalted 'night' extract/ml and between 0.02-0.18mg of desalted 'day' extract/ml. The results (Figure 6.7) show that as the 'day'/'night' ratio of the mixtures increases the PEP carboxylase kinase activity decreases. This observation suggests that the 'day' extracts contain an inhibitory substance.

Experiments using partially purified protein kinase and desalted 'day' extract were carried out. The results (Figure 6.8) indicate that the inhibitory material can also affect the partially purified protein kinase activity. In addition, the kinase activity was partially inhibited by the material present in an 0-85% ammonium sulphate precipitation from a 'day' extract. The inhibitory effect was almost completely removed from the desalted extract by boiling the sample for 2min prior to incubation. It is likely therefore that the inhibitor is a protein. Further experiments in which 'night' extracts prepared from leaves pretreated with 5 $x10^{-4}$M cycloheximide were incubated with partially purified protein kinase also revealed inhibition of PEP carboxylase kinase activity (Figure 6.9).

Compounds that activate PEP carboxylase kinase may also be present in mesophyll cells of *B. fedtschenkoi* , especially during the period of CO_2 fixation. However preliminary experiments showed no significant stimulation of partially purified PEP carboxylase kinase activity by incubation with desalted 'night' extracts (not shown).

Attempts to inactivate the partially purified protein kinase were made using protein phosphatases. The aim of the work was to investigate whether the protein kinase was activated during the 'night' via phosphorylation. The partially purified kinase (100µl)was preincubated in the presence of 3mU/ml of mammalian type 1 or type 2A protein phosphatase for 30min at 30°C . The phosphatase activity was inhibited by the addition of 10µM okadaic acid . The pretreated protein kinase was then assayed for activity as described in Section 2.5.2(ii). Treatment of the PEP carboxylase kinase with either protein phosphatase did not affect its activity.

6.3. Discussion

This work presents evidence for the involvement of protein synthesis in the regulation of

FIGURE 6.7. Effect of 'day' extracts on the PEP carboxylase kinase activity in 'night' extracts of *B. fedtschenkoi* leaves. Extracts from darkened and illuminated leaves of *B. fedtschenkoi* were prepared and desalted as described in Section 2.5.2 (i). 'Night' extract (0.5μg) was preincubated with increasing concentrations of 'day' extract (0.5μg, 1.0μg, 3.0μg, 3.5μg and 4.5μg) as described in Section 2.5.2(iii). The protein kinase activity was then determined by incubating in the absence or presence of purified PEP carboxylase (0.03units) and 4μCi/nmol ATP for 10min at 30°C (Section 2.5.2(i)). Denatured samples were separated on an SDS/polyacrylamide gel (a) stained with Coomassie Brilliant Blue and autoradiographed (b). Tracks 1,3,5,7,9,11,13, no exogenous PEP carboxylase added. Tracks 2,4,6,8,10,12,14, exogenous PEP carboxylase added. All incubations contained 0.5μg of 'night' extract, the 'day' extract was added as follows; tracks 1 and 2, no 'day' extract added; tracks 3 and 4, 0.5μg added ; tracks 5 and 6, 1.0μg added; tracks 7 and 8, 2.0μg added; tracks 9 and 10, 3.0μg added ; tracks 11 and 12, 3.5μg added; tracks 13 and 14, 4.5μg added.

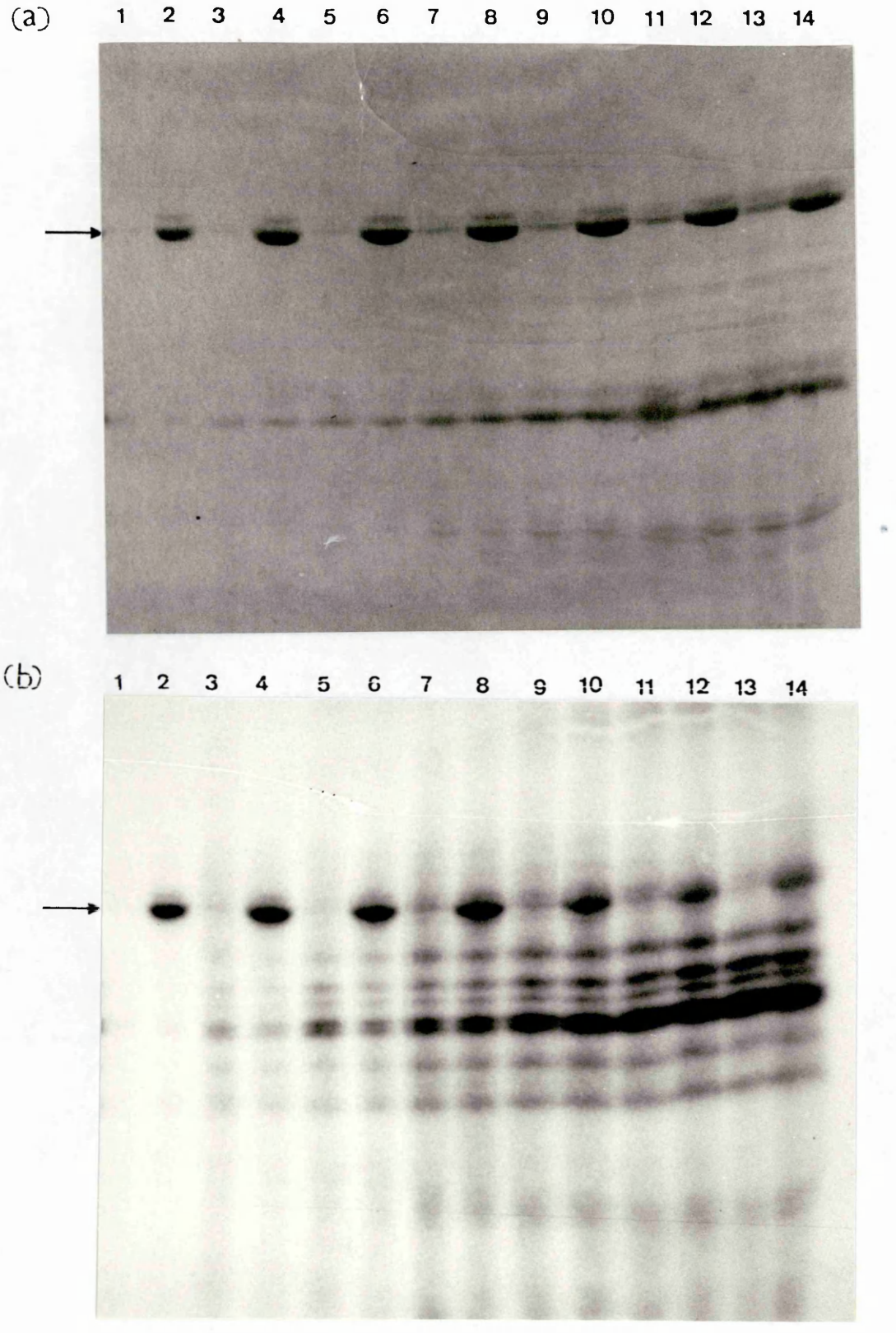

(a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
(b)
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIGURE 6.8. Effect of 'day' extracts on PEP carboxylase kinase activity. Leaves (30g) from illuminated *B. fedtschenkoi* plants were extracted and desalted as described in Section 2.5.2 (i). The extract was brought to 85% saturation with ammonium sulphate and the precipitate resuspended in 5ml of buffer B (Section 2.4.1). The effect of desalted 'day' extract (5µg and 7.5µg), boiled 'day' extract (equivalent volume to 7.5µg) and the desalted 0-85% ammonium sulphate fraction (10µg) was determined, on the partially purified PEP carboxylase kinase activity. The samples were pre-incubated with protein kinase (8.0µl), as described in Section 2.5.3(iii). The protein kinase activity was then determined by incubating in the absence or presence of purified 'day form' PEP carboxylase (0.03 units) and 4µCi/nmol ATP as described in Section 2.5.2 (i). Denatured samples were separated on an SDS/polyacrylamide gel (a) and autoradiographed (b). Tracks 1,6, no exogenous PEP carboxylase added. Tracks 2,3,4,5,7, exogenous PEP carboxylase added. Additions to the partially purified kinase incubation mixture were as follows; tracks 1 and 2, no additions; track 3, deaslted 'day' extract (5µg) added; track 4, desalted 'day' extract (7.5µg) added; track 5, boiled day extract added; tracks 6 and 7, desalted 0-85% ammonium sulphate fraction added. The arrow indicates the position of PEP carboxylase.

