The Regulation of Phosphoenolpyruvate Carboxylase in Stomatal Guard Cells of *Commelina communis*

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Abbreviations

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

ABA	Abscisic acid
BSA	Bovine serum albumin
CAM	Crassulacean acid metabolism
DTT	Dithiothreitol
GCP	Guard cell protoplast
G 6-P	Glucose 6-phosphate
MDH	Malate dehydrogenase
ME	Malic enzyme
OAA	Oxaloacetate
PEG	Polyethylene glycol
PEP	Phosphoenolpyruvate
PEPc	Phosphoenolpyruvate carboxylase
Pi	Orthophosphate, inorganic phosphate
PPDK	Pyruvate phosphate dikinase
RUBISCO	Ribulose bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine

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Summary

A great deal of work has been done recently to investigate mechanisms which control plant metabolism. In the CAM and C4 systems, it was found that phosphoenolpyruvate carboxylase which catalyses the primary fixation of CO_2 in these plants is controlled by a reversible phosphorylation mechanism, with the enzyme being more active in the phosphorylated form than the nonphosphorylated form. Stomatal guard cells exhibit a form of malate metabolism very similar to CAM, and require the pathway of malate synthesis to be active during stomatal opening, and inactive during closure. This pathway involves flux through phosphoenolpyruvate carboxylase. The main aim of the work described in this thesis was to investigate the regulation of phosphoenolpyruvate carboxylase in guard cells. In addition, signal transduction in stomatal guard cells has recently been investigated by many workers, and this system is often now regarded as a model for signal transduction in plants. An additional aim of this project, therefore, was to study aspects of the signalling process involved in regulation of guard cell phosphoenolpyruvate carboxylase.

The first part of the work involved the definition of conditions that would reproducibly cause stomatal movements. Stomata in epidermal strips from the C3 plant *Commelina communis* were found to open when incubated in the light on a solution containing 25mM K_2SO_4 and which had air with a reduced concentration of CO_2 bubbled through it. When the epidermis was placed into darkness and normal air was bubbled through the medium, the stomata closed. Stomata were also found to open in response to the fungal toxin fusicoccin when incubated on 25mM K_2SO_4 in the dark.

The possibility that guard cell phosphoenolpyruvate carboxylase is regulated by phosphorylation was investigated in several ways. The incubation of extracts of guard cell protoplasts with [γ-^{32}P] ATP, Mg^{2+} and cyclic AMP dependent protein kinase catalytic subunits showed that guard cell phosphoenolpyruvate carboxylase became phosphorylated *in vitro*. In the absence of added kinase, no phosphorylation of the enzyme was observed, even in the presence of 1mM free Ca^{2+}. Incubation of intact guard cell protoplasts with [^{32}P]-orthophosphate showed that the enzyme was also phosphorylated *in vivo*. The phosphorylation state of guard cell phosphoenolpyruvate carboxylase was increased when guard

cell protoplasts were incubated with 50mM K^+ in the light or with fusicoccin. The phosphorylation state of the enzyme did not increase when protoplasts were incubated in the presence of 50mM K^+ in darkness or with either abscisic acid or okadaic acid, an inhibitor of protein phosphatases. Light stimulated phosphorylation of the enzyme was reversed when protoplasts were placed in darkness. Protein phosphatase 2A activity, known to be responsible for the dephosphorylation of phosphoenolpyruvate carboxylase in the CAM plant *Bryophyllum fedtschenkoi*, was detected in extracts of guard cell protoplasts. Fusicoccin-stimulated phosphorylation was prevented by the protein synthesis inhibitor cycloheximide, a phenomenon similar to observations made in the CAM and C4 systems. It was also found that cycloheximide appeared to retard the stomatal opening that occors in response to both light and fusicoccin.

Assays were carried out to measure the activity and some of the kinetic parameters of guard cell phosphoenolpyruvate carboxylase. Enzyme activity was almost five fold higher at pH 8.0 than at pH 7.2, but the enzyme was relatively insensitive to the feedback inhibitor malate at higher pH. When measured at pH 7.8, increasing concentration of the inhibitor malate increased both apparent K_m and apparent V_{max} values, and at malate concentrations higher than 5mM, the enzyme appeared to depart from Michaelis-Menten kinetics. When guard cell protoplasts were incubated in the presence of K_2SO_4 in the light and dark or with and without fusicoccin, no significant differences in K_i (malate) from protoplasts in different conditions were observed. Further attempts to change K_i (malate) by the incubation of guard cell extracts with protein phosphatases and kinases also produced no significant change. When enzyme assays were carried out at pH 7.0 in the presence of glucose 6-phosphate, the enzyme extracted from guard cell protoplasts which had been incubated in the light had a K_m (PEP) of 4.7μM, whilst that from those incubated in the dark had a K_m (PEP) of 10.7μM, with a probability of identity between the two of less than 7%. Extracts of fusicoccin-stimulated protoplasts contained phosphoenolpyruvate carboxylase with a K_m (PEP) of 6.7μM, with a less than 18% probability of identity with the dark control. K_i (malate) was not found to change significantly when measured under these conditions, although V_{max} was decreased in protoplasts which were incubated in the light or with fusicoccin compared to the dark control.

These results provide strong evidence to suggest that guard cell phosphoenolpyruvate carboxylase is controlled by reversible phosphorylation, in a manner analagous to the CAM and C4 enzymes. In addition, consideration of these results leads to the suggestion that cytoplasmic K^+ concentration is an important signalling factor in guard cells.

Chapter 1

Introduction

1.1 General features of stomata

General stomatal physiology and structure have been extensively researched and are the subject of several major textbooks (Meidner and Mansfield, 1968; Jarvis and Mansfield, 1981; Weyers and Meidner, 1990).

Stomata are pores that are present on virtually all aerial surfaces of all land plants, most abundantly on the lower surfaces of leaves. These pores permit gas exchange between the plant and the atmosphere while regulating water loss. Control over these processes is achieved by alteration of pore aperture in response to environmental stimuli. Details of stomatal structure, size and position vary from species to species, but several general features are common to all stomata. Each individual stoma consists of two or more specialised epidermal cells. All stomata possess two guard cells, which lie next to each other, with their adjacent walls forming the border of the pore itself. Guard cells contain chloroplasts, mitochondria, vacuoles and all other organelles common to most plant cells; in this respect they are unlike epidermal cells which lack most types of organelle. There are two main groups of stomata, the eliptical type and the graminaceous type.

The elliptical stomata group is divided into two sub groups, those without subsidiary cells, for example *Vicia faba* stomata (Figure 1.1a) and those with subsidiary cells, for example *Commelina communis* (Figure 1.1b). Elliptical stomata have kidney shaped guard cells situated at the exterior end of an intercellular space known as the substomatal cavity (Figure 1.2). The guard cell wall is unattached on three sides - top and bottom and at the pore throat. A very important feature of guard cell structure is the distribution of cell wall material. In elliptical guard cells, the cellulose micellae radiate out from the centre of the pore (Figure 1.3). The pore side wall is much thicker than the rest of the guard cell wall, and the wall adjacent to the surrounding epidermal cells is of comparable thickness to epidermal cell walls. This arrangement facilitates the guard cell movements which occur during swelling (see section 1.2). The subsidiary cells associated with some elliptical stomata are specialised non-photosynthetic epidermal cells, arranged regularly around the guard

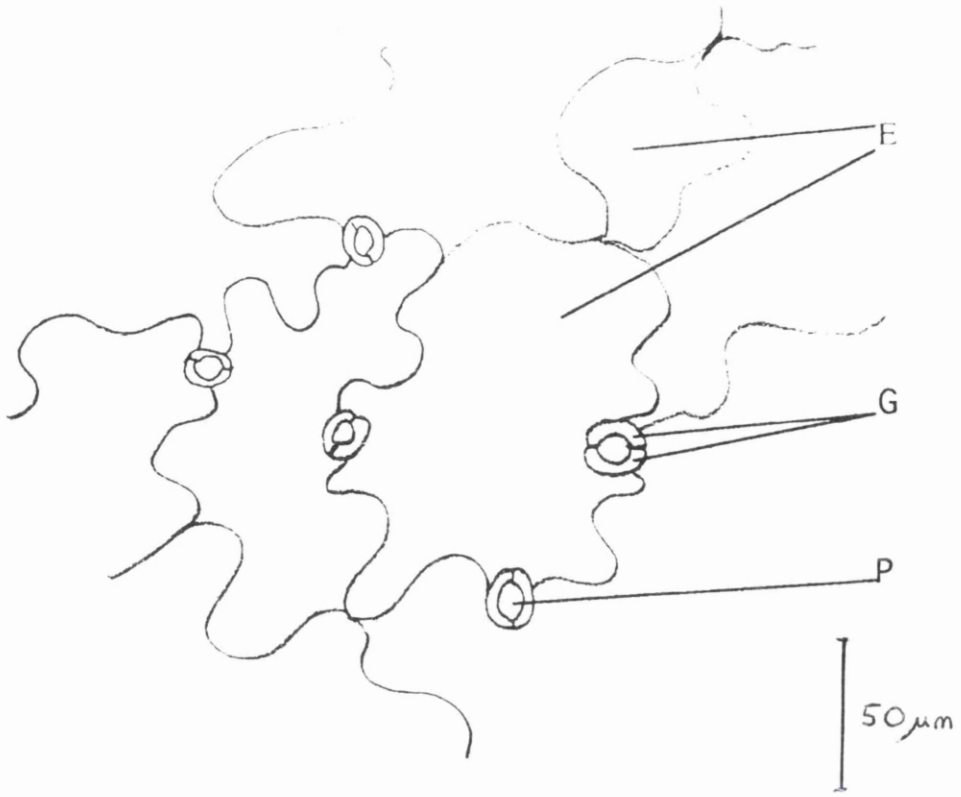


Figure 1.1 a ***Vicia faba*** **stomata**

G - stomatal guard cells E - epidermal cells P - stomatal pore

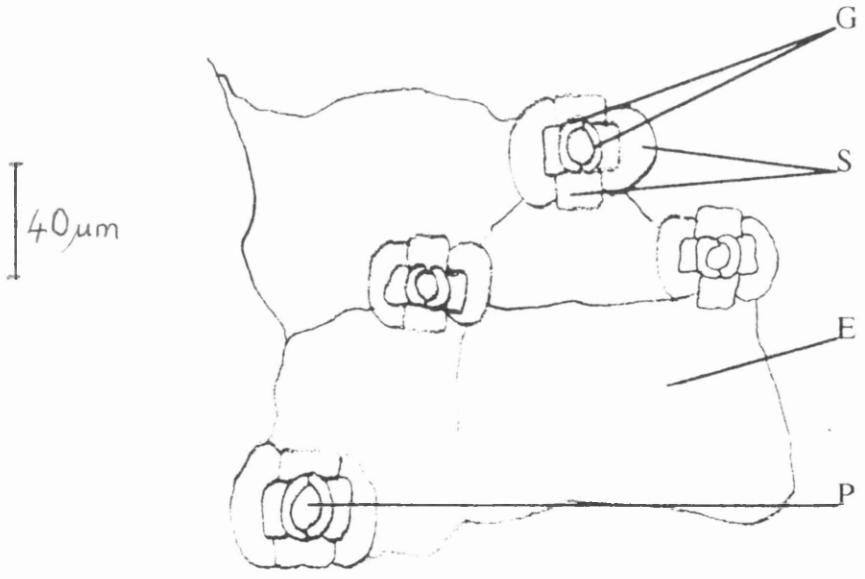


Figure 1.1b ***Commelina communis*** **stomata**

G - stomatal guard cell S - subsidiary cell E - epidermal cell P - stomatal pore

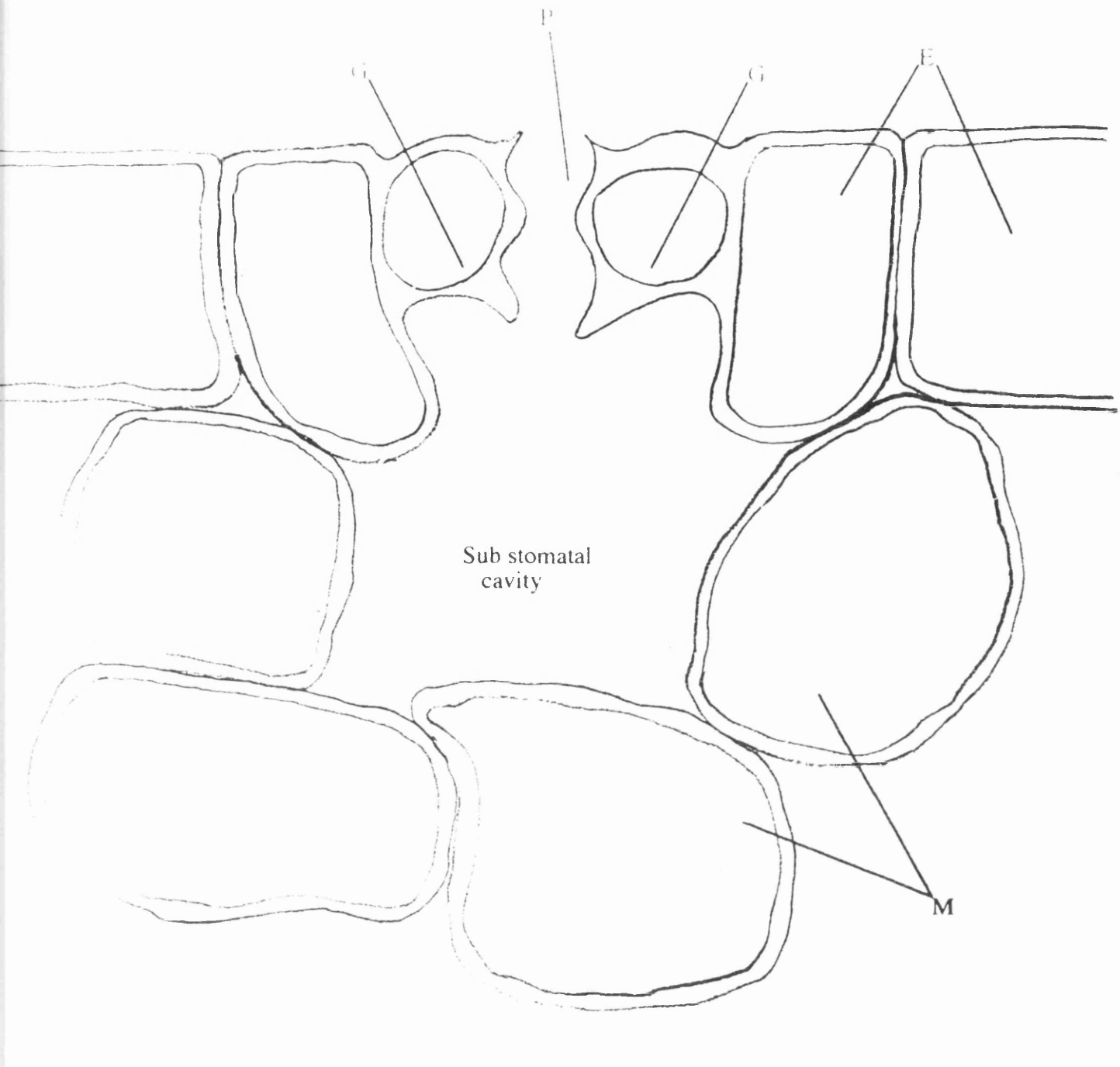


Figure 1.2 Transverse section through an elliptical stoma

G - stomatal guard cells P - stomatal pore throat E- epidermal cells
M- mesophyll cells

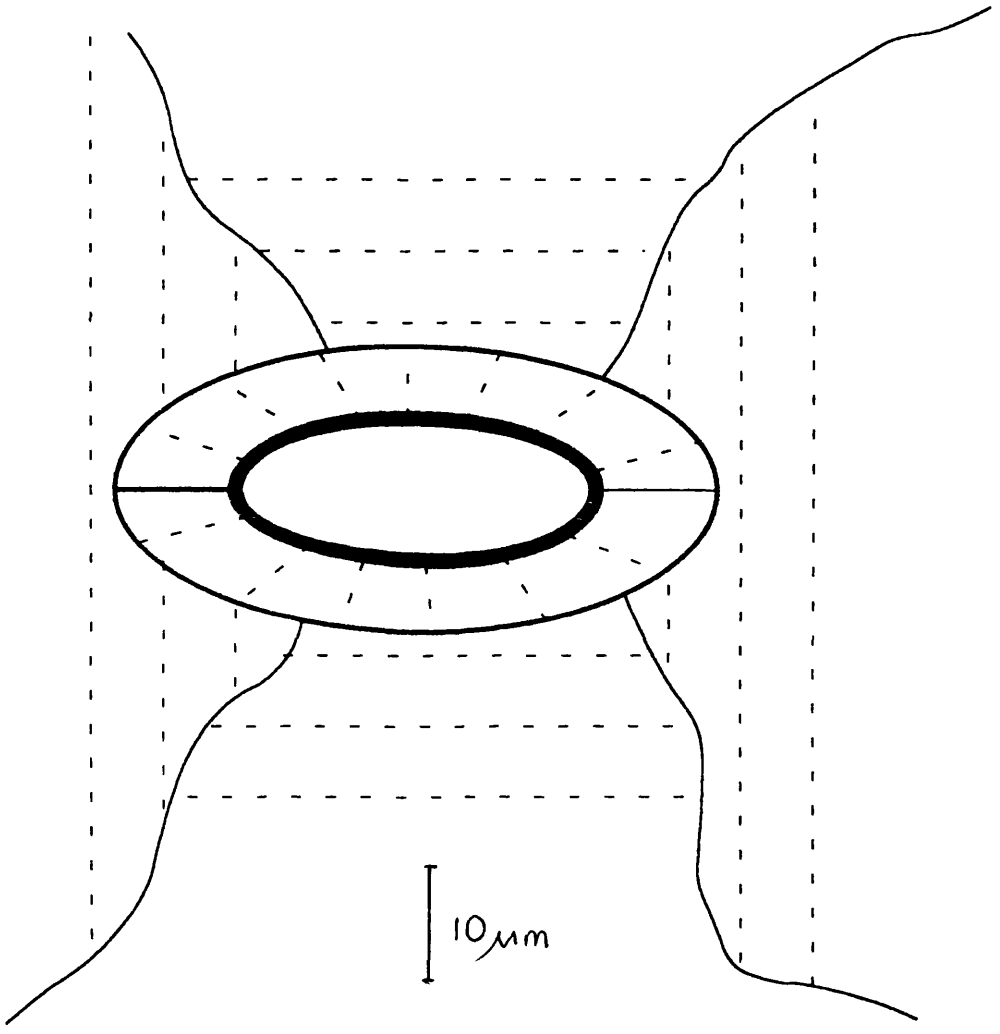


Figure 1.3 Distribution of cell wall micellae in elliptical stomata

Dashed lines indicate the direction in which cellulose micellae lie. Cell wall expansion is possible at right angles to the direction of the micellae.

cell pair. In *Commelina communis*, each guard cell pair has six subsidiary cells around it.

Graminaceous stomata possess dumbell shaped guard cells with two end chambers joined by a narrow channel of cytoplasm (Figure 1.4a). These guard cells, like those of elliptical stomata, are positioned above a substomatal cavity. Graminaceous stomata are arranged very regularly in rows on leaf epidermis, and do not have subsidiary cells. The region of wall forming a cylinder around the connecting channel is highly thickened and rigid. The micellae forming the wall around each end chamber radiate out from the point where the channel enters (Figure 1.4b). Micellae arrangement facilitates stomatal movents.

1.1.1 Stomatal regulation of photosynthesis and responses to environmental stimuli

Aspects of stomatal function involved in the control of photosynthesis and the responses of stomata to environmental stimuli have been reviewed by Zeiger (1983).

In order to photosynthesise and respire, plants must exchange gases with the atmosphere. Environmental variations in light, temperature, humidity and CO_2 concentration require that, in order to maintain photosynthesis whilst minimizing water loss, the rate of gas exchange must vary. This situation has given rise to the optimization theory of stomatal function (Zeiger, 1983).

Stomata achieve their modulation of photosynthetic rate by responding to a variety of environmental stimuli. Various authors such as Jewer (1982) and MacRobbie (1981) have demonstrated that at least some stimuli are detected by the guard cells themselves. Stimuli to which stomata are known to respond are outlined below.

<u>Responses to light</u>

Stomata open in the light. Stomatal aperture in whole leaves or isolated epidermis in CO_2 free air increases in an intensity dependent manner in white light (Fischer, 1968). The effect of light on stomata is dominant over the effect of CO_2 concentration (see below). Red light causes stomatal opening, whilst green does not. Blue light is up to five times more efficient in causing stomatal opening than is red light. It has been suggested that red light is detected by the photosynthetic pigments and that blue light is detected by either flavinoids or some as yet unidentified photoreceptor (Zeiger, 1983). Far red light alone or with

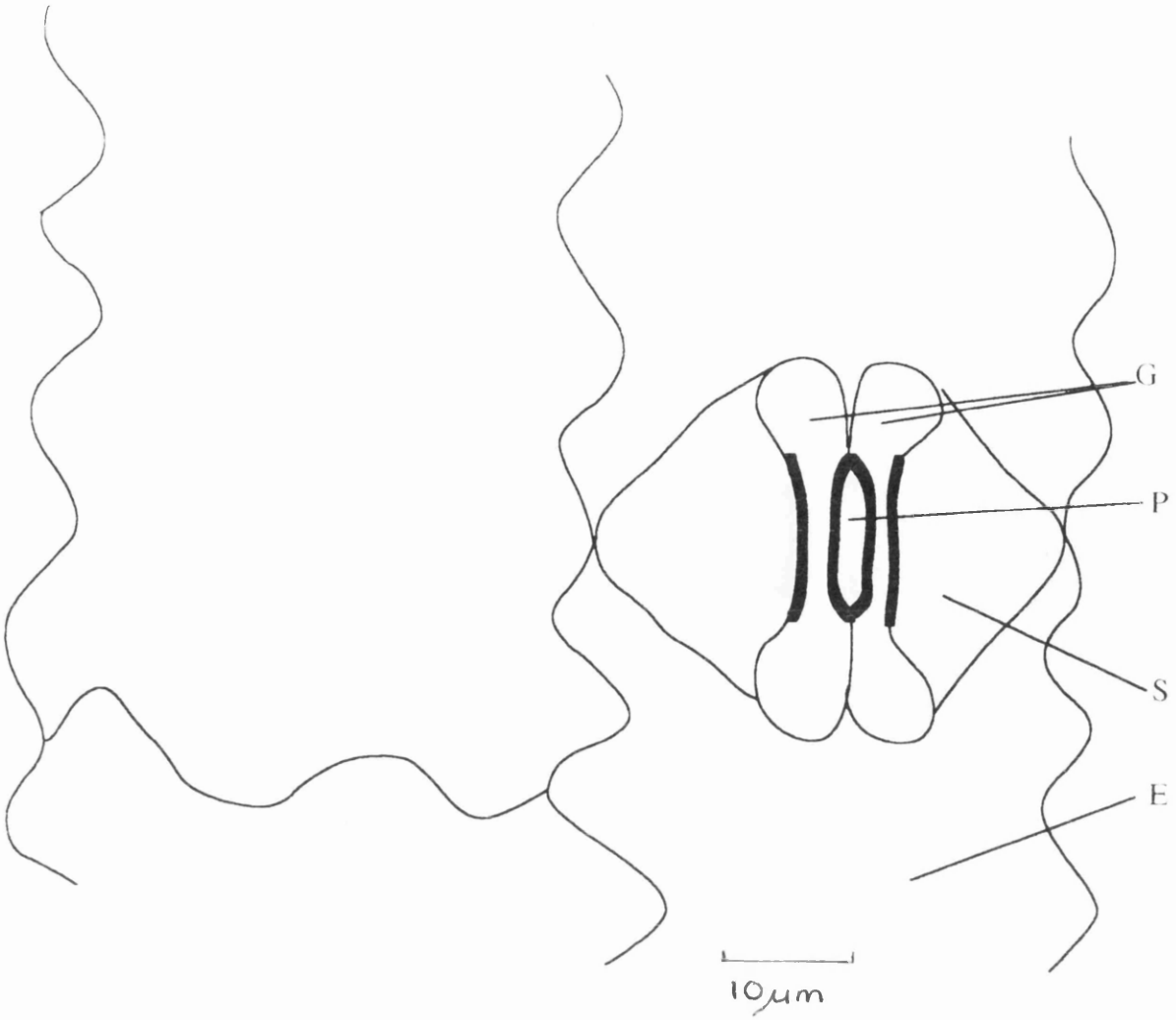


Figure 1.4a Graminaceous stomata of *Zea mays*

G - stomatal guard cell P - stomata pore E - epidermal cell S - subsidiary cell

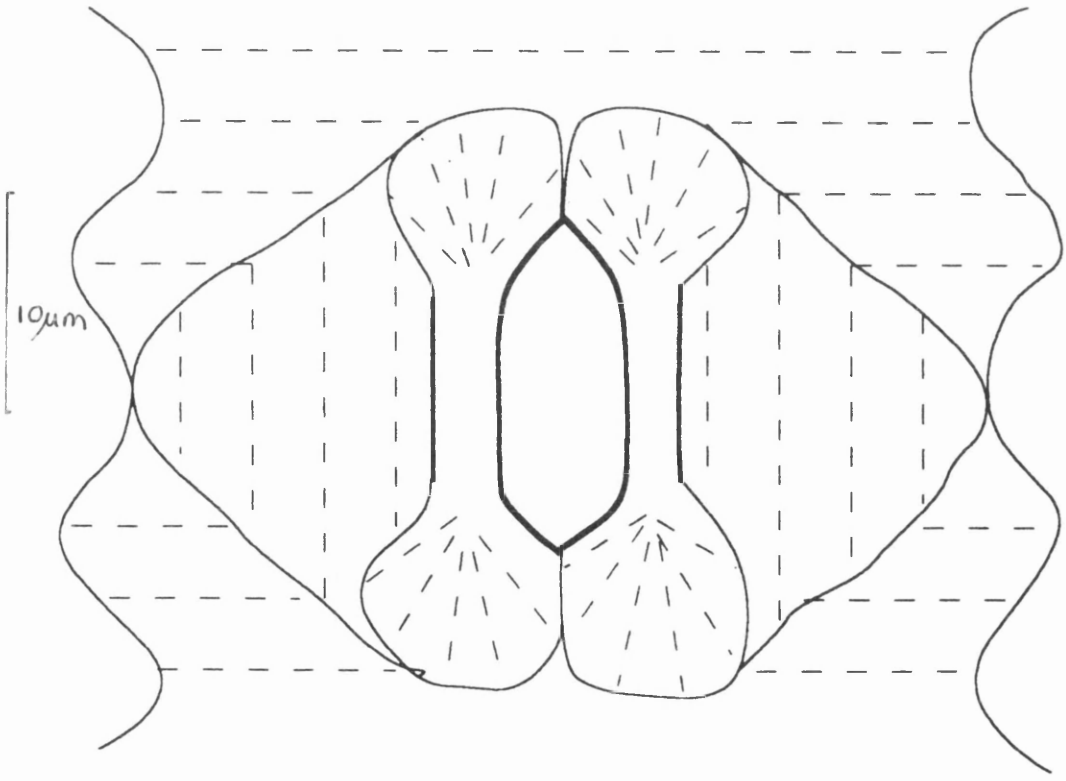


Figure 1.4b The distribution of micellae in cell walls of a graminaceous stoma

Dashed lines indicate the orientationof micellae in cell walls

near red light causes stomata to close. This suggests that phytochrome affects stomatal movement, and this system may be involved in entrainment of circadian rhythms.

Responses to carbon dioxide

Stomata open more as CO_2 concentration decreases. This observation is compatible with optimization theory, since more rapid gas exchange is required as CO_2 concentration decreases in order to maintain photosynthetic rate. CO_2 stimulated responses are rapid, occurring in a few seconds after a change in CO_2 concentration. In the absence of Cl^-, a CO_2 concentration of zero results in much reduced stomatal opening as malate synthesis requires CO_2 (Mansfield *et. al.* 1990). It has been proposed by Hedrich and Marten (1993) that guard cells detect elevated CO_2 indirectly. These workers have found that when guard cell protoplasts are incubated in solutions containing malate, anion efflux occurs. They suggest that *in vivo* malate, leaking from the guard cells themselves, could cause this response to happen, and that a high CO_2 concentration would lead to a greater loss of malate from the guard cells.

Responses to humidity and temperature

As vapour pressure density increases, stomata open more. This effect has been observed in intact leaves and in isolated epidermis. Temperature effects are closely related to humidity effects because vapour pressure density increases as temperature increases. In dry air, maximal opening in the light occurs at 35°C. Both higher and lower temperatures cause decreases in stomatal aperture. It is thought that aperture increases up to 35°C in order to allow leaf cooling. At higher temperatures the stomata become damaged and close.

Responses to abscisic acid

It is well known that abscisic acid causes stomatal closure, and this phenomenon has long been regarded as the mechanism by which water stress is detected in guard cells. However, the role of abscisic acid in water stress induced closure has been challenged. Observations have been made that stomatal closure occurs in response to water stress before abscisic acid levels rise detectably (Beardsell and Cohen, 1975; McMichael and Hanny, 1977; Henson, 1981). It has also been observed that stomata remain closed long after leaf abscisic acid has returned to prestress concentration (Henson, 1981). Harris and Outlaw (1990) have suggested that the concentration of abscisic acid in guard cells themselves rises in response to water stress. Trejo and Davies (1991) suggest that guard cells may be capable of detecting extremely low concentrations of abscisic acid. Both hypotheses are supported by the observation that the sensitivity of guard cells to abscisic acid is increased when there is a leaf water

deficit (Tardieu and Davies, 1992). Abscisic acid is known to act on stomata by stimulating efflux of K^+ ions, causing shrinking of the guard cells (Introduction section 1.2; MacRobbie, 1981).

Responses to auxins

Indoleacetic acid can act as an antagonist of both abscisic acid and CO_2 stimulated closure (Snaith and Mansfield, 1982). Auxin cannot, however, increase the degree to which stomata open in response to low CO_2 concentration (Snaith and Mansfield, 1985).

Responses to fusicoccin

Fusicoccin, a fungal toxin from *Fusicoccum amygdali* is a powerful agent promoting stomatal opening (Squire and Mansfield, 1972). Opening is thought to occur due to direct stimulation of the plasmalemma H^+/ATPase, causing influx of K^+ (Marre, 1979; see section 1.2.1). It is thought that fusicoccin causes a steric change in the C-terminal end of this ATPase (Johansson *et al.*, 1993). Fusicoccin reverses the effect of abscisic acid (Squire and Mansfield, 1972), and apears to induce malate formation in guard cells (Snaith and Mansfield, 1985; Muller *et al.*, 1991). In addition to its well known effects on the H^+/ATPase, it is thought that fusicoccin also interacts with a high affinity receptor, possibly a hormone receptor (DeBoer *et al.*, 1989; DeMichelis *et al.*, 1989; Meyer *et al.*, 1989).

Responses related to circadian rhythms

Circadian oscillations of stomatal aperture have been observed in many plant types. These oscillations persist in constant conditions for five or more days in detached epidermis. It has been proposed that phytochrome may be involved in entrainment of these rhythms (Zeiger, 1983). Whilst stomata in the "night" phase do open in response to environmental stimuli such as light, they do not do so to the same extent as those in the "day" phase (Snaith and Mansfield, 1985). There is some evidence to suggest that in Crassulacean acid metabolism (CAM) plants, whose stomata open at night, the circadian rhythm may be the dominant stimulus for stomatal movement.

1.1.2 The osmotic relations of guard cells

Opening and closure of stomata are caused by changes in the turgor of the guard cells relative to the epidermal cells. Because of the distribution of cell wall material, an increase in guard cell turgor relative to epidermal cell turgor causes an increase in stomatal aperture. Changes in stomatal aperture therefore result from changes in osmotic relations between the cells of the epidermis, and the maintenence of any given aperture requires the maintenence of osmotic gradients between cells.

The major cation involved in changes in guard cell turgor is K^+ (Penny and Bowling, 1974). The concentration of K^+ in guard cells of open stomata of *Commelina communis* was found to be 450mM, but it was only 95mM in the guard cells of closed stomata (Penny and Bowling, 1974). K^+ concentration has been found to rise in guard cells during stomatal opening in all species which have been studied. The large increases in K^+ concentration are not enough, however, to account for the changes in turgor. Increases in anion content make up the necessary osmoticum, and additionally decrease the energy requirement for the uptake of K^+ by decreasing the charge component of the electrochemical gradient (Zeiger, 1983). In various species, it has been observed that Cl^- and $malate^{2-}$ increase in concentration in guard cells as aperture increases. In almost all species studied, the relative amounts of Cl^- and $malate^{2-}$ which accumulate depend on the abundance of Cl^- available to the guard cells (Raschke, 1979; Allaway, 1981). In *Allium cepa*, which lacks starch in its guard cell chloroplasts, Cl^- is absolutely necessary for stomatal opening (Schnabl and Zeigler, 1977). The accumulation and loss of ions during stomatal movements are dealt with in Introduction section 1.2.

1.2 Guard cell ionic relations and metabolism.

Since it is generally accepted that the large turgor changes occurring in guard cells during stomatal movements are due to changes in intracellular amounts of K^+, $malate^{2-}$, Cl^- and possibly other osmotically active species (such as sugars), many studies have been carried out on the ionic relations of guard cells. Several studies have focussed on measuring the K^+ content of guard cells from various species (Table 1.1). Whilst comparison between guard cells from different species manipulated in different ways is of limited value, the data do serve to show the magnitude of K^+ fluctuations in these cells. In all of the cases where Cl^- content was measured, it was found to be insufficient to balance the observed K^+, although the degree of this shortfall varied between species and, in the case of *Vicia faba* , between the two sets of workers. Raschke and Schnabl (1978) found that, in *Vicia* when epidermis was incubated on 100mM KCl, Cl^- balanced 45% of the K^+, while malate balanced 50%. When no Cl^- was present in the incubation medium, malate balanced 91% of the K^+, only 6% being balanced by Cl^-. This has led to the conclusion that the type and amounts of anions

Species	K^+ concentration (mM)	
	Stomata open	Stomata closed
Zea Mays (Raschke and Fellows, 1971)	400	not available
Vicia faba (Humble and Raschke, 1971)	880	77
Vicia faba (Outlaw et al, 1979)	460 - 760	80
Commelina communis (Penny and Bowling, 1974)	448	95

Table 1.1 K^+ concentrations in guard cells of various species.

Raschke and Fellows used histochemical staining and scanning to measure total K^+ in guard cells. Humble and Raschke, and Penny and Bowling used microelectrodes, while Outlaw and Lowry used an enzymically linked assay on excised guard cells. In all cases K^+ concentrations were calculated by MacRobbie (1981) from the original authors data by assuming a volume of 4pl for guard cells.

accumulated by *Vicia* guard cells depends on the relative abundance of Cl^-. By contrast, guard cells in isolated epidermis of *Allium cepa* cannot open without Cl^- in the medium, nor do they accumulate any malate (Schnabl and Zeigler, 1977). The inability of *Allium* guard cells to synthesise malate has been attributed to their lack of starch (see section 1.2.3). Molecular mechanisms of accumulation and loss of the major solutes involved in stomatal movements have been widely studied.

1.2.1 K^+ uptake and efflux

K^+ ions pass into guard cells from the surrounding epidermis, crossing the guard cell plasma membrane. Two classes of plasma membrane K^+ channel have been identified in guard cells. These are the I_{K^+in} channels (permitting K^+ influx) and the I_{K^+out} channels (permitting K^+ efflux) (Hedrich and Schroeder, 1989). Both channel types have been shown, by patch clamp studies, to be voltage dependent. I_{K^+in} channels are activated by hyperpolarisation to values more negative than -100mV, I_{K^+out} channels are activated by depolarisation to values more positive than -40mV. A plasma membrane H^+ translocating ATPase, which is stimulated (in intact cells) by blue light and by fusicoccin, can hyperpolarise the membrane to -140mV, and as such can stimulate I_{K^+in} opening. H^+ currents have also been shown to be sufficient to drive K^+ uptake at observed rates (Schroeder, 1988). Al^{3+}, which blocks stomatal opening, but not closure, has been shown to block I_{K^+in} channels, but not I_{K^+out} channels.

K^+ transport in and out of the vacuole is much less well understood. Cation channels have been detected in the tonoplast by the patch clamp method (Colombo *et al.*, 1988; Hedrich *et al.*, 1988). Unspecific channels, permeable to both anions and cations, have also been detected by the same method (Hedrich *et al.*, 1986; Coyaud *et al.*, 1987). The regulation of these channels, and the thermodynamics of uptake and loss of various ions by the vacuole are poorly understood. K^+ channels have not been detected in the tonoplast of guard cells.

1.2.2 Cl^- uptake and efflux

Evidence for anion uptake channels permeable to Cl^- has come from work using the H^+ ATPase inhibitor vanadate. This anion inhibits the ATPase activity from the cytoplasmic side, and so must first be uptaken. Schwartz *et al* (1991) attempted to inhibit light stimulated

stomatal opening by floating epidermal peels of *Commelina communis* on KCl solutions containing V_2O_5. They found that when Cl^- concentration was higher than 50mM, no inhibition was seen. They also found that anion uptake channel blockers Zn^{2+} and anthracene-9-carbolic acid inhibited stomatal opening, and concluded that Cl^- interferes with the action of vanadate by competing for the anion uptake channels.

Voltage dependent chloride efflux channels have been detected in guard cells, which have maximal permeability at the same membrane potential (-40mV) at which I_{K^+out} channels are activated (Keller, 1989). Stretch activated ion channels have also been shown to have permeability to anions including Cl^- (Hedrich and Schroeder, 1989).

Two types of tonoplast anion channel, detected by the patch clamp method, could facilitate Cl^- uptake and loss by the guard cell vacuole (Hedrich *et al.* 1988). Fast vacuolar (F.V.) channels are activated by a decrease in cytosolic Ca^{2+} to less than 300nM at both -ve and +ve tonoplast membrane potentials. These can allow Cl^- uptake. Slow vacuolar (S.V.) channels, activated by an increase in cytosolic Ca^{2+} at -ve and low +ve potentials, can allow Cl^- efflux. In the CAM plant *Kalanchoe diagremontiana*, the tonoplast is positive inside (i.e. +ve membrane potential), and this is thought to drive anion uptake (see section 1.2.3).