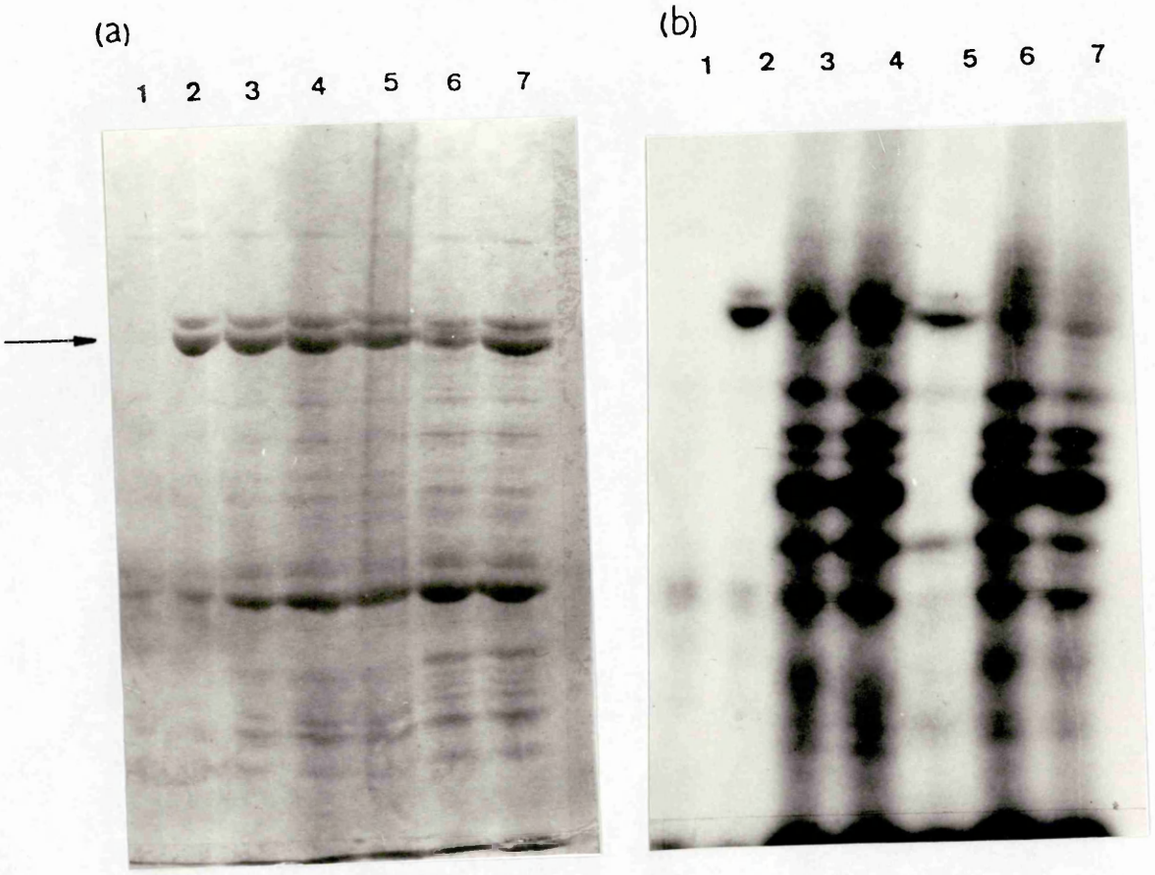
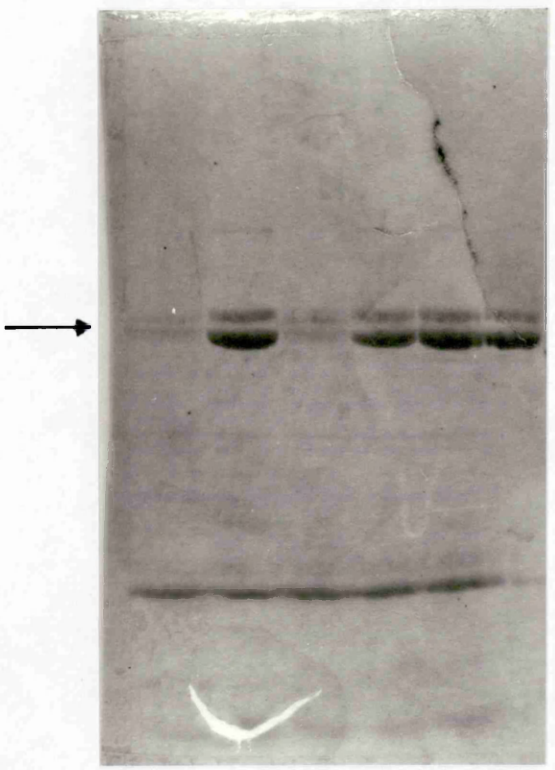

(a)
1 2 3 4 5 6 7
(b)
1 2 3 4 5 6 7

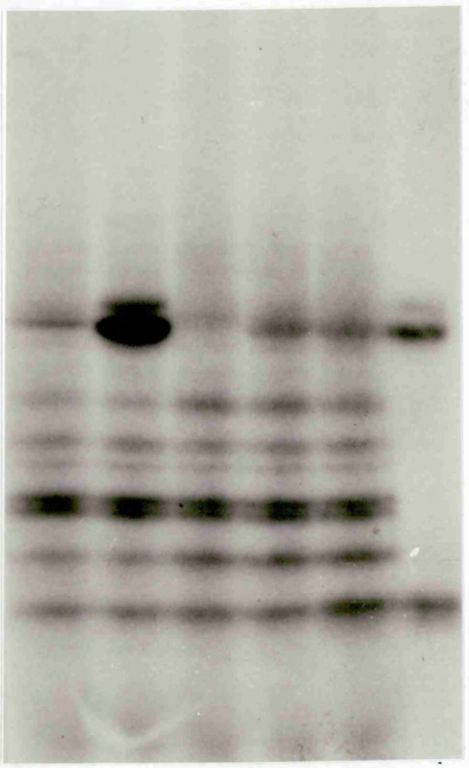
FIGURE 6.9. **Effect of cycloheximide pretreated leaf extracts on PEP carboxylase kinase activity.** Detached leaves were allowed to take up a solution of cycloheximide (5 x10^{-4}M) through the transpiration stream (Section 2.7). Treated and untreated leaves were extracted during the 'night' period and desalted into buffer C (Section 2.4.1). The PEP carboxylase kinase activity in the desalted extracts was determined by incubating in the absence or presence of purified 'day form' PEP carboxylase (Section 2.5.2(i)). The effect of the cycloheximide pretreated leaf extract (5µg) on the partially purified protein kinase activity (8.0µl) was then determined by pre-incubating the samples as described in Section 2.5.3(iii). The protein kinase activity was assayed by incubating with purified PEP carboxylase and 4µCi/nmol ATP for 10 min as described in Section 2.5.2 (ii). Tracks 1 and 2, untreated 'night' extract (5µg) incubated in the absence and presence of exogenous PEP carboxylase; tracks 3 and 4, cycloheximide pretreated 'night' extract (5µg) incubated in the absence and presence of exogenous PEP carboxylase; track 5, cycloheximide pretreated extract (5µg) incubated with the partially purified protein kinase in the presence of exogenous PEP carboxylase; track 6, partially purified protein kinase incubated in the presence of exogenous PEP carboxylase. The arrow indicates PEP carboxylase.

(a)



1 2 3 4 5 6

(b)



1 2 3 4 5 6

CAM. It also illustrates the existence of an inhibitor of kinase activity in *B. fedtschenkoi* leaves. This inhibitor appeaars to be present in leaves extracted during the 'day' or in 'night' leaves which were pretreated with protein synthesis inhibitors. Whether inhibition of protein synthesis has a direct effect on the inhibitor is unknown. The two observations may represent independent control mechanisms.

The regulation of PEP carboxylase is likely to be achieved at the level of the protein kinase and not via the protein phosphatase. This is suggested by the fact that protein phosphatases tend to have very broad substrate specifities in comparison to protein kinases (Cohen, 1989). It is also indicated by the variation in kinase activity that occurs over the diurnal cycle. The fact that some protein kinase could be partially purified from leaves in which the endogenous PEP carboxylase was in the dephosphorylated form (apparent K_i for malate of 0.3mM) indicates the complexity of the control mechanism. Two fundamentally different explainations are proposed to account for the various observations made in this work.

1. The PEP carboxylase kinase protein is synthesised *de novo* during the 'night'. In addition, a specific inhibitor of the protein kinase is present throughout the diurnal cycle. This inhibitor will remove any basal levels of protein kinase present during the 'day' ; but is insufficient to inhibit newly synthesised enzyme at 'night'. This interpretation may explain why a high concentration of 'day' extract is required to achieve significant inhibition of the PEP carboxylase kinase.

2. The PEP carboxylase kinase protein is present throughout the diurnal cycle, but its activity is controlled in a periodic manner by effector compounds. This may be achieved by:

(i) A tight-binding inhibitor, which is present throughout the 'day' period but is subsequently destroyed or inactivated during the 'night'. Removal of the inhibitor may be accomplished by the synthesis of a specific protease or regulatory enzyme. The appearance of some protein kinase activity during purification from 'day' leaves may be due to partial dissociation of this inhibitor.

(ii) A tight-binding activator, which is synthesised or activated during the 'night' period. Leaves extracted during the 'day' may contain a compound which antagonises this activator and hence removes protein kinase activity.

(iii) Covalent modification; the PEP carboxylase kinase may itself be regulated by a reversible phosphorylation.

PEP carboxylase kinase is clearly a highly regulated enzyme. It is evident from the proposed mechanisms that the concentration or specific activity of the effector compounds that prevail *in vivo* at any given time may be key regulatory factors in maintaining the CAM cycle. Cytoplasmic metabolites may also exhert a short-term control on the protein phosphatase type 2A activity, thereby controlling the phosphorylation state of PEP carboxylase.

Results obtained using protein synthesis inhibitors should be interpreted with great care (McMahon, 1975). Puromycin and cycloheximide were chosen in this study because they have very different structures and mechanisms of action. Puromycin inhibits protein synthesis by causing premature termination of nasent polypeptide chains. Cycloheximide appears to inhibit protein synthesis by decreasing the activity of transfer factor II, an enzyme that catalyses ribosomal translocation along mRNA. It is likely that the dissimilarity in the structure of these two drugs would reduce the possibility of their causing similar side-effects. Small doses of cycloheximide given in pulses induced phase-shifts in circadian rhythms in *Gonyaulax* (Walz and Sweeney, 1979), *Acetbularia* (Karakashin and Schweiger, 1976) and *Neurospora* (Nakashima *et al.*, 1981). In addition, both cycloheximide and puromycin caused a phase-shift in the circadian rhythm of neuronal activity in *Aplysia* (Rothman and Strumwasser, 1976). Two mutants of *Neurospora* whose 80S ribosomes showed cycloheximide-resistance were isolated (Pongratz and Klingmuller, 1973). These mutants displayed a normal or wild-type circadian rhythm which could not be perturbed (phase-shifted) by cycloheximide (Nakashima *et al.*, 1981). This result indicates that the target of cycloheximide is indeed 80S ribosomes and not some unknown alternative cellular target. The biochemical effects of cycloheximide and puromycin on *B. fedtschenkoi* are unknown, apart from the fact that they both inhibit the rhythm of CO_2 fixation. Thses drugs do not appear to significantly alter the leaf cell metabolism even after 3 days exposure. This is implied by the fact that the leaves are capable of photosynthesis upon illumination (see Figures 6.2 and 6.3). It is also evident from Figure 6.1b that cycloheximide and puromycin do not alter the phosphorylation of proteins other than PEP carboxylase. However, an additional phosphorylated protein (M_r approx. 50,000) was observed

in extracts of leaves that had been pretreated with cycloheximide. This phosphorylation was not observed in either puromycin pretreated leaf extracts or control leaf extracts (Figure 6.1b). In conclusion, the results presented in this study indicate control of the circadian rhythm of CO_2 fixation in *B. fedtschenkoi* is mediated at the level of protein synthesis or gene expression.

Chapter 7

General Discussion

Control of CAM is at least in part mediated by the reversible phosphorylation of PEP carboxylase. The work presented in this thesis highlights the fact that the phosphorylation state of PEP carboxylase determines its sensitivity to inhibition by malate, and thereby presumably establishes the relative activity of the enzyme *in vivo*. The characteristic circadian rhythm of CO_2-fixation in *B. fed--tschenkoi* which arises as a result of periodic PEP carboxylase activity (Nimmo *et al.*, 1984, 1987), is likely therefore to be maintained through regulation of the protein kinase and protein phosphatase responsible for the covalent modification of PEP carboxylase. Preliminary characterization of these regulatory enzymes has been achieved in this study (Carter *et al.*, 1990). In addition, attempts to understand the regulation of the protein kinase and protein phosphatase themselves, were undertaken with a view to identifying the basic oscillator of the circadian rhythm of CO_2-fixation (Carter *et al.*, 1991).

The protein kinase isolated in this work, phosphorylated PEP carboxylase at the same site(s) that are phosphorylated *in vivo* (Figure 4.10). The sequence of the phospho-peptide was not determined, but is likely to be equivalent to the Ser-15 phosphorylation site of maize PEP carboxylase (Jiao and Chollet, 1990). Phosphorylation of this site by either a soluble maize protein kinase (Jiao and Chollet, 1989) or the catalytic subunit of mammalian protein kinase A (Terada *et al.*, 1990) caused a change in the allosteric properties of the enzyme, as was observed with phosphorylation of the *B. fedtschenkoi* PEP carboxylase (see Figure 4.7). Comparison of the CAM (*Mesembryanthemum crystallinum*) and C_4 (maize) PEP carboxylase gene sequences, suggest that it is the Ser-11 residue of the CAM enzyme that is covalently modified (Jiao and Chollet, 1990).