1.2.3 Malate accumulation and removal.

Studies on rolled epidermis (in which epidermal cells are ruptured and their contents washed away) show that malate accumulates in guard cells of *Commelina communis* and *Vicia faba* as the stomata open in response to light, to such an extent that it is the most significant balancing ion to K^+ (Allaway, 1973). Malate accumulation is also stimulated by ammonia and fusicoccin (Muller *et al.*, 1991). By feeding $^{14}CO_2$ to isolated guard cell protoplasts of *Commelina* in the light, Schnabl (1980) and Willmer (1984) found 60% of label was fixed into malate, indicating that it is synthesised within the guard cells in a process involving fixation of CO_2 . Various studies have shown that epidermal tissue (Willmer *et al.*, 1973) and more specifically guard cells (Schnabl, 1981) contain the enzymes phosphoenolpyruvate carboxylase (PEPc), malate dehydrogenase (MDH), malic enzyme (ME) and pyruvate-phosphate-dikinase (PPDK). Phosphoenolpyruvate carboxylase and malate dehydrogenase fix CO_2 into phosphoenolpyruvate to form first

oxaloacetate then malate in C4 and CAM plants, whilst malic enzyme and pyruvate phosphate dikinase are involved in the breakdown of malate, forming phosphoenolpyruvate to feed into the gluconeogenic pathway (Figure 1.5). Phosphoenolpyruvate carboxylase activities in guard cell protoplasts of *Pisum sativum* have been calculated to be easily sufficient to supply the necessary oxaloacetate for malate production to balance K^+ uptake (Reckmann *et al.*, 1990). Very high levels of cytoplasmic NAD malate dehydrogenase and lower levels of chloroplastic NADP malate dehydrogenase have been detected in guard cells, suggesting that oxaloacetate is reduced in the cytoplasm (Scheibe *et al.*, 1990).

Malate production can be controlled at several steps in plant cells. Starch breakdown by amylase and phosphorylase can be controlled (Preiss, 1982), and subsequent glycolysis of glucose 6-phosphate may proceed by alternative energy and malate production routes, as proposed for CAM plants (Osmond, 1980). Because PEP has alternative fates resulting in either the breakdown or production of starch, the activities of PEP utilising enzymes must be regulated to direct flux in the appropriate direction. Phosphoenolpyruvate carboxylase is regulated in C4 and CAM plants by reversible phosphorylation (see section 1.3). Phosphoenolpyruvate carboxylase activity in guard cells has been widely studied. The rationale for control of phosphoenolpyruvate carboxylase activity in guard cells is simply that the enzyme must be active when guard cells are opening and accumulating malate, and inactive when they are closing and losing malate. Kottmeier and Schnabl (1986) reported a change in K_m for PEP of phosphoenolpyruvate carboxylase from guard cells of *Vicia faba*, decreasing from 0.20mM to 0.01mM as guard cell protoplast diameter increased. Unfortunately in this work the assay conditions were not described. Raschke *et al.* (1988) using guard cell protoplasts from *Pisum sativum* detected, at pH 7.0, a K_m (PEP) change from 0.57mM to 0.28mM after illumination. PEP concentration in *Vicia faba* guard cells has been calculated as 0.27mM (Outlaw and Kennedy, 1978). At this PEP concentration, both of the above Km changes would result in a significant (about two fold) change in phosphoenolpyruvate carboxylase activity, assuming Michaelis-Menten kinetics for the enzyme. Raschke *et al.* (1988) considered the feedback effects of malate build up and cytoplasmic pH change, and calculated that phosphoenolpyruvate carboxylase activity would not change greatly in

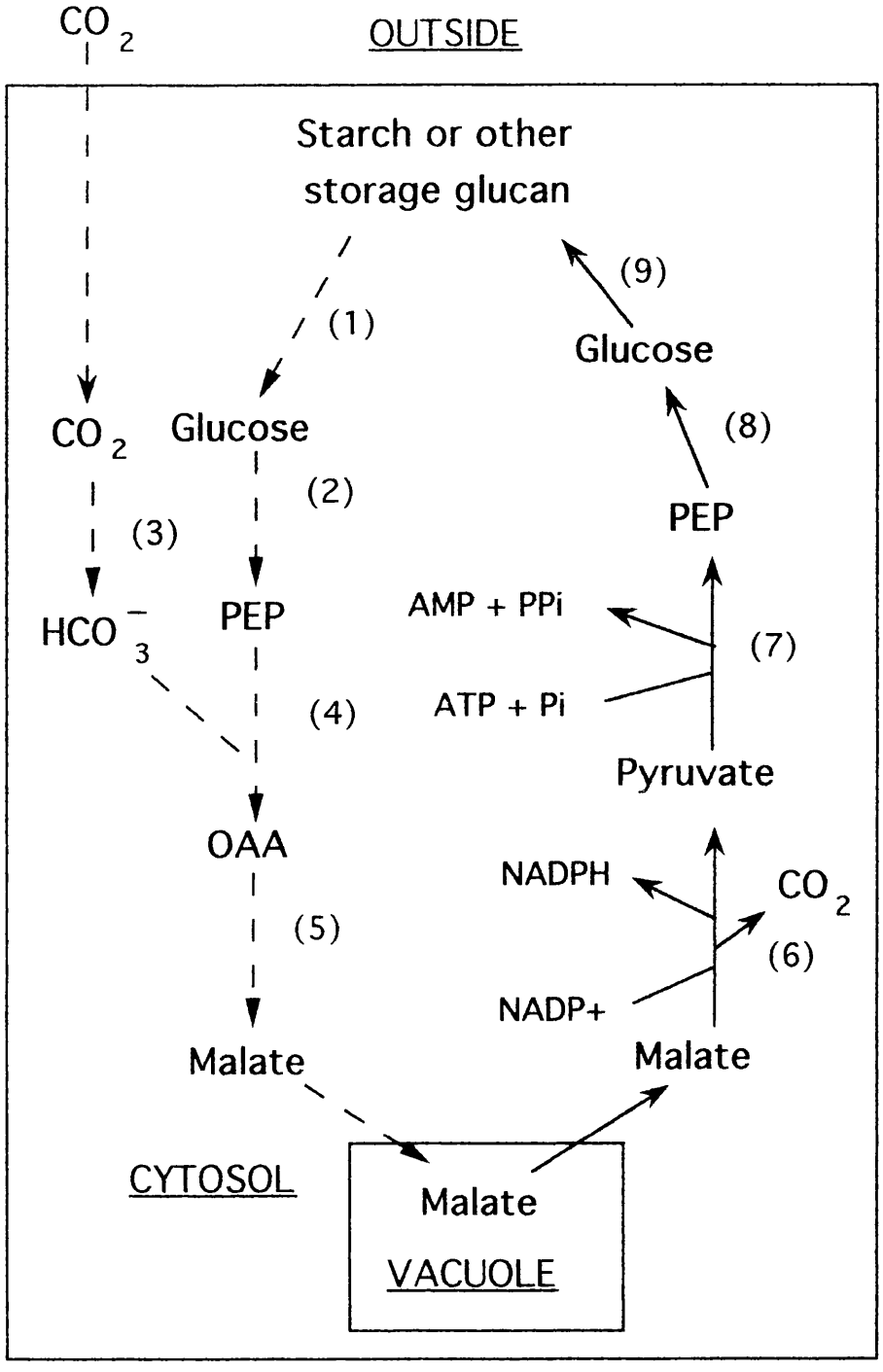


Figure 1.5 Malate metabolism in guard cells

Dashed lines indicate processes predominately occurring during opening, solid lines indicate those occurring during closure. Individual enzymes and processes are: (1) Starch/storage glucan breakdown; (2) Glycolysis; (3) Carbonic Anhydrase; (4) PEPc; (5) MDH; (6) ME; (7) PPDK; (8) Gluconeogenesis; (9) Starch/storage glucan synthesis.

response to cytoplasmic alkalinisation (as caused by proton extrusion) alone.

Outlaw and Tarczynski (1990) used microchemical techniques to assay phosphoenolpyruvate carboxylase in single *Vicia faba* guard cell pairs which had been freeze-quenched at various stages of opening, and then dissected from the epidermis. Their data indicated no assayable differences in phosphoenolpyruvate carboxylase from guard cells at different states of openness, but implied that changes in cytoplasmic pH could modulate activity, with the enzyme much more active at high pH, particularly in the presence of malate (see Table 1.2). Cytoplasmic malate content has been measured at 5.5mM for maize root cells by ^{13}C NMR spectroscopy (Chang and Roberts, 1989), but guard cell cytoplasmic malate concentration is unknown.

The effects of the well known phosphoenolpyruvate carboxylase activator glucose 6-phosphate (G 6-P) were not considered by the above authors, but Denecke *et al.* (1993) using guard cells from closed *Vicia faba* stomata found that inclusion of this effector in the assay causes the kinetics of the enzyme at pH 7.0 to resemble those at pH 8.3 (see Table 1.2). These data indicate that in the presence of glucose 6-phosphate, phosphoenolpyruvate carboxylase activity would be altered very little, even by quite large changes in pH. The nature of control of phosphoenolpyruvate carboxylase activity in guard cells remains controversial.

Uptake and loss of malate by the guard cell vacuole is poorly understood. Malate movements at the tonoplast of CAM plants has been much more widely studied. White and Smith (1989) found that H^+ pumping drives malate uptake into the vacuole of *Kalanchoe diagremontiana*, with two H^+ uptaken for each $malate^{2-}$. Both pyrophosphatase and ATPase type proton pumps have been found in the tonoplast of this plant. Three types of tonoplast channel are permeable to malate. The F.V. and S.V. channels discussed in section 1.2.2. transport malate in the same way as Cl^-, whilst a malate specific tonoplast channel has been detected in *Graptopetalum paraguayense* (Arata *et al.*, 1992). Malate utilisation or loss from the guard cells, which occurs during closure, is less well understood. The removal of malate during closure could occur via anion channels (Keller *et al.*, 1989), but $^{14}CO_2$ labelling studies suggest that malate is decarboxylated (by malic enzyme) and the

Kinetic data of Outlaw and Tarczynski (1990).

	Km_{PEP} (mM)	
	pH 7.0	pH 8.5
No malate	0.62	0.16
5mM malate	3.84	0.29

Kinetic data of Denecke *et al.*

	Km_{PEP} (mM)	
	pH 7.0	pH 8.3
	(-) G-6-P	(-) G-6-P
No malate	0.35	0.06
1mM malate	0.85	0.04
	(+) G-6-P	(+) G-6-P
No malate	0.06	0.05
1mM malate	0.06	0.04

Table 1.2 Kinetic properties of guard cell phosphoenolpyruvate carboxylase

Outlaw and Tarczynski carried out assays on freeze quenched excised guard cell pairs. Denecke *et al.* assayed guard cell phosp[hoenolpyruvate carboxylase purified from epidermal tissue.

resulting pyruvate used to regenerate PEP and ultimately starch via gluconeogenesis (Willmer, 1984).

1.2.4 Contribution of sugars to turgor changes in guard cells.

There are conflicting reports on the contribution of sugars to the changes in turgor of guard cells. Many workers have found very low activities of ribulose *bis*phosphate carboxylase / oxygenase (RUBISCO) and low incorporation of ^{14}C into hexoses in guard cells (Birkenhead and Willmer, 1984; Reckmann 1990). The activity of RUBISCO in guard cells of *Pisum sativum* has been calculated to be too small to make any significant contribution to guard cell turgor (Reckmann, 1990). Under conditions of low external K^+ and high external Ca^{2+}, when stomatal aperture is small, sugars may account for a more substantial proportion of the osmotic content of guard cells (Poffenroth *et al.*, 1992). It is uncertain whether these sugars are products of photosynthesis, or if they result from starch breakdown, which is thought to occur in order to supply glycolytic substrates for PEP, and ultimately, malate production.

In summary, ion channels have been found in the plasma membrane of guard cells which can permit influx and efflux of K^+ and Cl^-, and efflux of malate. Studies on the enzymic content of guard cells, and the pattern of metabolite labelling following a $^{14}CO_2$ pulse indicate that malate is synthesised and decarboxylated during opening and closure respectively, rather than transported across the plasma membrane. Malate synthesis could be controlled through control of the activity of phosphoenolpyruvate carboxylase, and this enzyme has been widely studied. Movement of ions at the guard cell tonoplast is poorly understood, though anion and cation channels have been detected in the tonoplast of CAM plants. A schematic diagram of possible movements of various metabolites and ions during guard cell opening and closure is shown in Figure 1.6.

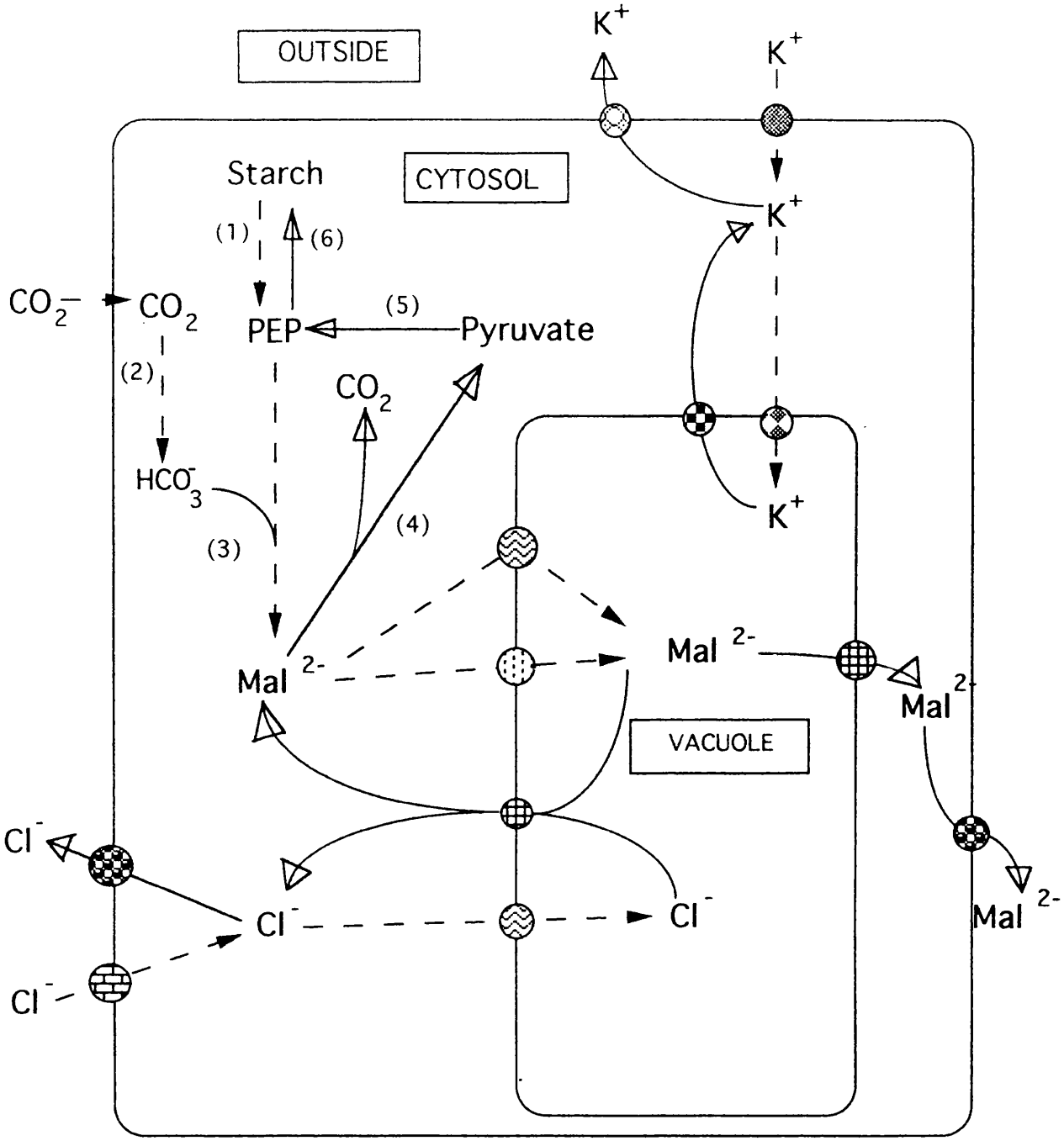


Figure 1.6 Ion fluxes in guard cells

Channel types.

- $I_{K^+ in}$
- $I_{K^+ out}$
- Anion uptake
- Anion efflux
- F.V. channel
- S.V. channel
- Malate uptake (M.U.) channel
- Putative tonoplast K^+ uptake channel
- Putative tonoplast K^+ efflux channel

Processes and enzymes.

(1) Starch breakdown and Glycolysis.

(2) Carbonic anhydrase.

(3) PEP carboxylase and Malate dehydrogenase .

(4) Malic enzyme.

(5) PPDK.

(6) Gluconeogenesis and Starch synthesis.

Dashed lines indicate processes occuring during stomatal opening, solid lines those occuring during closure

1.3 Phosphoenolpyruvate carboxylase - a key enzyme in plant metabolism.

1.3.1 Phosphoenolpyruvate carboxylase activity in plant tissues.

Phosphoenolpyruvate carboxylase [orthophosphate: oxaloacetate carboxylase (phosphorylation) E.C.4.1.1.31] activity is present in all plants, but is absent from mammalian tissues (O'Leary, 1982). The highly exergonic reaction catalysed by this enzyme is:

$$\underset{\text{(PEP)}}{{}^{-}O_2C\text{-}C(OPO_3^{2-})=CH_2} + HCO_3^{-} \text{ -----> } \underset{\text{(OAA)}}{{}^{-}O_2C\text{-}C(=O)\text{-}CH_2\text{-}CO_2} + HPO_4^{2-}$$

The enzyme requires a divalent cation for activity, and either Mg^{2+} or Mn^{2+} may be used *in vivo* (Nguyen *et al.*, 1987). Zn^{2+}, Ca^{2+}, Cu^{2+} and Cd^{2+} are all inhibtory (Andreo *et al.*, 1987). Phosphoenolpyruvate carboxylase from *Zea mays* is thought to have a random sequential mechanism. The preferred order of addition of reagents is first Mg^{2+}, then PEP and finally HCO_3^-. There is a high level of synergism in binding of substrates. Carboxyphosphate, the enolate of pyruvate and carbon dioxide have all been implicated as intermediates in the reaction (Janc *et al.*, 1992). Phosphoenolpyruvate carboxylase is unique as it can activate HCO_3^- by hydrolysis of a phosphate bond. Other carboxylases which utilise HCO_3^- rather than CO_2 need biotin (Wood and Bardon, 1977).