Attempts to modify PEP carboxylase kinase activity *in vitro* indicated that the enzyme from *B. fedtschenkoi* was insensitive to Ca^{2+} (Table 4.1). Partially purified maize PEP carboxylase kinase was also shown to be insensitive to Ca^{2+}/calmodulin (McNaughton *et al.*, 1991; Chollet, 1990). The effects of diacylglycerol and phospholipids on the protein kinase have not been investigated (see Introduction) although there is increasing evidence of their involvement in stimulating plant protein

kinases (Martiny-Baron and Scherer, 1989; Morre, 1990). Although other factors which affect the phosphorylation of PEP carboxylase by the partially purified protein kinase have been demonstrated *in vitro*, (for example malate, glucose 6-phosphate and the inhibitor present in 'day' extracts), the PEP carboxylase kinase remains to be characterized. One possible way of attempting to define the protein kinase would be to identify the site(s) it phosphorylates on mammalian glycogen synthase. This substrate can be phosphorylated *in vitro* at multiple sites by a number of different types of protein kinases (Cohen, 1988) and is a good substrate for the plant protein kinase isolated in this work (Figure 4.3). Further analysis of this phosphorylation was not carried out as it was likely that the partially purified protein kinase pool contained more than one protein kinase activity. The protein kinase which phosphorylates PEP carboxylase in *B. fedtschenkoi* may also have other physiological substrates. However, the transient presence of the protein kinase activity corresponds with the 8-10h period in which PEP carboxylase is phosphorylated (Figure 6.1), suggesting that it is a highly specific protein kinase. A prerequsite to further characterization of the PEP carboxylase will be isolation of homogenous enzyme.

An observation of major significance in this study was the diurnal variation in PEP carboxylase kinase activity in desalted extracts (Figure 6.1). The type 2A protein phosphatase activity measured using casein as a substrate did not fluctuate over the 24h period tested. This indicates the regulation of phosphorylation of PEP carboxylase occurs predominantly via the protein kinase. The activity of the protein phosphatase *in vivo* may however be modulated by various metabolic effectors. Such putative effectors could not be identified in this study, as only the catalytic subunit of the enzyme was isolated. Regulation of the protein kinase activity reflects the signal cascade mechanisms found in mammalian cells in response to hormones (Nimmo and Cohen, 1977). The question therefore arises as to what signal(s) control the plant protein kinase activity? In view of the fact that some of the more complex mammalian cascade systems involve covalent modification of protein kinases, such as glycogen phosphorylase kinase , it is possible that the plant protein kinase is also regulated in a similar manner. Attempts to dephosphorylate PEP carboxylase kinase (using mammalian protein phosphatases), however, gave no indication of such an event (see page 95). These preliminary studies require a more detailed analysis before the idea of a covlaently modified kinase can be ruled out.

The presence of an apparent inhibitor of PEP carboxylase kinase in leaf extracts deserves further investigation. This inhibitor was found in *B. fedtschenkoi* leaves which contained no PEP carboxylase kinase activity. The fact that high concentrations of leaf extracts were required to inhibit the protein kinase activity (Figure 6.7) suggests that either the inhibitor is only present in low abundance or that it is already tightly bound to another compound such as PEP carboxylase kinase. This inhibitor may be just one of several regulatory mechanisms controlling PEP carboxylase kinase activity. The regulation of RuBP carboxylase which has proven to be quite complex, is now known to involve among other things, a tight-binding inhibitor (Seravites, 1985,1990). This inhibitor is in fact a transition state analogue (carboxyarabinitol 1-phosphate) as opposed to a binding protein. The *B. fedtschenkoi* protein kinase inhibitor may, however, be a protein, judging by the fact that it was precipitated by ammonium sulphate (see Figure 6.8), and could prove analogous to the cAMP-dependent protein kinase inhibitor isolated from mammalian tissues (Demaille *et al.*, 1977; Glass *et al.*, 1989). The function of this mammalian protein kinase inhibitor remains unclear. It was shown to be present in very low concentrations but exhibits high affinity for the catalytic subunit of cAMP-dependent protein kinases, having K_i values in the nM range. The protein kinase inhibitor found in *B. fedtschenkoi* could be a protease. This protease may be synthesised towards the end of the 'night' period, when the protein kinase activity is known to decrease. Future studies should aim to determine whether the *in vitro* 'inactivation' of the protein kinase by the protein kinase inhibitor is time dependent.

The physiology of the circadian rhythm of CO_2-fixation had been investigated in some detail prior to the present studies (Wilkins, 1973, 1983, 1984; Anderson and Wilkins, 1987, 1989a, 1989b). Wilkins proposed an hypothesis for the generation of this rhythm based on the ability of the rhythm to be reset (phase-shifted) by various enviromental stimuli (see Introduction). This hypothesis involves the periodic inhibition of PEP carboxylase activity thereby giving rise to periods of CO_2-fixation and CO_2-output. This CO_2 rhythm is proposed to be a direct result of the accumulation and removal of malate in the cytoplasm, or more precisely in the micro-enviroment of the PEP carboxylase enzyme. PEP carboxylase fixes CO_2 with the subsequent accumulation of malate, to an extent whereby it

allosterically inhibits the enzyme. Further CO_2-fixation could therefore only be achieved by decreasing the malate content of the cytoplasm. Malate can be removed by different mechanisms depending on the enviromental conditions. Wilkins (1984) proposed that in continuous darkness and CO_2-free air the cytoplasmic malate is pumped into the vacuole. The CO_2-rhythm observed under these conditions persists for only 3 to 4 days after which time the vacuole is thought to be saturated with malate, hence the PEP carboxylase activity is permanently inhibited and the rhythm disappears. The rhythm of CO_2-fixation generated in continuous light and normal air is more complex in that it also involves CO_2-fixation by RuBP carboxylase. The malate which accumulates in the cytoplasm under these conditions may diffuse into the vacuole , but can also be metabolized by malic enzyme. The metabolism of malate thereby allows the rhythm of CO_2-fixation to persist for up to 12 days (Wilkins, 1984). The balance between the rate of malate synthesis and breakdown is no doubt significant in maintaining this latter rhythm and indeed may have an effect on the dark CO_2-rhythm (Anderson, 1987). Despite the importance of malate metabolism in CAM plants, studies in this area have largely been neglected. Although analysis of the total malate concentrations in leaves could in theory provide information on the PEP carboxylase activity, such measurements have often proved uninformative, due to the variability in malate content between different leaves (Nimmo *et al.*, 1987; Anderson and Wilkins, 1989a, 1989b). Conclusive proof of the validity of the hypothesis proposed by Wilkins (1983, 1984) requires the determination of malate levels in the different cellular compartments, especially in the vacuole. The vacuoles occupy a large volume of the mesophyll cells in CAM plants, which make it difficult to measure cytoplasmic metabolite concentrations. The vacuoles are known to act as 'malate-sinks' in CAM (Kenyon *et al.*, 1978; Buser-Suter *et al.*, 1982), but the extent to which they remove malate from the cytoplasm during the diurnal cycle is unknown.

Future work on the rhythms of CO_2-fixation in *B. fedtschenkoi* should be directed at analysis of the effects of phase-shifts on the phosphorylation state of PEP carboxylase. It is clear that phase-shifts alter PEP carboxylase activity (perhaps by malate inhibition); but does this cause changes in the protein kinase and protein phsophatase activities? Nimmo *et al.* (1987) studied the phosphorylation state of

PEP carboxylase *in vivo*, under constant enviromental conditions. However, these authors found no correlation between the circadian rhythm of CO_2-uptake in continuous light and of interconversion between the two forms of PEP carboxylase. In addition, when the constant conditions were started at the end of the 'night' period, no interconversion of PEP carboxylase was observed even though a persistent rhythm of CO_2-uptake was apparent. Since PEP carboxylase kinase and phosphatase can now be assayed relatively easily in leaf extracts it is important to determine whether these activities oscillate under constant enviromental conditions. It is possible that the protein kinase activity may not show as good an oscillation as was observed under a normal diurnal cycle (Figures 6.1a and 6.1b). Expression of the chlorophyll *a/b* binding protein (*Cab*) gene, which is known to be under circadian control (Meyer *et al.*, 1989; Fejes *et al.*, 1990) was shown to oscillate under constant enviromental conditions, albeit to a lesser extent. The effects of light and temperature treatments on the PEP carboxylase kinase and phosphatase activities in *B. fedtschenkoi* leaves kept in constant enviromental conditions may also provide an insight into the factors which regulate these enzymes. Apart from a possible endogenous control of the protein kinase, light may also affect the balance of the protein kinase and protein phosphatase activities. Wilkins (1973, 1984) provided evidence for the involvement of phytochrome in altering the CO_2-fixation rhythm. It is unlikely that phytochrome has a direct effect on the PEP carboxylase kinase, since its activity was shown to appear and disappear within the 16h dark period (see Figure 6.1). However light may alter the protein kinase activity indirectly by modifying the putative tight-binding effectors discussed in Section 6.3

In no case has PEP carboxylase kinase activity been found in desalted leaf extracts while the endogenous PEP carboxylase is in the dephosphorylated state (low K_i form), although some kinase activity has been partially purified from such leaves (Figures 6.5 and 6.6). The diurnal changes in the malate sensitivity of PEP carboxylase correlate with the presence or absence of the protein kinase (see Table 6.1 and Figure 6.1b). The factors which cause the PEP carboxylase kinase activity to diminish 2-3h before the end of the 'night' period are unknown. A high malate concentration in the cytoplasm will inhibit PEP carboxylase activity, but may also establish a metabolic state in the cell which favours protein phosphatase activity over protein kinase activity. Acidification of the cytoplasm may underlie

such an event.

Protein synthesis inhibitors were shown to block the nocturnal appearance of PEP carboxylase kinase under the normal diurnal cycle and inhibit the circadian rhythm of CO_2-fixation in continuous darkness and CO_2-free air (Figures 6.6 and 6.7). Interpretation of these results is complex and has been discussed in Chapter 6. However the results suggest that control of the CO_2-rhythm is influenced by protein synthesis of some essential component. Earlier interpretation of the inhibitory effects of cycloheximide on the CO_2-rhythm suggest it is caused by malate leakage from the vacuole into the cytoplasm (Bollig and Wilkins, 1979). This hypothesis is based on the notion of 'gated' channels in the tonoplast. These 'gates' are thought to be light and temperature sensitive (Wilkins, 1983, 1984). Hence treatment of leaves with cycloheximide under continuous light and normal air at 15°C should have no apparent effect on the CO_2-rhythm (as the tonoplast 'gates' will remain open in continuous light thereby allowing malate to distribute freely between the cytoplasm and vacuole). However preliminary studies by Armstrong and Wilkins (personal communication) showed that cycloheximide does inhibit this CO_2-rhythm. Therefore cycloheximide must act in another way in addition to any affect it may have on ion transport across the tonoplast. It is possible that cycloheximide alters the ion distribution in the stomatal guard cells, thereby causing them to close and thus prevent further CO_2-fixation. The role of stomata in generating the circadian rhythm of CO_2-output is not understood but is evidently important. Wilkins (1991) demonstrated that the removal of the epidermis from leaves maintained in continuous light and normal air at 15°C abolished the rhythm of CO_2-fixation, but had no effect on the CO_2-rhythm of leaves maintained in continuous darkness and CO_2-free air. By analogy with the cycloheximide and puromycin effects on the CO_2-rhythm observed in this thesis (continuous darkness and CO_2-free air at 15°C), it is increasingly likely that the rhythm of CO_2-output in continuous light is also maintained by synthesis of a key regulatory protein.

The idea of circadian control of protein synthesis has become evident in recent years (Morse *et al.*, 1990; Fejes *et al.*, 1990). *Gonyaulax*, an unicellular marine alga displays a circadian rhythm of

bioluminescence with a period of about 23h (Njus *et al.*, 1981). This luminescence is a result of the oxidation of luciferin. The rhythm of luminescence arises partly as a result of translational control of a luciferin binding-protein (LBP) mRNA (Morse *et al.*, 1989). The mechanism which causes the periodic translation of LBP is unknown but is thought to involve *trans*-acting cytoplasmic factors. Circadian oscillations in gene transcription have also been observed. The light harvesting chlorophyll *a/b* protein gene (*cab*) (Njus *et al.*, 1990) and the period (*per*) gene which controls eclosion and adult locomotor activity in *Drosophila* (Hardin *et al.*, 1990), are expressed in a rhythmic manner. Njus *et al.* (1990) have investigated the *cis* -acting sequences required for circadian rhythm-regulated expression of the *cab* gene. Since it has been established that PEP carboxylase kinase activity oscillates *in vivo* over the diurnal cycle, it is necessary to determine unambiguously if this is due to protein synthesis *per se*. If this proves to be the case then further analysis of transcriptional or translational control can be undertaken.

In summary, these studies on the regulation of PEP carboxylase are not only relevant to understanding CO_2-assimilation in CAM plants, but are equally relevant in that PEP carboxylase is a key enzyme involved in the generation of a unique circadian rhythm. The work described in this thesis, together with earlier work on the physiology of the CO_2-fixation rhythm in *B. fedtschenkoi*, provide a preliminary insight into the biochemical basis of the circadian rhythm. With the continuation of these studies and by comparison with other endogenous rhythms, an understanding of how biological systems measure time could be achieved.

Chapter 8

REFERENCES

Anderson, C.M. and Wilkins, M.B. (1989a) Planta **177**, 401-469

Anderson, C.M. and Wilkins, M.B. (1989b) Planta **177**, 456-469

Anderson, C.M. (1989) PhD Thesis, Botany Department, University of Glasgow

Andreo, C.S., Gonzalez, D.H. and Iglesias, A.A. (1987) FEBS Lett. **213**, 1-8

Balaga, B.S., Pronczuk, A.W. and Munro, H.N. (1969) J. Biol. Chem. **244**, 4480-4489

Ballou, L.M. and Fischer, E.H. (1986) The Enzymes **XVII** , 311-394 Boyer, P.D. and Krebs, E.G. ed., Academic Press, Inc.

Bennet, J. (1980) Eur. J. Biochem. **104**, 85-89

Bennet, J. (1984) Mol. Asp. Cell Reg. **3**, 227-246, Elsevier Biomedical Press Amsterdam.

Bennet, J. (1986) Eur. J. Biochem. **104**, 85-89

Berger, S., Groot, E.J., Neuhaus, G. and Schweiger, M. (1987) Eur. J. Cell Biol. **44**, 349-370

Black, C.C. (1973) Annu. Rev. Plant Physiol. **32**, 253-286

Bohnert, H.J., Ostrem, J.A., Cushman, J.C., Michalowski, C.B., Rickers, J., Meyer, G., deRocher, E.J., Vernon, D.M., Krueger, M., Velten, J., Hoefner, R . and Schmitt, J.M. Plant Mol. Biol. Reporter **6**, 10-28

Bollig, I.C. and Wilkins, M.B. (1979) Planta **145**, 105-112

Bowes, G., Ogren,L.W. and Hagemann, R.H. (1971) Biochem. Biophys. Res. Commun. **45**, 716-722

Boyer, P.D. and Krebs, E.G. (1986) The Enzymes **XVIII**, 3rd ed. Academic Press, Inc.