Ting and Osmond (1973) suggested that there are four molecular species of phosphoenolpyruvate carboxylase in higher plants: C3; C4 photosynthetic; CAM and non-autotrophic types. In addition, tissue specific forms of the enzyme may exist within single leaves. Schulz *et al.* (1992), detected three different phosphoenolpyruvate carboxylase subunits in leaves of *Vicia faba* , distinguishable by their molecular weight. All three subunits could be detected in guard cell protoplasts, but only the smallest could be detected in mesophyll and epidermal cell protoplasts, suggesting a specific isoform of the enzyme for guard cell function. It is thought that the C3 and non-autotrophic types serve an anaplerotic role, topping up the tricarboxcylic acid. cycle as intermediates

are removed for biosynthesis (Melzer and O'Leary, 1987; Schuller *et al.*, 1990). The C4 and CAM type enzymes are responsible for the primary fixation of atmospheric CO_2. In C4 plants phosphoenolpyruvate carboxylase is synthesised during greening of the tissue (Goatly and Smith, 1974; Perrot *et al.*, 1984). The C4 and C3 forms of the enzyme can be separated chromatographically and can be distinguished by molecular weight, immunochemical properties, tryptic digest peptide maps and kinetic properties (Ting and Osmond, 1973; Goatly and Smith, 1974; Vidal *et al.*, 1983). CAM phosphoenolpyruvate carboxylase has been studied in several plant species, including *Bryophyllum fedtschenkoi* (Jones *et al.*, 1981; Pays *et al.*, 1980; Nimmo *et al.*, 1984,1987) and the inducible CAM plant *Mesembryanthemum crystallinum* (Winter, 1981, 1982). Studies on guard cell phosphoenolpyruvate carboxylase are discussed in the previous section (1.2), whilst the much more extensive studies on C4 and CAM phosphoenolpyruvate carboxylase are discussed in the following sections.

1.3.2. Structure

Comparisons of phosphoenolpyruvate carboxylase protein by immunological methods or of mRNA transcripts encoding the enzyme have shown that there is some similarity between the C4 and CAM photosynthetic forms, as well as the C3 form and non-photosynthetic forms from C4 and CAM plants, and the guard cell enzyme. (Muller *et al.*, 1980; Matsuoka and Hata, 1987; Miller *et al.*, 1987; Thomas *et al.*, 1987; Schulz *et al.*, 1992). This indicates the possible existence of conserved regions in the protein from different sources. The genes encoding the photosynthetic forms of the enzyme from *Zea mays* and *Mesembryanthemum crystallinum* have been sequenced. The deduced amino acid sequences of the two consist of 970 and 966 residues respectively. There is 75% identity between these two enzymes, showing them to be more closely related to one another than to the *Escheichia. coli* and *Anacystis nidulans* enzymes, with which they have 30-40% identity. One 14 residue sequence, proposed as the PEP binding site (Ishijima *et al.*, 1985) is absolutely conserved between the C4 and CAM sequences, and highly conserved in the *Escherichia. coli* and *Anacystis nidulans* sequences. Additionally, this sequence is rich in glycine, and contains basic histidine and arginine residues which are conserved in all

four species (Rickers *et al.*, 1989). Histidine, arginine, cysteine and lysine residues have all been proposed as important in the active site of the enzyme (Stiborova and Leblova, 1983; Rustin *et al.*, 1988; Andreo *et al.*, 1987; Gonzalez and Andreo, 1989). Histidine residues have also been implied as important constituents of the malate binding site (Taghizadeh *et al.*, 1991).

Phosphoenolpyruvate carboxylase subunits from plant species are in the range 100-130kDa in size, depending on the species, and generally the holoenzyme appears as a tetramer, with the exception of the enzyme from *Crassula argentea*, which is a dimer (Weigend and Hincha, 1992). In most plant species, the subunits of the holoenzyme are identical, but in *Bryophyllum fedtschenkoi* and some species of *Sedum* and *Kalanchoe*, as well as in guard cells of *Vicia faba* , subunits dissimilar with respect to their molecular mass have been detected (Muller *et al.*, 1981; Nimmo *et al.*, 1986; Schulz *et al.*, 1992). The significance of these different subunits is not understood. Diurnal changes in oligomerisation state have been suggested as a mechanism for regulation of phosphoenolpyruvate carboxylase, and this, as well as other aspects of the regulation of the enzyme are discussed in the next section.

1.3.3 Regulation

Phosphoenolpyruvate carboxylase is regulated allosterically, the main effectors being glucose 6-phosphate and malate (O'Leary, 1982). Glucose 6-phosphate is an activator of both C4 and CAM phosphoenolpyruvate carboxylase. This effector has been shown to decrease the enzyme's K_m for PEP (Coombs *et al.*, 1973; Ting and Osmond, 1973; Huber and Edwards, 1975; Uedan and Sugiyama, 1976; Mukerji, 1977; Stibrova and Leblova, 1985; Rodrigues-Sotres *et al.*, 1987), although it has other, more complex effects. Maximal activation by glucose 6-phosphate is dependent on PEP concentration (Pays *et al.*, 1981; Nott and Osmond, 1982; Wedding *et al.*, 1989), and this effector can also reverse inhibition by malate (Pays *et al.*, 1980).

The inhibition of phosphoenolpyruvate carboxylase by malate plays a very important role in the control of the CAM and C4 enzyme. Large diurnal fluctuations in the malate sensitivity of phosphoenolpyruvate carboxylase have been demonstrated in *Bryophyllum fedtschenkoi* (Nimmo *et al.*, 1984, 1986), *Mesembryanthemum crystallinum* (Winter, 1980,1982), *Crassula*

argentea (Wu and Wedding, 1985; Wedding *et al.*, 1990), and *Sedum telephium* (Manetas, 1982). At high pH (pH 7.5-pH 8.0) malate acts as a competitive inhibitor, whilst at lower pH the type of inhibition varies, but the enzyme is more sensitive (Pays *et al.*, 1980; Nott and Osmond, 1982; Winter, 1982). It has also been found that proteolytic cleavage of a peptide from the N-terminus of phosphoenolpyruvate carboxylase can greatly reduce malate sensitivity (Nimmo *et al.*, 1986; McNaughton *et al.*, 1989). Sequence analysis of the enzyme reveals a proteolysis sensitive region 40 residues from the N-terminus which if cleaved would release a 4kDa peptide (Chollet, 1990).

Several other effectors of phosphoenolpyruvate carboxylase have been reported *in vitro*, but their physiological significance is not known. Glucose 1-phosphate and fructose 1-phosphate activate *Bryophyllum fedtschenkoi* phosphoenolpyruvate carboxylase at pH 7.8, while fructose 1,6-*bis*phosphate activates it at pH 5.8 (Pays *et al.*, 1980). Pi was found to be inhibitory in *Bryophyllum fedtschenkoi* (Jones *et al.*, 1978) and maize (Doncaster and Leegood, 1987) but Walker *et al.* (1988) observed Pi stimulation in maize. AMP and ADP stimulated phosphoenolpyruvate carboxylase activity in *Crassula*, while ATP was inhibitory (Rustin *et al.*, 1988). AMP stimulation and ATP inhibition have also been demonstrated in other plants (Wong and Davis, 1973; Lavergne and Champigny, 1983; Walker *et al.*, 1988).

In C4 and CAM plants there is clearly a need to regulate flux through phosphoenolpyruvate carboxylase, with flux being switched off when atmospheric CO_2 is not being fixed (at night in C4 plants, and during the day in CAM). Two major mechanisms for control of the enzyme have been proposed and investigated- diurnal changes in the oligomeric state of the enzyme and reversible phosphorylation.

The control of phosphoenolpyruvate carboxylase by a change in the oligomeric state between tetramer (active) and dimer (inactive) has been widely studied by a variety of methods but remains controversial. Wu and Wedding (1985) observed diurnal changes in the ratio of dimer : tetramer in *Crassula* , and the process of aggregation of dimers to form tetramers has been proposed to account for hysteretic lags in reaction rates for phosphoenolpyruvate carboxylase from *Crassula* (Ngam-ek, 1989) and maize (Lopez-Pozos, 1990). In the latter case, the observed lag was greater in phosphoenolpyruvate carboxylase extracted from plants which had been kept in the dark, suggesting that aggregation state

is important in the physiological regulation of the enzyme. In contrast, a number of studies have concluded that there are no diurnal cycles of aggregation / disaggregation in *Bryophyllum* (Nimmo *et al.*, 1986), *Kalanchoe* (Kruger and Kluge, 1988), or maize (McNaughton *et al.*, 1989). Some of the variation in observations may be caused by single authors only working on the enzyme from one particular species, with little cross referencing possible. One wide ranging study by Weigend and Hincha (1992) used high performance size exclusion liquid chromatography on phosphoenolpyruvate carboxylase from C3, C4, CAM and inducible CAM (in both physiological states). No diurnal variation in oligomeric state was found. The enzyme from all species tested was in the tetrameric form, except that from *Crassula*, which was in dimeric form throughout the diurnal cycle. In the absence of any study using other methods (such as light scattering) to measure the size of the holoenzyme from a wide range of sources, the evidence for variation in oligomeric state of phosphoenolpyruvate carboxylase as a regulatory factor is inconclusive.

Reversible phosphorylation of phosphoenolpyruvate carboxylase has been observed and studied in *Bryophyllum fedtschenkoi* (Nimmo *et al.*, 1984, 1986), *Kalanchoe* (Brulfert *et al.*, 1986), maize (Nimmo *et al.*, 1987; Jiao and Chollet, 1988), *Sorghum* (Guidici-Orticoni, 1988) and wheat (Van Quy and Champigny, 1992). In all cases the phosphorylated form of the enzyme is regarded as the active form. The nature of the activation brought about by phosphorylation is generally a reduction in the sensitivity to feedback inhibition by malate, although increases in V_{max} and decreases in K_m (PEP) have also been observed. Jiao *et al.* (1991) identified the regulatory phosphorylation site in phosphoenolpyruvate carboxylase from maize and *Sorghum* as a serine residue near the N-terminus of the polypeptide. Interestingly, C3 type phosphoenolpyruvate carboxylase from wheat has also been found to undergo activation by phosphorylation (Van Quy and Champigny, 1992). This has been proposed as a mechanism for increasing phosphoenolpyruvate carboxylase activity in C3 leaves during nitrogen assimilation, to top up the tricarboxcylic acid cycle.

The stimuli causing increased phosphorylation of phosphoenolpyruvate carboxylase vary from species to species. In *Bryophyllum*, phosphorylation is under circadian control (Nimmo *et al.*, 1987), whilst in maize it has been shown to be light-dependent

(Echevarra *et al.*, 1990). In wheat, phosphorylation is stimulated by light and nitrate availability (Van Quy and Champigny, 1992). The kinase responsible for the regulatory phosphorylation of phosphoenolpyruvate carboxylase (termed PEPc kinase) has been identified in several species (Jiao and Chollet, 1989; Carter *et al.*, 1991; McNaughton *et al.*, 1991; Bakrim *et al.*, 1992), and has been shown to be Ca^{2+} independent. Pierre *et. al.* (1992) found that phosphoenolpyruvate carboxylase phosphorylation increased when Sorghum mesophyll protoplasts were incubated with Ca^{2+}, and decreased when incubated with EGTA, however it is not clear wether this is a direct effect on the kinase, or some signalling process which induces the kinase. The phosphatase responsible for dephosphorylation has been identified as a type 2A protein phosphatase (Carter *et al.*, 1990; McNaughton *et al.*, 1991). In *Bryophyllum*, maize and wheat, the kinase level varies in response to stimuli, whilst the phosphatase level remains relatively constant (Carter *et al.*, 1991; McNaughton *et al.*, 1991; Van Quy and Champigny, 1992). Carter *et al.* (1991) showed that protein synthesis inhibitors prevented the appearance of kinase activity in intact *Bryophyllum* leaves, whilst many potential cytosolic effectors had little effect on the kinase activity *in vitro* (Jiao and Chollet, 1989; Carter *et al.*, 1991; McNaughton *et al.*, 1991). Cycloheximide has also been shown to block the light stimulated appearance of PEPc kinase in maize (Jiao *et al.*, 1991) and *Sorghum* (Bakrim *et al.*, 1992). Furthermore, inhibitors of the Calvin cycle and non cyclic electron flow can block both activation of phosphoenolpyruvate carboxylase (Karabourniotis *et al.*, 1983; Samaras *et al.*, 1988; McNaughton *et al.*, 1991) and the appearance of PEPc kinase in C4 plants (Jiao and Chollet, 1992). This indicates that both the action of the photosystems in the mesophyll cells and the Calvin cycle in the bundle sheath cells are necessary for the appearance of PEPc kinase. As the characteristics of phosphorylation and dephosphorylation of phosphoenolpyruvate carboxylase are elucidated, interesting comparisons and contrasts can be made with other protein phosphorylation systems in both plants and animals.

1.4 Phosphorylation as a control mechanism in higher plants

1.4.1 Protein kinases

Reversible phosphorylation is now well established as a key regulatory mechanism in animal cells (Krebs and Beavo, 1979), and it is becoming clear that the same mechanism is important in plant tissues (reviewed in Ranjeva and Boudet, 1987). Two reactions are involved in

reversible phosphorylation:

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

$$\text{Protein-P}_n + n\text{H}_2\text{O} \dashrightarrow \text{Protein} + n\text{Pi} \quad (2)$$

Reaction 1 (phosphorylation) is catalysed by protein kinase(s) and reaction 2 (dephosphorylation) by protein phosphatase(s). Most protein kinases use ATP as a phosphate donor. Phosphorylation occurs on serine, threonine or tyrosine residues, and is thought to affect enzyme activity via conformational changes. Protein kinases generally recognise amino acid motifs surrounding the target amino acids. Peptide substrates containing these motifs have been successfully used as substrates for kinases. Phosphorylation of an intact protein containing an appropriate motif depends, of course, on whether the motif is accessible, and can be modified by factors that affect the conformation of the protein.

Protein kinases usually contain a regulatory domain and a highly conserved catalytic domain, which can be on the same or different polypeptides, for example protein kinase C and protein kinase A respectively. The catalytic domain 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 pseudo-substrate sequence motifs which serve to inhibit the protein kinase. For example, activation of protein kinase C is thought to occur by removal of the pseudo-substrate 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 (CDPKs) are a large group, which include the Ca^{2+}-calmodulin dependent and Ca^{2+}-phospholipid dependent (protein kinase C) protein kinases. Other groups of protein kinases are activated by the cyclic nucleotides cAMP or cGMP. The "independent" kinases are a group which are named according to the substrate they specifically phosphorylate, for example casein kinase (Rosen and Krebs, 1981).

Protein phosphorylation is well documented in animal systems, and an ever increasing number of kinases is being described. In plants the documentation of protein phosphorylation and protein kinases is less well advanced, but is increasing rapidly. Molecular biological techniques, using oligonucleotide probes to highly conserved catalytic domains of mammalian protein kinases, have allowed isolation of

homologous sequences from plant gene libraries (Lawton *et al.*, 1990). These studies have indicated the presence of a variety of protein kinase genes in a large number of plant types, for example Elliot and Brennan, 1990; Zeilinski *et al.*, 1990; Lawton *et al.*, 1989; Walker and Zhang, 1990. Despite the identification of these protein kinase genes, no information is available concerning their substrate specificities or physiological function. The presence of a large number of plant protein kinases of various types has also been inferred from observations of labelling of proteins with ^{32}P after tissues or organelles have been allowed to take up either [^{32}P]- orthophosphate or [$\gamma\,^{32}P$]-ATP (Reymond *et al.*, 1993; Monroy *et al.*, 1993; Johnson and Chrispeels, 1993). In these cases, substrate specificity is again unknown, although the potential physiological role of the protein kinase can be inferred from the conditions under which phosphorylation occurs. A number of protein kinase activities with clear substrate specificities and physiological roles have been identified, and these are listed in section 1.4.2.

Protein kinases are integral components of intracellular signal transduction systems in animals, and it is likely that they play the same role in plants. No complete signal transduction pathway has as yet been elucidated in plants, but a number of signal transduction intermediates and second messengers, analagous to those found in animal cells, have been identified in plants. The study of signal transduction in plants is discussed in section 1.5.

1.4.2 Plant enzymes regulated by phosphorylation

There are currently eight plant enzymes known to be regulated by reversible phosphorylation. The pyruvate dehydrogenase complex (PDC) was the first plant enzyme to be shown to be controlled by this mechanism. PDC is inactivated by phosphorylation, and high ratios of ATP/ADP, acetyl CoA/CoA and NADH/NAD^+ in the mitochondria all enhance this inactivation (Randall *et al.*, 1981). Many photosynthetic processes are also regulated by phosphorylation. The light energy distribution between photosystems I and II is controlled by phosphorylation of the light harvesting chlorophyll a/b complex. This phosphorylation is carried out by a soluble light dependent protein kinase (Bennet, 1984). Both the small and large subunits of RUBISCO can be phosphorylated (Guitton and Mache, 1987). This example shows how protein phosphorylation can act as part of a multi-component regulatory

"package". As well as reversible phosphorylation, control mechanisms affecting RUBISCO include transcriptional control (Gilmartin *et al.*, 1990), carbamylation (by RUBISCO activase) (Salvucci *et al.*, 1987) and inhibition by *carboxy*arabinitol 1-phosphate (Gutteridge *et al.*, 1987).

Light/dark regulation of sucrose phosphate synthase is due to reversible phosphorylation (inactivation) / dephosphorylation (activation) of the enzyme. Sucrose phosphate synthase also contains other phosphorylation sites, which do not appear to be involved in the light/dark regulation of the enzyme (Huber and Huber, 1990).

The enzymes phosphoenolpyruvate carboxylase and pyruvate, phosphate dikinase, involved in CAM and C4 metabolism, are also known to be regulated by phosphorylation. Pyruvate, phosphate dikinase is inactivated by an unusual ADP dependent phosphorylation on a threonine residue, and activated by a Pi dependent dephosphorylation (Burnell and Hatch, 1984; Edwards *et al.*, 1985). A bifunctional protein is thought to catalyse both processes. Pyruvate, phosphate dikinase is activated in the light, but it is thought that the effects of light on the phosphorylation are indirect. Chollet *et al.* (1989) suggested that stromal metabolites may regulate the phosphorylation of pyruvate, phosphate dikinase. The regulation of the enzyme phosphoenolpyruvate carboxylase in various plant types is discussed in section 1.3.

Other enzymes, 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 reductase, which is involved in isoprenoid biosynthesis (Russel *et al.*, 1985).

1.4.3 Protein phosphatases

The phosphorylation process is reversed by hydrolysis of phosphate groups from the protein, and this process is catalysed by protein phosphatases. Systems which are controlled by reversible phosphorylation require both a protein kinase and a protein phosphatase, and the relative activities of these two enzymes defines the level of phosphorylation of the target protein. This concept is neatly illustrated by Champigny and Foyer (1992) in their proposal for a mechanism of coordinated control of sucrose phosphate synthase and

phosphoenolpyruvate carboxylase with nitrogen metabolism in plant leaves.

Protein phosphatases are one of the most highly conserved groups of enzymes throughout evolution (Cohen and Cohen, 1989), and this has allowed close comparisons to be drawn between plant and mammalian protein phosphatases. Four major classes of protein phosphatase catalytic subunit have been identified in mammals (reviewed in Cohen, 1989), and plant protein phosphatases are currently being found which fall into these classes. Classification is based on substrate specificity and sensitivity to activators and inhibitors.

Type 1 protein phosphatases preferentially dephosphorylate the ß-subunit of phosphorylase kinase. They do not require metal ions and are inhibited by the thermostable proteins inhibitor-1 and inhibitor-2 (Cohen *et al.*, 1988). The type 2 protein phosphatases preferentially dephosphorylate the α-subunit of phosphorylase kinase. There are three type 2 protein phosphatases, distinguishable by their requirement for metal ions. Type 2A does not require metal ions, while type 2B requires Ca^{2+}/calmodulin and type 2C requires Mg^{2+} (Cohen, 1989). Identification and quantification of different protein phosphatases is assisted by the tumor promoter okadaic acid. This compound inhibits type 1 and 2A protein phosphatases, but is a much more potent inhibitor of type 2A (K_i approximately 1nM) than type 1 (K_i approximately 10-15nM) (Cohen *et al.*, 1989). Other inhibitors of type 1 and 2A protein phosphatases have been discovered, including microcystin-LR, which is a cyanobacterial compound and tautomycin, which is a polyketide from *Streptomyces* (MacKintosh *et al.*, 1990; MacKintosh and Klumpp, 1990).

A number of protein phosphatases have been identified in plants, some by molecular biological techniques, others by direct assay of activity. The gene for type 1 protein phosphatase has been detected in maize (Smith and Walker, 1991), and the genes for type 1 and type 2A protein phosphatases have been detected in *Brassica oleracea* (Rundle and Nasrallah, 1992). In *Arabidopsis thaliana*, a family of at least 6 genes encoding protein phosphatase 2A has been found (Arino *et al.*, 1993).

Type 1 and 2A protein phosphatase activities have been measured in extracts of *Brassica napus* (MacKintosh and Cohen, 1989), and the 2A activity has been found to dephosphorylate several important

phosphoproteins, including sucrose phosphate synthase (Siegl *et al.*, 1990); phosphoenolpyruvate carboxylase (Carter *et al.*, 1990) and quinate dehydrogenase (MacKintosh *et al.*, 1991). In addition, type 2C protein phosphatase has been detected in a variety of plants (MacKintosh *et al.*, 1991), and a chloroplast protein phosphatase activity has been discovered which may fall outside the standard classification system (Sun and Markwell, 1992).

It is clear, therefore, that plants contain protein phosphatases and kinases which can carry out enzyme regulation, and possibly other functions, in an analagous manner to those in mammalian systems. The presence in plants of other components of signal transduction pathways, and their similarities and and dissimilarities to their mammalian counterparts will be discussed in section 1.5.

1.5 Stimulus-response coupling in guard cells

1.5.1 Signal transduction pathways

Many authors have proposed that, since guard cells respond to a large number of stimuli in an easily reproducible and measureable way (either by swelling or shrinking), they are a suitable cell type for investigation into signal transduction in plants (Serrano and Zeiger, 1989; McAinsh *et al.*, 1992; Lee *et al.*, 1993; Parmar and Brearly, 1993). The literature regarding signal transduction in guard cells has been reviewed by Assmann (1992). The coupling of a stimulus (such as an environmental or hormonal signal) to a response (such as a change in metabolic state of a cell or tissue) requires a signal transduction pathway. In mammalian systems, these pathways have been found to consist of many components. The signal is perceived by a receptor - often a membrane bound protein. This receptor transduces the signal into one or more internal signals, either by chemically modifying cellular proteins or by producing second messengers. Second messengers are small molecules whose cytosolic concentrations change on reception of certain stimuli. Internal receptors for these second messengers become altered on binding, changing their biological activity. Protein kinases and phosphatases as well as ion channels and pumps can all be altered in this way, resulting in the observed response. The receptors, second messengers and targets of second messengers are all components of signal transduction.

Whilst a great deal is known about the signal transduction components present in mammals, much less is known about any most stimulus-response processes in plants. Many enzyme activities and compounds - known to be components of signal transduction pathways in mammals - have now been identified in plants, and stimulus response coupling is being studied particularly exhaustively in guard cells. One signal transduction mechanism known to exist in mammals - the Ca^{2+}/phosphoinositide system - is currently being investigated by many workers, and the evidence for the existence of this system in plants, as well as its relevance to stomatal function, are discussed in sections 1.5.2 and 1.5.3. Protein kinase and phosphatase activities have already been discused in section 1.4, and ion channels and pumps have been discussed in section 1.2.

1.5.2 Evidence for Ca^{2+} as a signalling component in plant cells, and its relevance to stomatal function

The literaure on the regulation of cytosolic calcium concentration in plants has been reviewed by Bush (1993), and a review of the literature concerning the calcium modulated proteins calmodulin and calcium dependent protein kinase in plants has also been published (Roberts and Harmon, 1992).

Many workers have measured cytosolic calcium concentrations in living plant cells using fluorescence imaging techniques. Cytosolic calcium concentration has been found to vary in response to stimuli in a number of ways. Some stimuli (such as touch and temperature change) cause a transient increase in calcium concentration lasting a number of minutes. The plant growth regulators gibberelic acid and cytokinin have been found to increase calcium concentration to a steady state level, whilst in some tissues other stimuli (such as auxin) can cause oscillations in cytosolic calcium concentration (Bush, 1993). Cytosolic calcium and cytosolic pH were found to vary simltaneously by Gehring *et al.* (1990). This group incubated coleoptile, hypocotyl and root tissues with acetoxymethyl esterified forms of the Ca^{2+} indicator Fluo-3 and the pH indicator 2',7'-*bis*(2'carboxyethyl)-5(6)-carboxyfluorescein. The tissues were then incubated with either abscisic acid or an auxin analougue and cytosolic Ca^{2+} concentration and pH values were measured after 4 min. Both treatments resulted in an approximately 1.5 fold increase in cytosolic calcium concentration. Abscisic acid increased cytosolic pH by

0.05 - 0.1 pH units, while the auxin analogue reduced pH by 0.1 to 0.2 pH units. The authors suggest that, while both stimuli cause the same increase in Ca^{2+} concentration, the different changes in pH mediate the profound differences in the responses of the tissues to abscisic acid and auxin.

It has been known for some time that when epidermis is floated on a solution containing calcium, stomatal opening is inhibited (Inoue and Katoh, 1987). Fluorescence imaging methods have been used to investigate changes in cytosolic calcium concentration in response to a variety of stimuli in guard cells. Gilroy *et al.* (1990) used *Commelina communis* stomata in a study which employed the Ca^{2+} indicator indo-1. These authors found that the release of injected caged Ca^{2+} or caged inositol(1,4,5)*tris*phosphate in guard cells of open stomata caused a transient increase in cytosolic calcium concentration, and when this exceeded a threshold value (of between 500 to 600nM, or 2.5 to 3 times resting calcium concentration) stomatal closure occurred. Gilroy *et al.* (1991), again using the same experimental system, stimulated stomata to close by transferring the epidermis from a solution containing a high concentration of K^+ to one containing a low K^+ concentration, or from a solution containing a low concentration of Ca^{2+} to one containing a higher concentration, or by adding the Ca^{2+} ionophore Br-23187. The authors found that in all cases where closure occurred there was a transient increase in cytosolic calcium concentration, although this was unevenly distibuted throughout the cell, being greatest at the vacuolar and nuclear membranes. When stomata in this study were stimulated to close by abscisic acid, an increase in cytosolic calcium concentration occurred in only 26% of cases, whilst closure always occurred.

More reproducable increases in cytosolic Ca^{2+} concentration in response to abscisic acid were observed by McAinsh *et al.* (1992). This group also used *Commelina communis* guard cells and indo-1 for their study. In 68% of cases where open stomata were stimulated to close by incubation with abscisic acid, transient increases in cytosolic Ca^{2+} were observed. The distribution of Ca^{2+} was similar to that observed by Gilroy *et al.* (1991).

Irving *et al.* (1992) measured cytosolic Ca^{2+} concentrations in guard cells from the orchid *Paphiopedilum tonsum* using the indicator fluo-3. This group also observed cytosolic pH using 2',7'-*bis*(2'carboxyethyl)-5(6)-carboxyfluorescein. When open stomata were

stimulated to close by incubation of epidermis on a solution containing abscisic acid, there was a 1.5 to 3 fold transient increase in Ca^{2+} concentration in 70% of cases, and an increase in pH of 0.04 to 0.3 units in 93% of cases. These workers also stimulated closed stomata to open using auxin, kinetin and fusicoccin. The resulting changes in Ca^{2+} and pH (similar for all three, but typified by the results for auxin) were a 1.5 to 2 fold increase in Ca^{2+} concentration in all cases, and a decrease in pH of 0.2 to 0.4 units (frequency not given).

The results of these studies suggest that Ca^{2+} transients accompany both opening and closing responses, but that the differences may be mediated by different changes in cytosolic pH. In all of the above studies, where Ca^{2+} transients are not observed in 100% of cases where opening or closure is caused, there is the suggestion that due to the timing of sampling, the transient might be missed in some cases.

1.5.3 Evidence for the presence of the phosphoinositide system in plants, and its relevance to stomatal function

A great deal of work has been carried out to investigate wether the phosphoinositide system - a major signalling mechanism in mammalian cells - also operates in plant cells. This work has been reviewed by Drøbak (1992).

Polyphosphoinositides do exist in plant cell membranes (Sandelius and Sommarin, 1990), and membrane associated phospholipase C activity has also been detected (Melin *et al.*, 1987). There is conflicting evidence as to wether G proteins are involved in the activation of phospholipase C. Some workers have found no activation of phospholipase C by GTPγS (Melin *et al.*, 1987; McMurray and Irvine, 1988; Tate *et al.*, 1989; Biffen and Hanke, 1990), whilst Einsphar *et al.*, (1989) stimulated phospholipase C activity in plant vesicle preparations using GTPγS. Dillenschneider *et al.* (1986) did not measure phospholipase C activity, but did find that sycamore cell membranes released increased levels of inositol phosphates when incubated with guanine nucleotides. Protein kinase C activity has not been detected in plant cells (Drøbak, 1992).

Strong evidence that the phosphoinositide system operates in guard cells was put forward by Gilroy *et al.* (1990) who found that guard cells injected with caged inositol(1,4,5)*tris*phosphate exhibited a transient increase in cytosolic Ca^{2+} concentration after it was released, and when

this concentration exceeded a threshold value, stomatal closure occurred. Inositol phosphates have been identified in guard cells, including inositol *tris*phosphates, although inositol(1,4,5)*tris*phosphate has not yet been rigorously identified (Parmar and Brearly, 1993). Lee and Assmann (1991) found that incubation of epidermis in medium containing diacylglycerol caused an enhancement of opening in response to light and and inhibition of closure in response to darkness. In addition, proton pumping at the plasma membrane was found to be enhanced. This suggests the involvement of protein kinase C. Lee *et al.* (1993) found that GTPγS and pertussis toxin both induced stomatal opening on the epidermis of *Commelina communis*. This is difficult to explain, since GTPγS activates G proteins, which then activate phospholipase C, whilst pertussis toxin inhibits this process. The observation that diacylglycerol and inositol(1,4,5)*tris*phosphate have opposite effects is also difficult to explain, since if an inositol phospholipid is cleaved by phospholipase C, both compounds will be produced, although diacylglycerol can be produced from other phospholipids also.

In summary, Ca^{2+} transient increases and pH changes appear to accompany stomatal movements. Many of the components necessary for the operation of the phosphoinositide system are present in guard cells, which also appear to be able to respond to various intermediates and inhibitors of the phosphoinositide system. The question of whether Ca^{2+} transients (and possibly pH changes) result from the operation of the phosphoinositde system, and how the phosphoinositide system can coordinate multiple (and often opposite) stimuli requires more investigation. Observations of changes in calcium and pH have certainly provided much information on signal transduction in plants, and while it is interesting to observe the effects of various signal transduction intermediates on stomatal aperture, it must be remembered that the change in aperture is the result of a complex series of events including for example changes in enzyme activity, ion channel activity and the utilisation of intracellular storage glucan. As a result, it is difficult to tell whether the alteration of the cytosolic concentration of a single signalling intermediate will produce a competent response - indeed this may be the cause of some of the contrary observations various workers have made. A valuable option, then, is the study of an enzyme activity which is different during opening than it is during closure due to a covalent modification. The factors causing such a modification (for example

protein kinases and phosphatases) could then be studied, allowing the signal transduction pathway to be elucidated. For this reason, the study of the control of phosphoenolpyruvate carboxylase is important to the study of signal transduction in plants.

1.6 Objectives

Stomatal opening and closure result from changes in the cellular concentrations of K^+ and balancing anions, most importantly malate and Cl^-. Malate synthesis in guard cells occurs via the fixation of CO_2 by phosphoenolpyruvate carboxylase to form oxaloacetate, which is subsequently reduced to form malate. Malate is synthesised during stomatal opening, and decarboxylated during closure (Zeiger, 1983). Because of the central position of PEP in both malate production and gluconeogenesis, it is necessary to control the activity of phosphoenolpyruvate carboxylase in order to avoid futile cycling. In CAM and C4 plants, in which phosphoenolpyruvate carboxylase is the primary carbon-fixing enzyme, its activity is controlled by reversible phosphorylation. At the time this study was undertaken, it was not known whether guard cell phosphoenolpyruvate carboxylase could be phosphorylated, either *in vivo* or *in vitro*. The major aim of this study was therefore to determine whether this enzyme is controlled by reversible phosphorylation in guard cells; and, if it is, whether phosphorylation is stimulated by opening stimuli (such as light and fusicoccin), and dephosphorylation is stimulated by closing stimuli (such as darkness and abscisic acid). The other major aim of this study was to determine whether changes in the activity of phosphoenolpyruvate carboxylase occur in response to opening and closing stimuli and if phosphorylation were shown to occur, whether it was accompanied by changes in enzyme activity. In addition, some aspects of the signalling process involved in stimulating the putative phosphorylation were investigated in order to aid understanding of stimulus/response coupling in plant cells.

Chapter 2

Materials and Methods

2.1. Materials

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

Abscisic acid (ABA), antipain hydrochloride, benzamidine hydrochloride, bromophenol blue, bovine serum albumin (BSA), Coomassie brilliant blue G250, cycloheximide, fusicoccin, glucose 6-phosphate (monosodium salt), leupeptin (hemisulphate salt), L-malate (disodium salt), M_r marker proteins for SDS/polyacrylamide gel electrophoresis, percoll, silver nitrate and Tween 20 were obtained from the Sigma Chemical Co. (London), Poole, Dorset, UK.

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

Acrylamide monomer, ammonium persulphate, ethanol, glycine, hydrogen peroxide, 2-mercaptoethanol, N',N'-methylene*bis*acrylamide, sodium dihydrogen orthophosphate, sodium dodecyl sulphate (SDS), N,N,N',N',-tetramethylethylenediamine (TEMED) and trichloroacetic acid were 'AnalaR' grade materials from BDH Chemicals, Poole, Dorset, UK.

Normal donkey serum and peroxidase conjugate mouse anti rabbit IgG were obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland.

Okadaic acid was from Moana Bioproducts, Hawaii, USA.

Seeds of *Commelina communis* were originally a gift from Dr. C.M. Willmer, University of Stirling, Bridge of Allan, Scotland.

Mammalian protein phosphatase types 1 and 2A were gifts from

Dr. C. MacKintosh and Prof. P. Cohen of the Department of Biochemistry, University of Dundee, Dundee, Scotland.
[^{32}P]-phosphocasein was a gift from Dr. J. McKee, Glasgow University.
All other reagents used were of the highest grade commercially available.

2.2. Experimental tissues

2.2.1 Plant material

The C3 plant *Commelina communis* was grown from seed in a glasshouse under a 16h photoperiod, maintained throughout the year with mercury-vapour lamps. Five to eight week old plants were selected and used either immediately or after one day of storage in a controlled environment growth room with a 12h photoperiod. The light was provided by white fluorescent lamps and tungsten lamps giving a light intensity of 500μE/m^2/sec at the top of the plant canopy. Seeds were collected from plants which had been allowed to flower (approximately 10 weeks old) and subsequently set seed. Once collected, seed was stored in a sealed container for at least 8 weeks before being sown.

2.2.2 Preparation of epidermal strips and guard cell protoplasts

The methods described are based on those of Fitzsimmons and Weyers (1983).

1. Epidermis was peeled by hand from the abaxial surface of the three youngest fully expanded leaves on each branch on the plants, and floated cuticle uppermost in 180mm diameter petri dishes on 150mM mannitol, 10mM Mes/NaOH pH 6.2. This is a preplasmolysis treatment designed to reduce damage which may be caused during plasmolysis as the plasma membranes of the cells are pulled away from the cell walls. Epidermal strips were incubated on this buffer for 15-30min.
2. Epidermis was transferred to 90mm petri dishes containing protoplast suspension buffer (300mM mannitol, 20mM Mes/NaOH pH 6.2) using a seeker, and incubated on this medium for 30min in order to plasmolyse the cells.

3. The protoplast suspension buffer was removed and replaced by 5ml per dish of cell wall digestion buffer (300mM mannitol, 2% (w/v) cellulysin, 0.05% (w/v) pectolyase, 0.5% (w/v) bovine serum albumin and 20mM Mes/NaOH pH 5.5). The dishes were then placed on a gently shaking tray in a 30°C warm room.

4. After approximately 1h of this incubation, the cell wall digestion buffer was removed using a 10ml plastic syringe, and centrifuged at 400g for 5min to remove epidermal cell protoplasts which were released early in the digestion. The supernatant from this centrifugation was added back to the dishes, which were then replaced in the 30°C warm room.

5. The release of guard cell protoplasts from the epidermis was monitored by observation under the microscope. Release normally occurred 3 to 5h after the dishes were placed back in the 30°C room after the first centrifugation (indicated by a spherical appearance of guard cells, and empty guard cell "ghosts" in the epidermis). Cell wall digestion buffer containing guard cell and other protoplasts was removed and centrifuged at 100g for 5min. The supernatant was removed and discarded and the pellet of protoplasts was retained. The dishes containing the remaining epidermis were then rinsed with protoplast suspension buffer and tapped on the benchtop for several minutes to dislodge any protoplasts which may have been attached to the epidermis. The buffer from this rinse was then used to resuspend the protoplast pellet which was then recentrifuged at 100g for 5min. This rinsing procedure was repeated, and finally a pellet of mixed protoplasts was obtained.

6. A stepwise percoll density gradient was prepared in a centrifuge tube as follows: bottom layer - 90% percoll, 300mM mannitol, 20mM Mes/NaOH pH 6.2; top layer - 28% percoll, 300mM mannitol, 20mM Mes/NaOH pH 6.2.

7. The mixed protoplast pellet was resuspended in approximately 1ml of protoplast suspension buffer, and placed on top of the percoll step gradient. This was then centrifuged at 100g for 5min.

8. After centrifugation, the guard cell protoplasts appeared at the interface between the 90% and 28% percoll phases. These protoplasts were removed and washed twice in protoplast suspension buffer to remove the percoll.

9. The guard cell protoplasts were finally suspended in protoplast suspension buffer and were ready for use.

2.3 General biochemical methods

2.3.1 pH calibrations were done using a Russell pH probe. The pH of buffers and solutions were adjusted at either 5°C (for buffers which were to be used cold, for example extraction buffer) or room temperature.

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

2.3.3 Spectrophotometric assays were carried out in semi-micro quartz cuvettes or plastic disposable cuvettes (1cm path, 1ml). Enzyme assays and apparent K_i (malate) determinations were carried out on a Philips PU8720 UV/VIS scanning spectrophotometer.

2.3.4 Centrifugation was carried out using a Beckman model TJ-6 centrifuge. Small volume samples were centrifuged using a Beckman microfuge model E.