Braam, J. and Davis. R. (1990) Curr. Top. Plant Biochem. Physiol. **9**, 85-100

Bradford, M.M. (1976) Anal. Biochem. **72**, 248-254

Brandon, P.C. (1967) Plant Physiol. **42**, 977-984

Brulfert. J., Guerrier, D. and Queiroz, O. (1973) Plant Physiol. **51**, 202-222

Brulfert, J., Vidal, J., Le Marechal, P., Gadal, P., Queiroz, O., Kluge, M. and Kruger, I. (1986) Biochem. Biophys. Res. Commun. **136**, 151-159

Buchanan-Bollig, I.C. (1984) Planta **160**, 264-271

Buchanan-Bollig, I.C., Fischer, A. and Kluge, M. (1984) Planta **161**, 71-80

Buchanan-Bollig, I.C. and Smith, J.A.C. (1984) Planta **161**, 314-319

Budde, R.J.A. and Chollet, R. (1986) Plant Physiol. **82**, 1107-1114

Bunning, E (1958) Nature, **181**, 1169-1171

Bunning, E. and Moser, I. (1972) Proc. Nat. Acad. Sci. USA. **69**, 2732-2736

Bunsow, R. (1953) Planta (Berl.) **42**, 220-252

Burnell, J.N. and Hatch, M.D. (1984) Arch. Biochem. Biophys. **237**, 490-503

Burnell, J.N. and Hatch, M.D. (1985) Trends in Biochem. Sci. **115**, 288-291

Buser-Suter, C., Wiemken, C. and Matile, P. (1982) Plant Physiol. **69**, 456-459

Carter, P.J., Nimmo, H.G., Fewson, C.A. and Wilkins, M.B. (1990) FEBS Lett. **263**, 233-236

Carter, P.J., Nimmo, H.G., Fewson, C.A. and Wilkins, M.B. (1991) EMBO J. (in press)

Chollet, R., Budde, R.J.A., Jiao, J-A. and Roeske, C.A. (1989)Physiol. Plant. **76**, suppl., 35

Chollet, R. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 344-356

Cleveland, D.W., Fischer, S.G. and Laemmli, U.K. (1977) J. Biol. Chem. **252**, 1102-1106

Cockburn, W. and McAulay, A. (1977) Plant Physiol. **59**, 455-458

Cohen, P. (1988) Mol. Asp. Cell Reg., **5**, 123-193

Cohen, P. (1989) Annu. Rev. Biochem. **58**, 453-508

Cohen, P., Klummp, S. and Schelling, D.L. (1989) FEBS Lett. **250**, 596-600

Cohen, P., Schelling, D.L. and Stark, M.J.R. (1989) FEBS Lett. **250**, 601-606

Cohen, P., Alemay, S., Hemmings B.A., Resink, T.J., Stralfors, P and Tung, H.Y.L. (1988a) Meths in Enzymol. **159**, 390-409

Cohen, P., Gordon, J.F., Holmes, F.B., Nimmo, G.A. and Tonks, N.K. (1988b) Meths in Enzymol. **159**, 427-437

Cohen, P., Holmes, C.F.B. and Tsukitani, Y. (1990) Trends in Biochem. Sci. **15**, 98-102

Coombs, J., Maw, S.L. and Baldry, C.W.(1975) Planta, **117**, 279-292

Cushman, J.C., Michalowski, C.B. and Bohnert, H.J. (1990) Plant Physiol. **94**, 1137-1142

Demaille, J.G., Peters, K.A. and Fischer, E.H. (1977) Biochemistry **16**, 3080-3086

Dittrich, P. (1975) Plant Physiol. **57**, 310-314

Doncaster, H.D. and Leegood, R.C. (1990) J. Plant Physiol. **136**, 330-335

Echevarria, C., Vidal, J., LeMarechal, P., Brulfert, J., Ranjeva, R. and Gadal, P. (1988) Biochem. Biophys. Res. Commun. **155**, 853-840

Echevarria, C., Vidal, J., Jiao, J-A. and Chollet, R. (1990) FEBS Lett. **275**, 25-28

Edwards, G.E. and Huber. S.C. (1981) The Biochemistry of Plants. A comprehensive treatise, 8, Hatch, M.D. and Boardman, N.K., ed., Academic Press, New York

Edwards, G.E., Nakamoto, H., Burnell, J.N. and Hatch, M.D. (1985) Annu. Rev. Plant Physiol. **36**, 255-286

Elliot, D.C. and Brennan, P.A. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 383-397

Ellis, R.J. and MacDonald, I.R. (1970) Plant Physiol. **46**, 227-232

Endo, M. and Ikusima, I. (1989) Plant Cell Physiol. **30**, 43-47

Ettlinger, C. and Lehle, L. (1988) Nature **331**, 176-178

Fejes,E., Pay, A., Kanevsky, I., Szell, M., Adam, E., Kay, S. and Nagy, F. (1990) Plant Mol. Biol. **15**, 921-932

Felle, H. (1988) Planta **174**, 495-499

Fischer, A. and Kluge, M. (1985) Physioligia. Plantarum. **63**, 327-331

Gadal, P., Perrot-Rechenmann, C and Vidal, J. (1983) Physiol. Veg. **21**, 1055-1062

Garnier-Dardat, J. and Queiroz, O. (1974) Phytochemistry **13**, 1695-1702

Glass, D.B., Cheng, H-C., Mueller-Menda, L., Reed, J. and Walsh, D.A. (1989) J. Biol. Chem. **264**, 8802-8810

Goatly, M.B. and Smith, H. (1974) Planta **117**, 67-73

Gonzalez. D.H. and Andreo, C.S. (1989) Trends in Biochem. Sci. **14**, 24-27

Gorton, H.L., Williams, W.E., Binns, M.E., Gemmell, C.N., Leheny, E.A. and Sheperd, A.C. (1989) Plant Physiol. **90**, 1329-1334

Gregory, R.P.F. (1990) The Biochemistry of Photosynthesis. 178-188 3rd ed., Wiley-Interscience publications, Inc.

Guitton, C. and Mache, R. (1987) Eur. J. Biochem. **166**, 249-254

Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) Science **241**, 42-52

Hardin, P.E., Hall, J.C. and Rosbash, M. (1990) Nature (London) **343**, 536-540

Harmon, A.C. (1990) Inositol Lipid Metabolism in Plants. Morre, D.J. Boss, W.F. and Loewus, F. eds., Wiley-Liss, Inc., New York

Harris, P.J. and Wilkins, M.B. (1978) Planta **143**, 323-328

Hatch, M.D. and Osmond, O. (1976) Encyclopedia of Plant Physiol., New series, **3**, Stocking, C.R. and Huber, U., ed., Springer-Verlag, Berlin

Hatch, M.D. (1977) Trends in Biochem. Sci. **2**, 199-202

Haystead, T.A.J., Sim, A.T.R., Carling, D.L., Honnor, R.C., Tsukitani, Y., Cohen, P. and Hardie, D.G. (1989) Nature (London) **337**, 78-81

Hepler, P.K. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 1-9

Holdsworth, M. (1971) Can. J. Bot. **49**, 1520-1522

House, C., Robinson, P.J. and Kemp, B.E. (1989) FEBS Lett. **249**, 243-247

Huber, S.C. and Sugiyma, T. (1986) Plant Physiol. **81**, 674-677

Huber, S.C., Sugiyma, T. and Akazawa, T. (1986) Plant Physiol. **82**, 550-554

Huber, J.L.A., Huber, S.C. and Nielsen, T.H. (1989) Arch. Biochem. Biophys. **270**, 681-690

Huber, S.C. and Huber, J.L.A. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 329-343

Hudspeth, R.L., Glackin, C.A., Bonner, J. and Grula, J.W. (1986) Proc. Nat. Acad. Sci. USA. **83**, 2884-2888

Hudspeth, R.L. and Grula, J.W. (1989) Plant Mol. Biol. **12**, 579-589

Hunter, T. (1987) Cell **50**, 823-829

Ishijima, S., Katagiri, F., Kodaki, T., Izui, K., Katsuki, H., Nishikawa, K. and Ooi, T. (1985) Biochem. Biochem. Res. Commun. **133**, 436-441

Jiao, J-A. and Chollet, R. (1988) Arch. Biochem. Biophys. **261**, 409-417

Jiao, J-A. and Chollet, R. (1989) Arch. Biochem. Biophys. **269**, 526-535

Jiao, J-A. and Chollet, R. (1990a) Plant Physiol. (suppl.) **93**, 153

Jiao, J-A. and Chollet, R. (1990b) Arch. Biochem. Biophys. **283**, 300-305

Jones, R., Wilkins, M.B., Coggins, J.R., Fewson, C.A. and Malcolm, A.D.B. (1978) Biochem. J. **175**, 391-406