2.3.4 Micropipetting (5μl - 1ml) 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).

2.4. Methods for the extraction of enzyme activities

2.4.1 Epidermal strip extracts

Epidermis, prepared as described in section 2.2.2, was ground to a fine powder under liquid nitrogen in a mortar and pestle with a little sand. This powder was then added to ice cold extraction buffer (50mM Tris/HCl pH 7.5, 10mM EDTA, 1mM DTT, 2% PEG 20000, antipain (5μg/ml), leupeptin (5μg/ml), 1mM benzamidine) in the ratio 1g powder to 3ml extraction buffer. The resulting mixture was stirred thoroughly and centrifuged at 10000g for 15min. The supernatant was then taken and used for subsequent experiments.

2.4.2 Protoplast extracts

Protoplasts in suspension, prepared as described in section 2.2.2, were centrifuged for a few seconds in a microcentrifuge. The supernatant was removed and ice cold extraction buffer added. The protoplasts were then resuspended in the extraction buffer and drawn back and forth through a hypodermic needle (Becton Dickinson Microlance 2, 0.5 x 16) at least eight times. The subsequent homogenate was then centrifuged for 3min at 13000g to remove chloroplasts and any unbroken protoplasts. The supernatant was removed and used in subsequent experiments.

2.5 Assay procedures

2.5.1 Phosphoenolpyruvate carboxylase activity

The standard spectrophotometric assay mixture (assay buffer A) contained, in 1ml, 50mM Tris/HCl, pH 7.8, 5mM $MgCl_2$, 2mM PEP, 0.2mM NADH, 10mM $NaHCO_3$, 5 units of MDH and the enzyme sample. Assays used to determine the difference in kinetics of putative phosphorylated and non phosphorylated phosphoenolpyruvate carboxylase (assay buffer B) contained, in 1ml, 50mM Hepes/KOH, pH 7.0, 5mM $MgCl_2$, 0.2mM NADH, 10mM $NaHCO_3$, 5mM glucose 6-phosphate, 0.5 mM PEP, 5 units of MDH and the enzyme sample. In both cases the decrease in A_{340} at 25°C caused by the oxidation of NADH by the coupling enzyme MDH was proportional to the phosphoenolpyruvate carboxylase concentration.

The malate sensitivity of phosphoenolpyruvate carboxylase was determined using an appropriate range of L-malate concentrations in the 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.

K_m (PEP) determinations were made by using an appropriate range of PEP concentrations in the assay buffer. Apparent K_m values were calculated from the direct linear plot (Cornish-Bowden) using the Enzpack program (Elsevier-Biosoft, Cambridge, UK) which then used direct linear plots to calculate the apparent K_m values.

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

2.5.2 Protein phosphatase activity

Protein phosphatase activity was quantified by measuring the release of ^{32}Pi from ^{32}P-labelled casein by the method of Cohen *et al.* (1988). The dephosphorylation of this substrate was limited to $\leq$ 30% to ensure linearity, hence samples to be assayed were diluted appropriately.

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

Each sample (10μl, diluted in 50mM Tris/HCl pH 7.0, containing 0.1mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 1mg BSA/ml) was preincubated for 10min at 30°C with 10μl of the dilution buffer containing 0.03% Brij-35 in place of BSA. The reaction was initiated by the addition of 4μM casein to a total volume of 30μl. The reaction was terminated by the addition of 0.2ml of 10% (w/v) TCA. Samples were kept on ice for 2min followed by centrifugation for 5min at 12000g. A 0.2ml sample of the supernatant was added to 1ml Ecoscint and the ^{32}Pi released determined by scintillation counting.

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% 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. Electrophoresis was carried out at 45mA until the tracking dye had reached the end of the separating gel. Molecular mass standard markers were rabbit muscle myosin (205 kD), E. Coli β-galactosidase (116 kD), rabbit muscle phosphorylase b (97.4 kD), bovine albumin (66 kD) egg albumin (45 kD) and bovine erythrocyte carbonic anhydrase (29 kD).

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 one or two days in 50% methanol. The staining solution was prepared by adding solution A (0.8g $AgNO_3$ in 4ml H_2O) to solution B (1.4 ml 14.8M NH_4OH and 21ml 0.36% (w/v) NaOH) dropwise while stirring vigorously. This mixture was made up to 100ml with distilled water. The gel was then shaken gently for 10min in the staining solution and rinsed for 1h with 6 changes of distilled water. To develop the stain the gel was immersed in a solution comprising 2.5ml 1% (w/v) citric acid and 0.25 ml of 38% (v/v) formaldehyde in 500ml 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 or a Drygel Sr. slab gel drier model 1160 (Hoefer Scientific Instruments, San Fransisco) 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 and 5 days. The X-ray film was developed using a Kodak X-OMAT Processor Model ME-3.

2.6.4 Scanning of autoradiographs

Autoradiographs were scanned at 420nm using a Shimadzu Model CS-9000 flying spot laser densitometer on absorbance/transmittance mode. Autoradiograph tracks were scanned from 1cm above the band of interest to 1cm below it, and zero absorbance was set at the start of the scan. Output of the system was given as a plot of absorbance units against distance along the track, and the system also calculated the area below each peak on this plot.

2.6.5 Immunoblotting of polyacrylamide gels

The method used for immunoblotting is based on that of Towbin *et al.* (1970) as modified by Batteigner *et al.* (1982).

Stock solutions

Buffer 1 (Transfer buffer)

25mM Tris
190mM Glycine
20% Methanol
0.02% SDS

Buffer 2 (Blocking buffer)

20mM Tris/HCl pH 7.2
150mM NaCl

Buffer 3 (Chloronapthol stain)

50ml 10mM Tris/HCl pH 7.4
30mg chloronapthol in 10ml methanol
150µl 4% v/v H_2O_2

H_2O_2 was mixed immediately before use.

Procedure

SDS polyacrylamide gel electrophoresis was carried out (Method 2.6.1). When electrophoresis was complete, the proteins were transferred to Hi-bond C nitrocellulose filter (Amersham UK) using an electrophoretic transfer unit 2051 (LKB Bromma, Sweden) containing Buffer 1. The transfer was run at 150mA for 3h, after which the nitrocellulose filter was placed in buffer 2 containing 5% (v/v) normal donkey serum at 4°C overnight. The nitrocellulose was then rinsed twice in Buffer 2. The paper was then soaked in Buffer 2 containing 5% (v/v) normal donkey serum, 0.5% (v/v) Tween 20 and 0.5% (v/v) antiserum at 25°C for 90 min. The nitrocellulose filter was then washed four times for 12 min each in Buffer 2 containing 0.5% (v/v) Tween 20 and then once for 12 min in Buffer 2 alone. The nitrocellulose filter was then soaked in Buffer 2 containing 5% (v/v) normal donkey serum and 0.05% peroxidase conjugate mouse anti rabbit IgG at 25°C for 90 min. The filter was

then washed five times for 12min each in Buffer 2 and developed by soaking in Buffer 3 for 5min. The developed blot was then washed in distilled water and dried.

2.7 Experimental manipulation of tissue

2.7.1 Incubation of epidermal strips and the measurement of stomatal apertures

During experiments in which epidermal strips were incubated under various conditions and stomatal apertures measured, the following methods were used.

1. Plants were placed in the dark at room temperature for 2h prior to peeling, and samples of epidermis were examined to ensure that all stomata were closed before peeling began.
2. Epidermis was peeled by hand and floated on 0.1mM $Ca(NO_3)_2$. The epidermal strips were then cut into pieces of approximately 5mm x 5mm using scissors.
3. Any preincubations (with cycloheximide for example) were carried out whilst the pieces of epidermis were floating on the $Ca(NO_3)_2$ solution, and the dishes were placed on a shaking tray.
4. Immediately prior to incubation in the light or with fusicoccin, the pieces of epidermis were transferred to 2.5cm diameter petri dishes containing K^+ solution as detailed in the relevant Results sections.
5. When stomatal movements were stimulated by light and darkness, the petri dishes were placed in the apparatus illustrated in Figure 2.1. Petri dishes (D) were placed on a support frame (F) with their bases in the water bath (B) to maintain them at 25°C. Holes pierced in the petri dish lids admitted hypodermic needles (N) which were connected via air lines (A) to an air pump (P). Air could be pumped directly to the needles, or via a $Ca(OH)_2$ column (C) to remove CO_2. Illumination at 400μE/m^2/s was applied from above, and additional illumination was obtained by reflection from the reflective surface (R) at the base of the water bath. The whole apparatus could be covered to omit light.

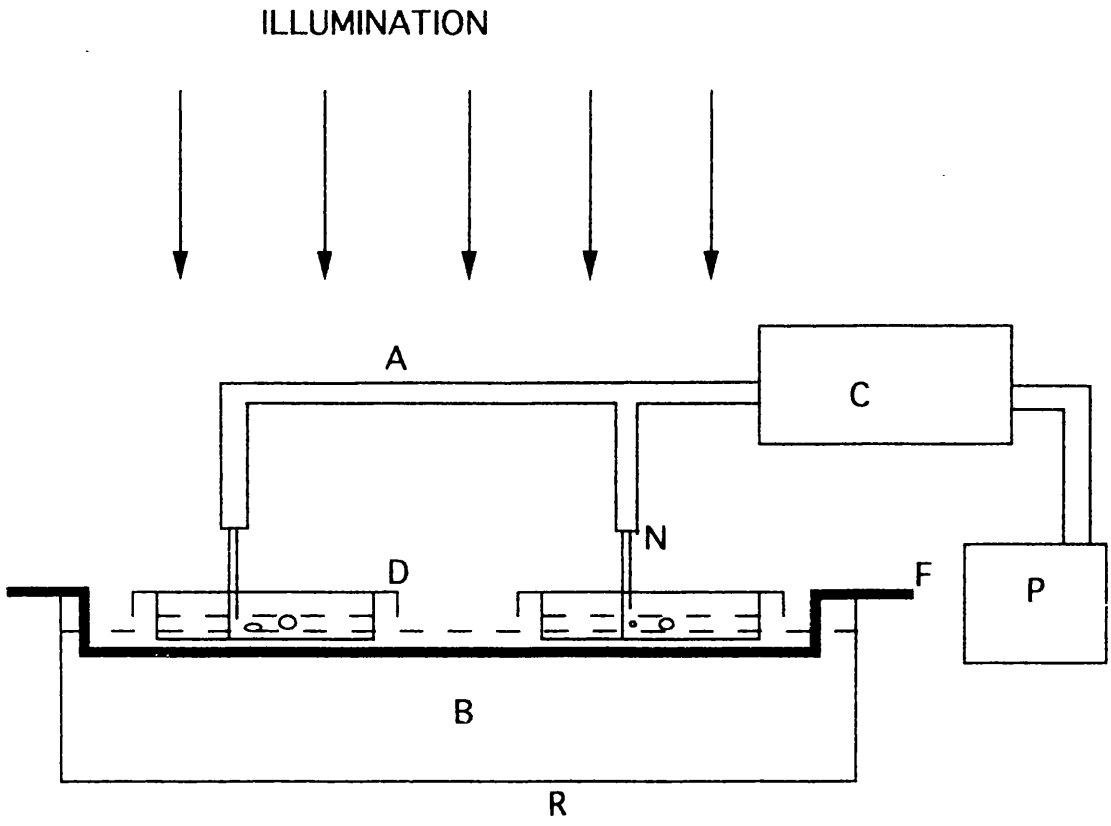


Figure 2.1 Epidermis incubation apparatus

A- Air lines

B- Water bath

C- Column containing $Ca(OH)_2$

D- Petri dish

F- Support frame

N- Hypodermic needle

P- Air pump

R- Reflective surface

6. When stomatal opening was stimulated by fusicoccin, the petri dishes containing epidermal pieces were placed on a shaking tray in the dark and the fusicoccin was added.
7. At times indicated in the relevant results sections, three pieces of epidermis were removed from the incubation and mounted, in 0.1mM $Ca(NO_3)_2$, on a microscope slide. Stomata were observed under the x50 objective and the apertures of alternate stomata accross the width of each piece of epidermis measured using an eyepiece micrometer NE1 (Graticules Ltd, Tonbridge, Kent). After measurement, the pieces of epidermis were discarded.

2.7.2 Incubation of guard cell protoplasts

1. Any preincubations were carried out in the dark in protoplast suspension buffer without K^+. In ^{32}P labelling experiments, guard cell protoplasts were preincubated with [^{32}P]-orthophosphate and 50μM NaH_2PO_4 as a carrier. The specific activity was 4μCi/nmol. The protoplasts were incubated in the phosphate for 3h, after which an equal volume of protoplast suspension buffer containing 50mM K_2SO_4 was added (see point 2 below).
2. Immediately prior to the commencement of incubations in the light or with fusicoccin, K_2SO_4 was added to a concentration of 25mM.
3. Incubations in which guard cell protoplasts were illuminated used the apparatus illustrated in Figure 2.2. Illumination was provided by a strip bulb (B). A filter (F), filled with flowing water, screened the heat from the lamp. The guard cell protoplasts were placed in a pyrex tube (T) which was fixed to the filter in the position shown, at which the light intensity was 200μE/m²/s. A reflective surface placed behind the tube increased the light intensity somewhat. During incubations in the dark, the same apparatus was used but the light was not switched on and the sample tube was covered in aluminium foil to omit light. Protoplasts were kept in suspension throughout the experiments by occasional agitation.
4. Incubations with fusicoccin were carried out in the dark. Protoplasts were kept in suspension in Eppendorf tubes throughout experiments by occasional agitation.

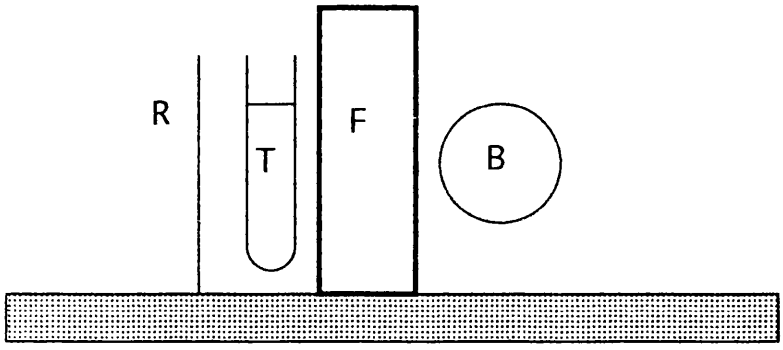


Figure 2.2 Apparatus for illumination of guard cell protoplasts

B- Strip bulb

F- Water filled filter

R- Reflective surface

T- Sample tube

2.8 Miscellaneous methods

2.8.1 Immunoprecipitation of phosphoenolpyruvate carboxylase

Anti-*Bryophyllum* phosphoenolpyruvate carboxylase serum was added to guard cell protoplast extracts in the ratio 10µl of serum to 0.03U of phosphoenolpyruvate carboxylase activity. The mixture was vortexed, placed on ice for 2h and then centrifuged for 3min at 13000g. The supernatant was removed and the pellet resuspended in ice cold phosphate buffered saline (0.15M NaCl, 7.2mM Na_2HPO_4, 1mM NaH_2PO_4) and centrifuged at 13000g for 3min. The supernatant was removed and the pellet resuspended in sample buffer (Method 2.6) and boiled for 2min.

2.8.2 Vital staining of protoplasts

Fluoresceine diacetate buffer consisted of 3ml of fluoresceine diacetate (5mg/ml dissolved in acetone) mixed with 20ml of protoplast suspension buffer. The fluoresceine diacetate buffer and the protoplast suspension were mixed 1:1 and incubated at room temperature for 5min. The protoplasts were then mounted on a microscope slide and counted. Blue light was then used to illuminate the protoplasts and those fluorescing green were counted.

2.8.3. Labelling of phosphoenolpyruvate carboxylase in extracts of guard cell protoplasts

Extracts of guard cell protoplasts were prepared as described in Methods 2.4.2 and divided into several parts of 100µl each. To these parts were added 0.01mM [γ-^{32}P] ATP (1µCi), 20mM $MgCl_2$, 5nM okadaic acid and either 10µl of protein kinase solution or extraction buffer as detailed in the relevant Results section. The final volume was 120µl. Incubations were carried out for 20min at 30°C, after which the samples were placed on ice. Immunoprecipitation followed by denaturation and gel electrophoresis were then carried out.

2.8.4. Non radioactive incubations of guard cell extracts with protein phosphatases and kinases

Guard cell protoplasts were prepared as described in Methods 2.2.2 and extracted as described in Methods 2.4.2. In each case the

extract was split into several equal parts, and the following additions made:

1. Protein phosphatase incubations; 3mU phosphatase activity added to 0.03U phosphoenolpyruvate carboxylase activity.
2. Protein kinase incubations; to guard cell extracts containing approximately 0.03U of phosphoenolpyruvate carboxylase activity were added 0.5mM ATP, 20mM $MgCl_2$, okadaic acid as indicated in the relevant Results section, and a quantity of protein kinase as indicated in the relevant Results sections.

In both cases, control incubations were carried out with the relevant volume of extraction buffer in place of either the kinase or the phosphatase, and sampling times are indicated in the relevant Results sections. Incubations were carried out on the bench at room temperature.

Chapter 3

RESULTS

3.1 Introduction

Phosphoenolpyruvate carboxylase is one of a number of enzymes that has been shown to be regulated by reversible phosphorylation (see Introduction section 1.3). The phosphorylation and dephosphorylation of phosphoenolpyruvate carboxylase in C4 and CAM plants corresponds to changes in the physiological function of the tissue (Nimmo, 1993). General criteria have been proposed by Nimmo and Cohen (1977) which must be satisfied if reversible phosphorylation is to be shown to be responsible for any observed physiological effects. These criteria are:

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 a 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 should occur *in vivo* at the same site(s) that is / are phosphorylated by the purified kinase *in vitro*.

In order to study guard cell phosphoenolpyruvate carboxylase with a view to satisfying these criteria, it was necessary to design experiments to study changes in the enzyme during defined physiological processes. To this end, experiments were carried out to measure the responses of *Commelina communis* stomata to various stimuli under different experimental conditions. After establishing the appropriate conditions, they were used to investigate changes in the phosphorylation state of guard cell phosphoenolpyruvate carboxylase in response to the same stimuli. Finally, changes in the kinetic properties of the enzyme in relation to its phosphorylation state and the physiological state of the tissue were investigated.

3.2 Responses of *Commelina communis* stomata to various stimuli

It is well known that *Commelina* stomata open in response to light and low CO_2 concentration and close in response to darkness and normal CO_2. (Zeiger, 1983). The size and rate of these aperture changes measured using our apparatus is shown in Figure 3.1. The stomata opened steadily when epidermis was incubated in the light and air which had its CO_2 content reduced by passing through $Ca(OH)_2$ was bubbled through the incubation medium, and began closing almost immediately they were placed in darkness and normal air was bubbled through the medium. Each point in the scattergram is the mean of 36 individual measurements of stomata in 3 pieces of epidermis. The aperture sizes making up each data point have an approximately normal distribution (Figure 3.2). As described in Methods section 2.7.1, all stomata were closed to begin with and as the stomata opened a gaussian distribution of apertures became established. As the mean aperture increased, this curve moved along the aperture axis and became broader, indicating that at higher mean aperture, the variation in the actual apertures increased. Reasons for the variability in apertures during experiments of this type include differences in size and responsiveness of stomata from different leaves on a single plant and even between different parts of the epidermis on a single leaf (Weyers and Meidner, 1990). Despite the obvious spread in the data, since 36 measurements were made for each point, the differences between mean apertures were statistically significant. In the case of the data in Figure 3.2, all three mean apertures were statistically different from each other with a probability of identity of less than 0.5%. These results therefore show that the stomata responded as expected in our experimental conditions and that aperture measurements could be used to report on the physiological state of the stomata.

The ability of stomata to respond to environmental stimuli depends on the availability of K^+ ions (Zeiger, 1983). The results of experiments carried out to determine the effect of concentration of K^+ ions in the incubation medium are shown in Figure 3.3. Quite clearly, while *Commelina* stomata opened rapidly in 100mM and 75mM KCl in the light, they did not appear to be able to close in the dark. In 50mM and 25mM KCl, *Commelina* stomata could still open rapidly in response to light, but could also close in the dark. A K^+ concentration of 50mM was

Figure 3.1 The responses of mean stomatal aperture to opening and closing stimuli

This scattergram shows the results of three separate experiments which were carried out identically. Small pieces of epidermis were prepared and set up on different occasions as described in Methods section 2.7.1. At zero time, the epidermal strips were transferred to 50mM KCl which had air which had been passed through $Ca(OH)_2$ to remove CO_2 bubbled through it as described in Methods section 2.7.1. The strips were then incubated under a tungsten lamp (at 400 μmol / m^2 / sec) at 25°C with continual bubbling. Samples were taken at appropriate times and apertures measured using an eyepiece micrometer as described in Methods section 2.7.1. After 140 minutes, air with normal CO_2 concentration was bubbled through the medium and the strips were placed in darkness. Further samples were taken at appropriate time points. Each point plotted is a mean of 36 individual apertures from three pieces of epidermis. Units of aperture are eye piece units (epu).

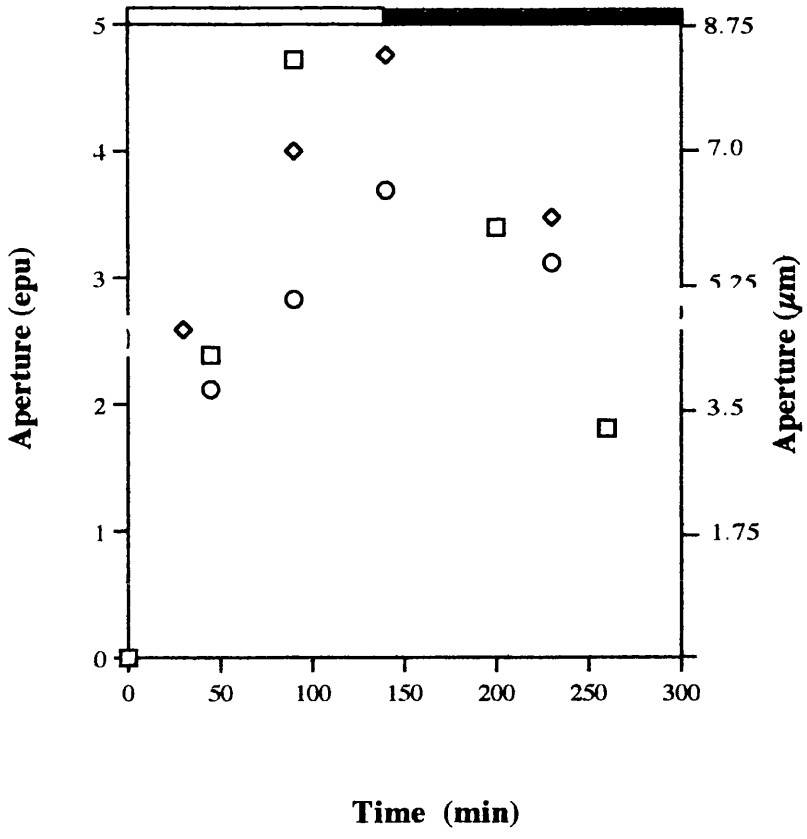

Aperture (epu)
5
4
3
2
1
0
8.75
7.0
5.25
3.5
1.75
Aperture (μm)
0
50
100
150
200
250
300
Time (min)
Experiment 1
Experiment 2
Experiment 3

Figure 3.2 Frequency distribution of stomatal apertures in stomatal populations of different mean apertures

The graph shows aperture data for three representative aperture measurements with different means. The total number of stomata measured in each case was 36. Means and standard deviations in each case were:

Squares: mean = 1.14 epu ; standard deviation = 0.96 epu

Diamonds: mean = 3.36 epu ; standard deviation = 0.80 epu

Circles: mean = 5.30 epu ; standard deviation = 1.04 epu.

Use of Student's t tests showed that all three mean apertures were different from each other with a probability of identity of less than 0.5%.

Units of aperture are eye piece units (epu).

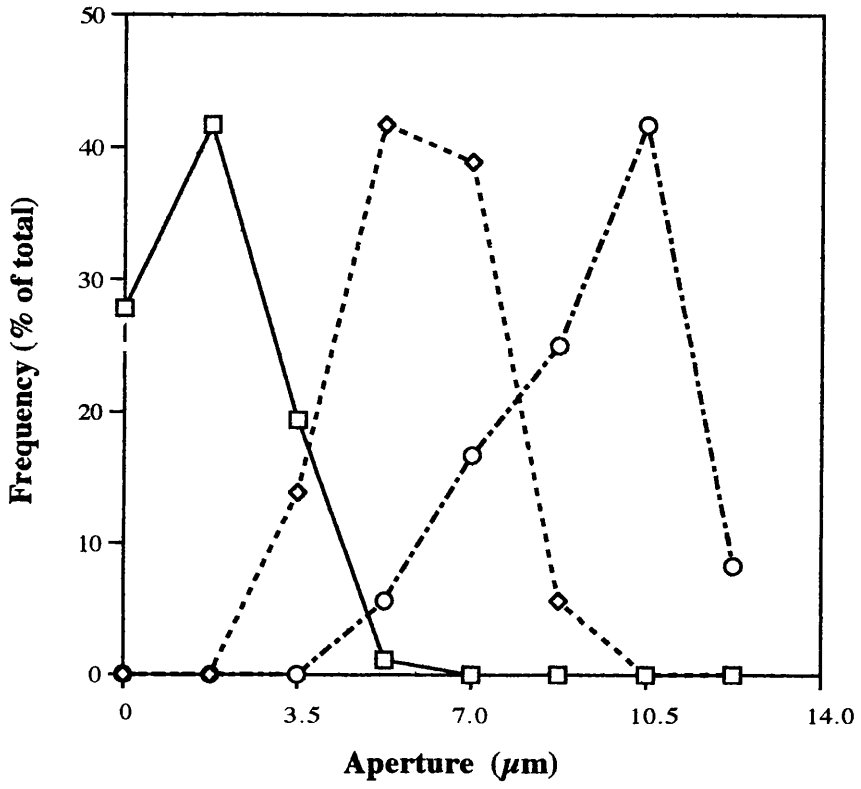
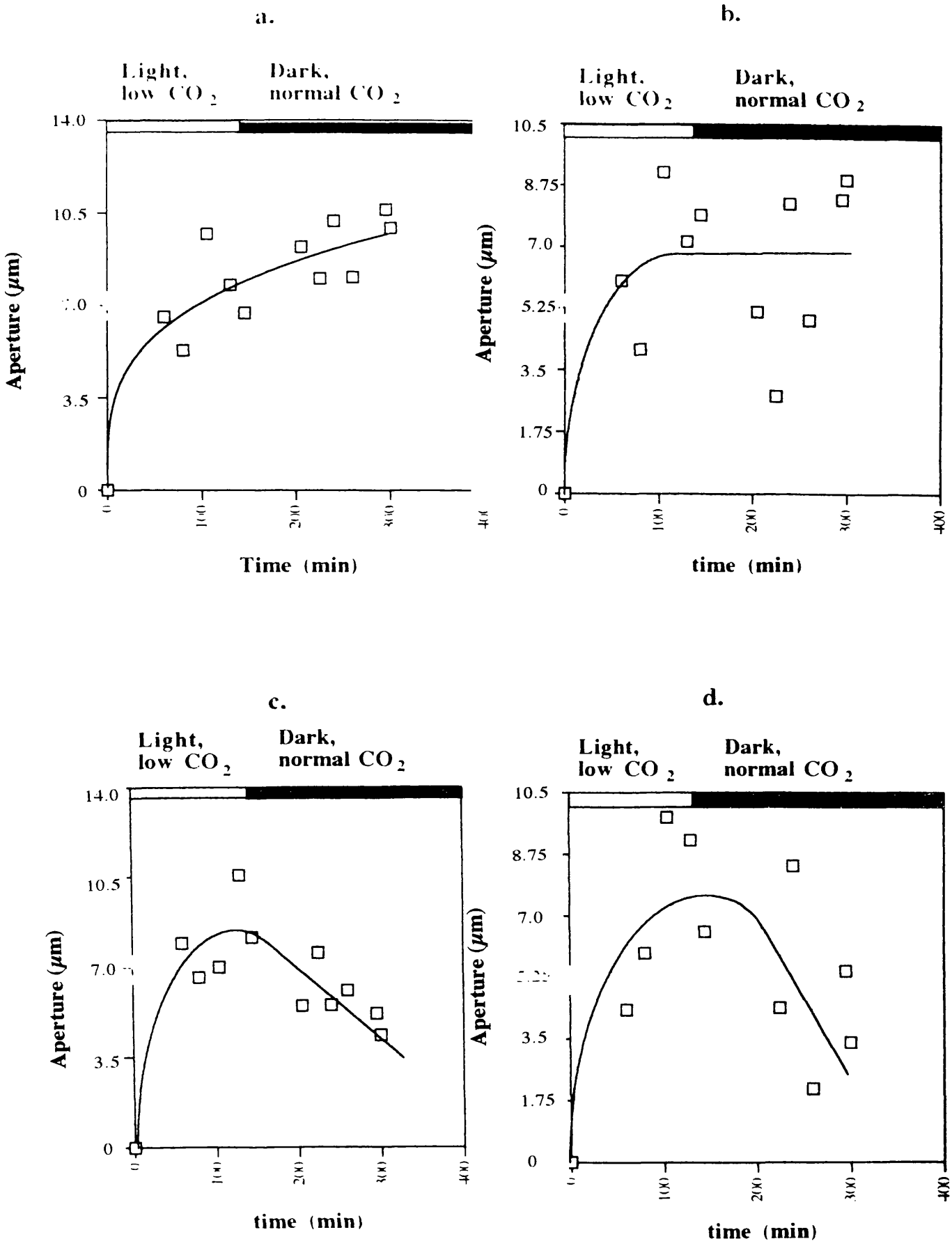

50
40
30
20
10
0
Frequency (% of total)
0
3.5
7.0
10.5
14.0
Aperture (μm)
Mean aperture 1.14 epu
Mean aperture 3.36 epu
Mean aperture 5.30 epu

Figure 3.3 Effect of KCl concentration on opening and closure of stomata in response to light/low CO_2 and darkness/ normal CO_2

Small pieces of epidermis were prepared as described in Methods section 2.7.1. At time zero, strips were transferred to the appropriate KCl solution, which had low CO_2 air bubbled through it, and illuminated at 400μE / m^2 / s. Strips were removed and apertures measured at appropriate times. After 130 minutes, normal air was bubbled through the medium and the strips were placed in darkness. Again, strips were removed and apertures measured at appropriate times up to 300 minutes. The KCl concentrations were

a. 100mM **b**. 75mM **c**. 50mM **d**. 25mM. Each point plotted is the mean of 36 individual aperture measurements. Units of aperture (epu) are eye piece units. Lines were fitted by hand.


a.
Light, low CO 2
Dark, normal CO 2
14.0
10.5
7.0
3.5
0
Aperture (μm)
Time (min)
b.
Light, low CO 2
Dark, normal CO 2
10.5
8.75
7.0
5.25
3.5
1.75
0
Aperture (μm)
time (min)
c.
Light, low CO 2
Dark, normal CO 2
14.0
10.5
7.0
3.5
0
Aperture (μm)
time (min)
d.
Light, low CO 2
Dark, normal CO 2
10.5
8.75
7.0
3.5
1.75
0
Aperture (μm)
time (min)

chosen as the most appropriate to use in the subsequent experiments. Because the concentration of Cl^- in the medium had been shown to affect the accumulation of malate in opening stomata (Raschke and Schnabl, 1978), it was decided to use the impermeant anion SO_4^{2-} for subsequent work.

In order to gain information on factors that might affect the phosphorylation of guard cell phosphoenolpyruvate carboxylase, it was decided to investigate another, quite different opening stimulus, namely fusicoccin. *Commelina* stomata responded very rapidly to fusicoccin, even in the dark without perfusion (Figure 3.4).

3.3 The phosphorylation of guard cell phosphoenolpyruvate carboxylase

3.3.1 Introduction

The phosphorylation of phosphoenolpyruvate carboxylase in maize and *Bryophyllum* was first studied by feeding inorganic ^{32}P phosphate to leaves (Nimmo *et al.*, 1984; Nimmo *et al.*, 1987). Extracts of these leaves were made and analysed by gel electrophoresis and autoradiography. This type of analysis is very useful, as the change in phosphorylation state of the enzyme *in vivo* can be followed during, for example, a diurnal cycle. In order to carry out this type of analysis, it is necessary to separate the enzyme of interest (in this case phosphoenolpyruvate carboxylase) from proteins of a similar size, as these might interfere with the autoradiography. When studying phosphoenolpyruvate carboxylase from guard cells, it is also necessary to eliminate phosphoenolpyruvate carboxylase from other tissue types. This requirement makes the study of guard cell phosphoenolpyruvate carboxylase difficult, since only very small amounts of tissue can be isolated. When working with epidermal strips or protoplasts made from epidermal strips, the major contaminant cells are epidermal cells. Table 3.1 shows the distribution of different cell types within the epidermis of *Commelina communis*, and the distribution of phosphoenolpyruvate carboxylase among these cell types. Since almost half of the phosphoenolpyruvate carboxylase in intact epidermal strips is contained within the epidermal cells, this tissue is not suitable to use for labelling studies. It has been reported that it is possible to isolate guard cells

Table 3.1 Distribution of phosphoenolpyruvate carboxylase between different cells in the epidermis of *Commelina communis*

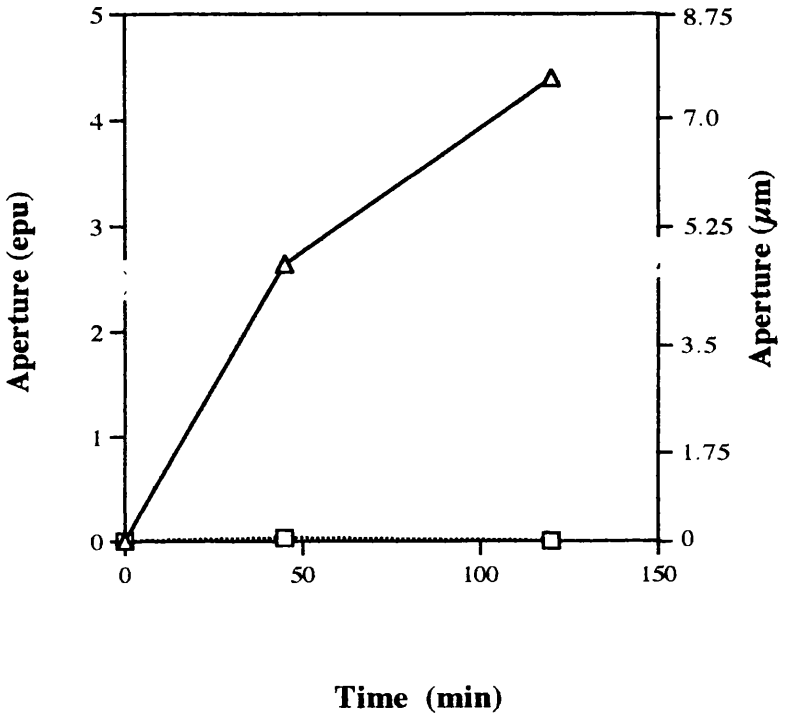
Guard cells and epidermal cells were counted in 4 representative areas of the epidermis of *Commelina communis* , and their proportions recorded as percentages of the total number of cells counted. Subsidiary cells were counted as epidermal cells for this purpose.

Protoplasts were prepared from the epidermis, and epidermal cell protoplasts were separated from guard cell protoplasts by centrifugation on a 28% / 90% Percoll step gradient as described in Methods section 2.2.2. Each population of protoplasts was then resuspended in 300mM mannitol, 20mM Mes/NaOH buffer, pH 6.2 and a sample counted so that the number of protoplasts/ml could be determined. In each case a known number of protoplasts was extracted and the activity of phosphoenolpyruvate carboxylase determined (Methods section 2.5.1). From this, activity of phosphoenolpyruvate carboxylase per cell was calculated, and subsequently the distribution of the enzyme between the two cell types in the epidermis was established.

	Guard cells.	Epidermal cells.
Proportion of epidermis	18%	82%
PEP carboxylase activity / cell (U / cell)	448×10^{-9}	77×10^{-9}
Proportion of PEP carboxylase activity in epidermis	56%	44%

Figure 3.4 The effect of fusicoccin on stomatal aperture.

Small pieces of epidermis were prepared as described in Methods section 2.7.1. At zero time, strips were transferred to 25mM K_2SO_4 with either 10nM fusicoccin and 0.2% ethanol (triangles) or with 0.2% ethanol alone (squares). The strips were kept in the dark without aeration and were agitated using a shaker tray. Each point is the mean of 36 individual aperture measurements from 3 strips. Units of aperture (epu) are eye piece units.


5
4
3
2
1
0
Aperture (epu)
8.75
7.0
5.25
3.5
1.75
0
Aperture (μm)
0
50
100
150
Time (min)

within epidermal strips by incubating the strips at pH 3.7 to 4.5 (Weyers and Meidner, 1990). This treatment kills the epidermal cells, but not the guard cells. Epidermal cell contents can then be washed away, leaving strips containing only guard cells and epidermal cell "ghosts". This material was deemed unsuitable for our studies from a practical point of view. In order to observe phosphoenolpyruvate carboxylase on a denaturing slab polyacrylamide gel, approximately 0.03 U of enzyme are required. When preparing protoplasts, we found that to prepare over 10^6 guard cell protoplasts, the amount of epidermis required was enough to completely cover the surface area of six standard 90mm petri dishes. We found that such a preparation gave us sufficient phosphoenolpyruvate carboxylase to run a maximum of five tracks on SDS PAGE after losses during guard cell preparation, phosphoenolpyruvate carboxylase extraction and immunoprecipitation. This meant that approximately one petri dish of epidermis gives one track of phosphoenolpyruvate carboxylase. Incubation of epidermis in this type of dish requires 5ml of medium to float the strips. In order to attain satisfactory labelling of guard cell proteins, we found it necessary to use phosphate at a concentration of 50 μM, and a specific activity of 4μCi/nmol, i.e. 200μCi/ml. As a result, using "isolated" guard cells, we would have needed to use 1mCi per track, but this was considered excessive. More than 10^6 guard cell protoplasts could be incubated in 1ml total volume, allowing 5 tracks on SDS PAGE to be run using only 200μCi, rather than 5mCi, as would have been necessary using "isolated" guard cells. Consequently all studies on the phosphorylation of guard cell phosphoenolpyruvate carboxylase were carried out using guard cell protoplasts.