Jones, R., Buchanan, I.C., Wilkins, M.B., Fewson, C.A. and Malcolm, A.D.B. (1981) J. Exp. Bot. **32**, 427-441

Karabourniotis, G., Manetas, Y. and Gavalas, N.A. (1983) Plant Physiol. **73**, 735-739

Karakashian, M.W. and Hastings, J.W. (1963) J. Gen. Physiol. **47**, 1-12

Karakashian, M. and Schweiger, H.G. (1976) Exp. Cell Res. **98**, 303-312

Kemp, B.E., Byland, D.B., Huang, T.S. and Krebs, E.G.(1975) Proc. Nat. Acad. Sci. **72**, 3448-3452

Kelly, G.J., Latzko, E. and Gibbs, M. (1976) Annu. Rev. Plant Physiol. **27**, 181-205

Kemp, B.E. and Pearson, R.B. (1990) Trends in Biochem. Sci. **15**, 342-346

Kenyon, W.H., Kringstad, R. and Black, C.C. (1978) FEBS Lett. **94**, 281-284

Kloppstech, K. (1985) Planta **165**, 502-506

Kluge, M. and Osmond, C.B. (1971) Naturwissenschaften **58**, 414-415

Kluge, M. and Ting, I.P. (1978) Crassulacean Acid Metabolism. Analysis of an Ecological Adaption. Springer-Verlag, Berlin, Heidelberg, New York.

Kluge, M. and Fischer, A. (1984) Planta **160**, 121-128

Kluge, M., Maier, P., Brulfert, J., Faist, K. and Wollny, E. (1988) J. Plant Physiol. **133**, 252-256

Knoetzel, J. and Resing, L. (1990) J. Plnat Physiol. **136**, 280-288

Knopf, O. and Kluge, M. (1979) Plant Cell Environ. **2**, 73-78

Krebs, E.G. and Beava, J.A. (1979) Annu. Rev. Biochem. **48**, 923-959

Kruger, I. and Kluge, M. (1988) Bot. Acta. **101**, 32-35

Ku, M.S.B., Schmitt, M.R., Edwards, G.E. (1979) J. Exp. Bot. **30**, 89-98

Laemmli, U.K. (1970) Nature(London) **227**, 680-685

Lavergne, D. and Champigny, M.L. (1983) Physiol. Veg. **21**, 1021-1029

Lawton, M.A., Yamamoto, R.T., Hanks, S.K. and Lamb, C.J. (1989) Proc. Nat. Acad. Sci. USA. **86**, 3140-3144

Lawton, M.A. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 373-382

Lin, P.P.C., Key, J.L. and Mori, T. (1980) Plant Physiol. **66**, 368-374

Lorrimer, G.H. and Andrews, T.J. (1981) The Biochemistry of Plants. A comprehensive treatise, **8**, 329-347, Hatch, M.D. and Boardman, N.K., ed., Academic Press, New York

Luttge, U. and Ball, E. (1978) Z. Pflanzenphysiol. **90**, 69-77

Luttge, U. and Ball, E. (1979) J. Memb. Biol. **47**, 401-422

Luttge, U., Smith, J.A.C., Marigo, G. and Osmond, C.B. (1981) FEBS Lett. **126**, 81-84

MacKintosh, C. and Cohen, P. (1989) Biochem. J. **262**, 335-339

* MacKintosh, C. and Klumpp, S. (1990) FEBS Lett. **277**, 137-140

Manetas, Y. (1982) Photosyn. Res. **3**, 321-333

Martiny-Baron, G. and Scherer, G.F.E. (1989) J. Biol. Sci. **264**, 18052-18059

Marquardt, G. and Luttge, U. (1987) J. Plant Physiol. **129**, 269-286

Marquardt-Jarczyk, G. and Luttge, U. (1990) J. Plant Physiol. **136**, 129-136

McGowan, C.H. and Cohen, P. (1988) Meths. in Enzymol. **189**, 416-426

McMahon, D. (1975) Plant Physiol. **55**, 815-821

McNaughton, G.A.L., Fewson, C.A., Wilkins, M.B., and Nimmo, H.G. (1989) Biochem. J. **261**, 349-355

McNaughton, G.A.L., Mackintosh, C., Fewson, C.A., Wilkins, M.B., and Nimmo, H.G.(1991) in press

Melkonian, B., Burchert, M., Kreimer, G. and Latzko, E. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 38-46

Melzer, E. and O'Leary, M. (1987) Plant Physiol. **84**, 58-60

Memon, A., Rincon, M. and Boss, W.F. (1989) Plant Physiol. **91**, 477-480

Meyer, H., Thienel, U. and Birgit, P. (1989) Planta **180**, 5-15

Morre, D.J. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 47-65

Morse, M.J., Crain, R.C. and Satter, R.L. (1987) Proc. Nat. Acad. Sci. USA. **84**, 7075-7078

Morse, D.S., Milos, P.M., Roux, E. and Hastings, J.W. (1989) Proc. Nat. Acad. Sci. USA. **86**, 172-176

Morse, D.S., Fritz, L. and Woodland-Hastings, J.W. (1990) Trends in Biochem. Sci. **15**, 262-265

Muller, D., Kluge, M. and Groschel-Stewart, U. (1980) Plant Cell Environ. **5**, 223-230

Myto, S. and Shimogawa, K. (1985) FEBS Lett. **193**, 88-92

* MacKintosh, C., Beatie, K.A., Klumpp, S., Cohen, P. and Codd, G.A. (1990) FEBS Lett. **264**, 187-192

Nagy, F., Kay, S.A. and Chua, N.H. (1988) Genes Dev. **2**, 376-382

Nakashima, H., Perlman, J. and Feldman, J.F. (1981) Science **212**, 361-362

Neales, T.F. (1973) Aust. J. Biol. Sci. **26**, 539-546

Nguyen, T.T., Ngam-Ek, A., Jenkins, J. and Grover, S.D. (1988) Plant Physiol. **86**, 104-107

Njus, D., Gooch, V. and Hastings, J.W. (1981) Cell Biophys. **3**, 223-231

Nimmo, H.G. and Cohen, P (1977) Hormonal Control of Protein Phosphorylation. In: Advances in Cyclic Nucleotide Research **8**, 145-266, Greengard, P. and Robinson, G.A. ed. Raven Press, New York

Nimmo, G.A., Nimmo, H.G., Fewson, C.A. and Wilkins, M.B. (1984) FEBS Lett. **178**, 199-203

Nimmo, G.A., Nimmo, H.G., Hamilton, I.D., Fewson, C.A. and Wilkins, M.B. (1986) Biochem. J. **239**, 213-220

Nimmo, G.A., McNaughton, G.A.L., Fewson, C.A., Wilkins, M.B. and Nimmo, H.G. (1987) FEBS Lett. **213**, 18-22

Nimmo, G.A., Wilkins, M.B., Fewson, C.A. and Nimmo, H.G. (1987) Planta **170**, 408-415

Nishida, K. and Tominaga, O. (1987) J. Plant Physiol. **127**, 385-393

Nott, D.L. and Osmond, C.B. (1982) Aust. J. Plant Physiol. **9**, 409-422

O'Leary, M.H. (1982) Annu. Rev. Plant Physiol. **33**, 297-315

O'Leary, M.H. (1990) The Enzymes **20**, Boyer, P.D. and Krebs, E.G. ed., 3rd ed., Academic Press, Inc.

Osmond, C.B. (1978) Annu. Rev. Plant Physiol. **29**, 379-414

Osmond, C.B. and Holtum, J.A.M. (1981) The Biochemistry of Plants. A comprehensive treatise, **8**, 283-329, Hatch, M.D. and Boardman, N.K., ed., Academic Press, New York

Ostrem, J.A., Olson, S.W., Schmitt, J.M. and Bohnert, H.J. (1987) Plant Physiol. **84**, 1270-1275

Pays, A.G.G., Jones, R., Wilkins, M.B., Fewson, C.A. and Malcolm, A.D. (1980) Biochem. Biophys. Acta **614**, 151-162

Perrot, C., Vidal, J., Brulfert, J. and Gadal, P. (1981) Planta **151**, 226-231

Perrot-Rechenmann, C., Vidal, J., Brulfert, J., Burlet, A. and Gadal, P. (1982) Planta **155**, 24-30

Pierre, J.N. and Queiroz, O. (1979) Planta **44**, 143-151

Pilon-Smits, E.A.H., Hart, H. and van Brederode, J. (1990) J. Plant Physiol. **136**, 155-160

Podesta, F.E. and Andreo, C.S. (1989) Plant Physiol. **90**, 427-433

Polya, G.M. and Haritou, M. (1988) Biochem. J. **251**, 357-363

Pongratz, M. and Klingmuller, W. (1973) Mol. Gen. Genet. **124**, 359-363

Randall, D.D., Williams, M. and Rapp, B.J. (1981) Arch. Biochem. Biophys. **207**, 437-444

Ranjeva, R. and Boudet, A.M. (1987) Annu. Rev. Plant Physiol. **38**, 73-93

Rickers, J., Cushman, J.C., Michalowski, C.B., Schmitt, J.M. and Bohnert, H.J.(1989) Mol. Gen. Genet. **215**, 447-454

Ritz, D., Kluge, M. and Veith, H.G. (1987) Plant Physiol. Biochem. **25**, 391-399

Rocha, V. and Ting, I.P. (1970) Plant Physiol. **46**, 754-756

Rosen, O.M. and Krebs, E.G. (1981) Protein Phosphorylation. Cold-Spring Harbour Conference on Cell Proliferation, **8**

Russell, D.W., Knight, J.S. and Wilson, T.M. (1985) Curr. Top. in Plant Biochem. **4**, 191-206

Rustin, P., Meyer, C.R. and Wedding, R.T. (1988) J. Biol. Chem. **263**, 17611-17614

Sanders, D., Miller, A.J., Blackford, S., Brosnan, J.M. and Johannes, E. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 20-37

Satter, R.L. and Galson, A.W. (1981) Annu. Rev. Plant Physiol **32**, 83-110

Scherer, G.F.E., Andre, B. and Martiny-Baron, G. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 190-218

Schnabl, H. and Kottmeier, C. (1984) Planta **162**, 220-225

Schnarrenberger, C., Gross, D., Burkhard, C.H. and Herbert, M. (1985) Plant **147**, 477-484

Schuller, K.A., Plaxton, W.C. and Turpin, D.H. (1990) Plant Physiol. **93**, 1303-1311

Servaites, J.C. (1985) Plant Physiol **78**, 839-843

Servaites, J.C. (1990) Plant Physiol **92**, 867-870

Smith, J. A.C., Vribe, E.G., Ball, E. and Luttge, U. (1984) Planta **162**, 299-304

Soll, J. and Buchanan, B.B. (1983) J. Biol. Chem. **258**, 6686-6689

Srivasta, A., Pines, M. and Jacoby, B. (1989) Plant Physiol. **77**, 320-325

Stiborvora, M. and Leblova, S. (1983) Physiol. Veg. **21**, 939-942

Sugiyma, T. and Laetsch, W.M. (1975) Plant Physiol. **56**, 605-607

Sutton, B.G. (1975a) Aust. J. Plant Physiol. **2**, 337-388

Sutton, B.G. (1975b) Aust. J. Plant Physiol. **2**, 389-402

Sweeney, B.M. (1974) Plant Physiol. **53**, 337-341

Tavladoraki, P., Kloppstech, K. and Argyroudi-Akoyunogiou, J. (1989) Plant Physiol. **90**, 665-672

Terada,K., Kai,T., Okuno, S., Fujisawa, H. and Izui, K. (1990) FEBS Lett. **259**, 241-244

Ting, I.P. and Osmond, C.B. (1973a) Plant Physiol. **51**, 439-447

Ting, I.P. and Osmond, C.B. (1973b) Plant Physiol. **51**, 448-453

Ting, I.P. (1985) Annu. Rev. Plant Physiol. **36**, 595-622

Trautschold, I., Lamprect, W. and Schweitzer, G. (1983) Methods of Enzymatic Analysis (3rd edition), 346-357, Bergmeyer, H.U. ed., VCH publishers

Vidal, J., Cretin, C. and Gadal, P. (1983) Physiol Veg. **21**, 977-986

von Willert, D.J., Treichel, S., Kirst, G.O. and Curdts, E. (1976) Phytochem. **15**, 1435-1436

von Willert, D.J. and von Willert, E. (1979) Z. Pflanzenphysiol. **95**, 43-49

Walker, G.H. and Huber, S.C. (1987) FEBS Lett. **213**, 375-380

Walker, G.H., Ku, M.S.B. and Edwards, G.E. (1988) J. Plant. Physiol. **133**, 144-151

Walker, J.L. and Zhang, R. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 398-406

Walsh, D.A., Perkins, J.P., Brostrow, C.O., Ho, E.S. and Krebs, E.G. (1971) J. Biol. Chem. **246**, 1968-1971

Walz, B. and Sweeney, B.M. (1979) Proc. Nat. Acad. Sci. USA. **76**, 6443-6447

Warren, D.M. and Wilkins, M.B. (1961) Nature **191**, 686-688

Wedding, R.T. and Black, M.K. (1983) Plant Physiol **72**, 1021-1028

Wedding, R.T., Black, M.K. and Meyer, C.R. (1989) Plant Physiol. **90**, 648-652

Wedding, R.T., Black, M.K. and Meyer, C.R. (1990) Plant Physiol. **92**, 456-461

Wilkins, M.B. (1959) J. Exp. Bot. **10**, 377-390

Wilkins, M.B. (1960) J.Exp. Bot. **11**, 269-274

Wilkins, M.B. (1962) Proc. of Royal Soc. **156**, 220-241

Wilkins, M.B. (1967) Planta **72**, 66-77

Wilkins, M.B. (1973) J. Exp. Bot. **24**, 488-496

Wilkins, M.B. (1983) Planta **157**, 471-480

Wilkins, M.B. (1984) Planta **161**, 381-384

Wilkins, M.B. (1991) Planta (in press)

Wilkins, M.B. and Holowinski, D. (1965) Plant Physiol. **40**, 907-909

Willmer, C.M. (1983) Physiol. Veg. **21**, 943-953

Winter, K. (1980) Plant Physiol. **65**, 792-796

Winter, K. (1981) Aust. J. Plant Physiol. **8**, 115-119

Winter, K. (1982) Planta **154**, 298-308

Winter, K., Foster, J. G., Edwards, G. and Holtum, J.A.M. (1982) Plant Physiol. **69**, 300-307

Wong, K.F. and Davies, D.D. (1989) Biochem. J. **131**, 415-458

Wood, H.G. and Barden, R.E. (1977) Annu. Rev. Biochem. **46**, 385-413

Wray, W., Boulakis, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. **118**, 197-203

Wu, M-X. and Wedding, R.T. (1987) Plant Physiol. **84**, 1080-1083

Yunghans, W.N. and Morre, D.J. (1977) Plant Physiol. **60**, 144-149

Zielinski, R.E., Ling, V. and Perera, I. (1990) Curr. Top. in Plant Biochem. Physiol. **9**, 420