Immunoprecipitation of guard cell phosphoenolpyruvate carboxylase was carried out using antiserum raised against *Bryophyllum* phosphoenolpyruvate carboxylase. This antiserum precipitates two guard cell phosphoenolpyruvate carboxylase bands (masses 114kD and 110kD as estimated by SDS gel electrophoresis) from extracts of purified guard cell protoplasts. A gel stained with Coomassie Blue and a Western blot of immunoprecipitated guard cell phosphoenolpyruvate carboxylase are shown in Figure 3.5, and these show that phosphoenolpyruvate carboxylase is precipitated by anti-phosphoenolpyruvate carboxylase serum. Assays performed before and after precipitation (Table 3.2) showed that phosphoenolpyruvate carboxylase activity was removed

Figure 3.5 Immunoprecipitation of guard cell phosphoenolpyruvate carboxylase protein

Guard cell protoplasts were prepared and purified as described in Methods section 2.2.2, and extracted as described in section 2.4.2. The extract was then divided into two parts. One part had 40µl of anti-*Bryophyllum* PEP carboxylase serum added, the other had 40µl of pre-immune serum added. Both were kept on ice for two hours, then centrifuged at 13000g for 5 min. Both pellets were washed in phosphate buffered saline (Methods section 2.8.1), and centrifuged for a further 5min at 13000g. The pellets were then boiled in 50µl of SDS sample buffer (Methods section 2.6.1). An aliquot (20µl) of each sample was then loaded twice onto separate halves of the gel. After the gel had been run, one half was stained with Coomassie Blue, the other half was blotted onto nitrocellulose filter as described in Methods section 2.6.5. The blot was then probed with *Bryophyllum* anti phosphoenolpyruvate carboxylase serum followed by mouse (anti rabbit IGg) HRP IGg conjugate. The immunoreactive bands were then observed using chloronaphthol.

The left panel shows a gel stained with Coomassie Blue. Tracks;

M- Molecular weight markers

1- Pellet from guard cell protoplast extract precipitated with pre-immune serum

2- Pellet from guard cell protoplast extract precipitated with anti-phosphoenolpyruvate carboxylase antiserum

Arrows indicate phosphoenolpyruvate carboxylase bands.

Right panel shows Western blot. Tracks:

1- Pellet from guard cell protoplast extract precipitated with pre-immune serum

2- Pellet from guard cell protoplast extract precipitated with anti-phosphoenolpyruvate carboxylase serum.

Arrows indicate phosphoenolpyruvate carboxylase bands.

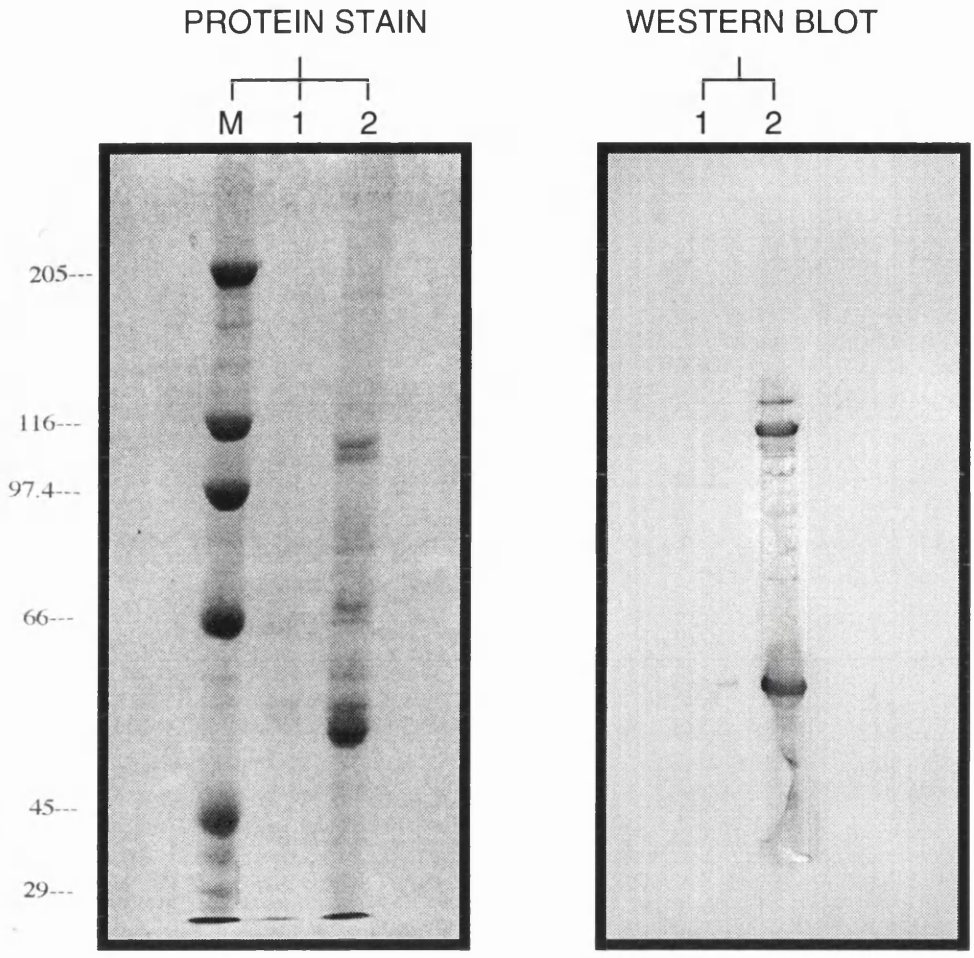

PROTEIN STAIN
M 1 2
205---
116---
97.4---
66---
45---
29---
WESTERN BLOT
1 2

Table 3.2 Immunoprecipitation of phosphoenolpyruvate carboxylase activity from guard cell extracts.

Guard cell protoplasts were prepared as described in Methods section 2.2.2, and extracted as described in Methods section 2.4.2. Once the extract had been made, phosphoenolpyruvate carboxylase acivity was measured at pH 7.8 as described in Methods section 2.5.1. The extract was then split into two parts for immunoprecipitation, which was carried out as described in Methods section 2.8.1; one of the two halves was precipitated with anti-*Bryophyllum* phosphoenolpyruvate carboxylase serum, while the other was precipitated with pre-immune serum. After two hours on ice, the two samples were centrifuged for 5min at 13000g. The supernatant from each was then assayed for phosphoenolpyruvate carboxylase activity at pH7.8.

The table indicates the activities (in mU/ml) and proportions of the original (in %) of phosphoenolpyruvate carboxylase activity in extracts of guard cell protoplasts before and after incubation with antisera followed by high speed centrifugation.

	mU/ml	% non-precipitated value
Crude extract	41	100
Supernatant from extract precipitated with pre-immune serum	45	109
Supernatant from extract precipitated with anti-PEP carboxylase antiserum	6	15

from the extract by anti-phosphoenolpyruvate carboxylase antiserum, but not the preimmune serum. All experiments in which phosphoenolpyruvate carboxylase was precipitated from extracts were subsequently performed using anti-*Bryophyllum* phosphoenolpyruvate carboxylase antiserum.

3.3.2 Phosphorylation of phosphoenolpyruvate carboxylase in guard cell extracts

In order to determine whether guard cell phosphoenolpyruvate carboxylase could be phosphorylated, guard cell extracts were incubated with [γ-^{32}P] MgATP in the presence and absence of protein kinase A catalytic subunits (PKA) . This protein kinase has been shown to phosphorylate maize and *Sorghum* phosphoenolpyruvate carboxylase at the same regulatory site as that which becomes phosphorylated *in vivo* (Terada *et al.*, 1990; Arrio-Dupont *et al.*, 1992). Figure 3.6 shows that guard cell phosphoenolpyruvate carboxylase is phosphorylated by PKA, but that incubation of an extract with [γ-^{32}P] MgATP alone does not bring about phosphorylation of the enzyme. This implies that either there is no phosphoenolpyruvate carboxylase kinase present in the guard cell extract, or that the kinase is inactive. Inclusion of Ca^{2+} in the incubation did not stimulate phosphorylation of phosphoenolpyruvate carboxylase in guard cell extracts (Table 3.3). It was concluded that these guard cells, which had not received any stimulation by light or other opening stimuli, did not contain detectable levels of PEPc kinase.

3.3.3. Time course of labelling of the ATP pool in guard cell protoplasts by incubation of the protoplasts in [^{32}P]-orthophosphate

When developing the conditions for experiments in which guard cell protoplasts would be incubated with [^{32}P]-orthophosphate, it was necessary to know the following:

1. A suitable concentration and specific activity of [^{32}P]-orthophosphate.
2. The length of incubation necessary to fully label the ATP pool in guard cell protoplasts.
3. The effects on labelling of the ATP pool of factors known to cause stomatal opening.

Figure 3.6 The Phosphorylation of phosphoenolpyruvate carboxylase in guard cell extracts

Guard cell protoplasts were prepared as described in Methods section 2.2.2, extracted as described in section 2.4.2 and assayed as described in section 2.5.1. Samples of this extract containing 0.03U of phosphoenolpyruvate carboxylase were taken and incubations with $\gamma[^{32}P]$ MgATP in the presence or absence of protein kinase A catalytic subunits were carried out as described in Methods section 2.8.3.

The protein stained gel and autoradiograph are shown. The tracks are:

Protein stained gel;

M - high molecular weight markers

1 - incubation without protein kinase A

2 - incubation with protein kinase A

Autoradiograph;

1 - incubation without protein kinase A

2 - incubation with protein kinase A

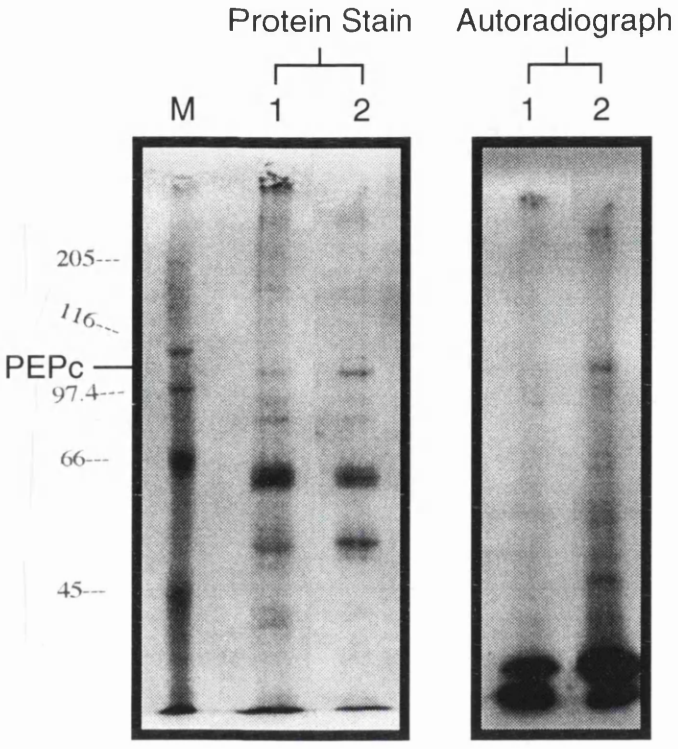

Protein Stain
Autoradiograph
M
1
2
1
2
205---
116---
PEPc
97.4---
66---
45---

Table 3.3 Ca^{2+} has no effect on phosphorylation of phosphoenolpyruvate carboxylase in guard cell extracts

An experiment was carried out exactly as in Figure 3.6, with one additional sample which contained 1mM free Ca^{2+}. The autoradiograph from this experiment was scanned as described in section 2.6.4.

Sample	peak area (arbitrary units)
Guard cell extract icubated with γ^{32}P MgATP alone	zero
Guard cell extract incubated with γ^{32}P MgATP and 1mM Ca^{2+}	zero
Guard cell extract incubated with γ^{32}P MgATP and PKA	25622

Carter *et al.* (1991) had previously used [^{32}P]-orthophosphate at a concentration of 50μM and specific activity of 4μCi/nmol to label phosphoenolpyruvate carboxylase in intact *Bryophyllum* leaves. The same concentration and specific activity were tried with guard cell protoplasts. In order to determine the length of incubation required to label the ATP pool to equilibrium, it would have been possible to make extracts of the guard cell protoplasts at different times during the incubation and analyse the ATP by HPLC. Because the quantities of tissue available were so small, a different approach was taken. The degree of labelling of total cell protein was used as an indication of the degree of labelling of the ATP pool. This is a valid method because the phosphate in phosphoproteins is constantly turned over by protein kinases and phosphatases to achieve a steady state of phosphorylation. As the ATP pool in a cell is becoming labelled with radioactive phosphate, the degree of labelling of the proteins in the cell will increase. After the ATP pool has reached equilibrium with the phosphate the cell is being incubated in, the degree of labelling of proteins will come to an equilibrium with the ATP pool.

Time course experiments such as that shown in Figure 3.7 were therefore carried out to determine the rate of labelling of the ATP pool and whether fusicoccin would affect this. Figure 3.7 shows that the phosphorylation state of protoplast proteins is higher after 3h than after 1.5h of incubation in [^{32}P] orthophosphate. The degree of labelling after 6h was highest of all (data not shown). This indicates that the phosphoprotein pool in guard cell protoplasts takes a considerable length of time to become fully labelled. Figure 3.7 also shows that there is no difference in the overall level of protein labelling when the cells are incubated with or without fusicoccin, although a few bands do appear to be present in one treatment but not the other.

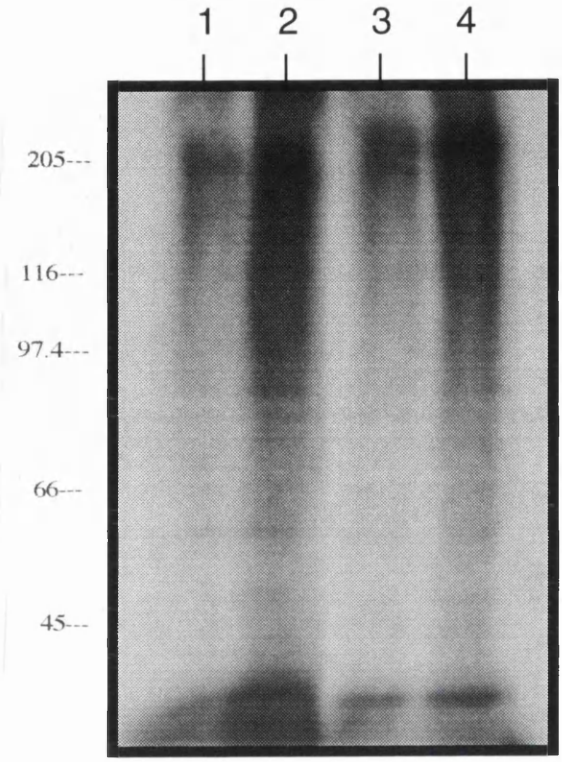
After 3h, the guard cell proteins were labelled to a degree which was easily detectable by autoradiography. Whilst the labelling of proteins still appeared to be increasing after 6h, it was felt that such a long preincubation would be excessive. Since fusicoccin did not appear to stimulate labelling of the total guard cell phosphoprotein pool, it was felt that the guard cell protoplasts could remain in the [^{32}P]-orthophosphate during experimental incubations. Any differences observed in the phosphorylation of phosphoenolpyruvate carboxylase could be attributed

Figure 3.7 Time course of labelling of total protein in guard cell protoplasts

Guard cell protoplasts were prepared as described in Methods section 2.2.2, and suspended in 500µl of protoplast suspension buffer. This suspension had [^{32}P] orthophosphate added to 50µM concentration and 4µCi/nmol specific activity. The suspension was split into two equal parts. One part had fusicoccin dissolved in ethanol added to 10nM concentration, the other had a control quantity of ethanol added. At various times, 50µl samples were removed from each incubation. Each sample was centrifuged for 3min at 13000g, and the pellets were resuspended in sample buffer and boiled for 5min. SDS polyacrylamide gel electrophoresis and autoradiography were then carried out as described in Methods section 2.6.1..

The first two samples from both the control and +fusicoccin incubations are shown. The later samples are not shown, as the autoradiograph tracks were very dark and did not reproduce well. Tracks:

1. – **fusicoccin, 1.5h**
2. – **fusicoccin, 3h**
3. + **fusicoccin, 1.5h**
4. + **fusicoccin, 3h**


1
2
3
4
205---
116---
97.4---
66---
45---

to differences in activity of PEPc kinase, rather than differences in ATP pool labelling.

3.3.4. The effect of fusicoccin on the phosphorylation state of phosphoenolpyruvate carboxylase

As described in section 3.2, fusicoccin greatly stimulated stomatal opening in *Commelina*. Experiments were carried out in order to determine whether phosphoenolpyruvate carboxylase would become phosphorylated in response to fusicoccin.

Guard cell protoplasts were incubated with [^{32}P]-orthophosphate and then had either 0.2% ethanol or 10nM fusicoccin plus 0.2% ethanol added. The gel and autoradiograph of this experiment (Figure 3.8) clearly show that fusicoccin stimulated phosphorylation of phosphoenolpyruvate carboxylase.

This result implies that the signal which switches on phosphoenolpyruvate carboxylase phosphorylation is not a primary stimulus such as light, but may be a cytosolic signal, such as K^+ or pH change. This is discussed more fully in the Discussion chapter section 4.5.

3.3.5 Time course of phosphorylation of guard cell phosphoenolpyruvate carboxylase

The time course of phosphorylation of phosphoenolpyruvate carboxylase is of interest since this can suggest which methods of signal transduction may be involved (see Discussion section 4.5). It is also necessary to show that the rate of phosphorylation of guard cell phosphoenolpyruvate carboxylase is comparable to the rate of change in physiological state of the tissue.

By preincubating guard cell protoplasts with [^{32}P] orthophosphate, then transferring them to the light and adding K^+ ions to 50mM concentration, the time course of phosphorylation of guard cell phosphoenolpyruvate carboxylase could be followed. Figure 3.9 shows the gel and autoradiograph of just such a time course, and demonstrates that the phosphorylation state of the enzyme increased with time in the light. In all, three such time course experiments were carried out, and control experiments in which the guard cell protoplasts were maintained in the dark throughout the time course were also carried out. The autoradiographs were scanned and the data from these scans are presented in Figure 3.10. In all cases where guard cell protoplasts were

Figure 3.8 Effect of fusicoccin on phosphorylation of guard cell phosphoenolpyruvate carboxylase.

Guard cell protoplasts were prepared as described in Methods section 2.2.2, and incubated for 3h with [^{32}P] orthophosphate as described in Methods section 2.7.2. The suspension was then diluted 1:1 with protoplast suspension buffer with 50mM K_2SO_4 added. This suspension was then split. One part (the control) had 0.2% EtOH added, the other had 10nM fusicoccin added. Both were incubated at room temperature for 1h in the dark. The protoplasts were kept in suspension throughout the experiment by intermittent agitation. At the end of the incubation, the guard cell protoplasts were centrifuged and extracted as described in Methods section 2.4.2, and phosphoenolpyruvate carboxylase was immunoprecipitated and run on an 8% gel. This gel was silver stained (Methods section 2.6.2), dried and autoradiographed. Tracks:

M- molecular weight markers

1- control

2- +fusicoccin.

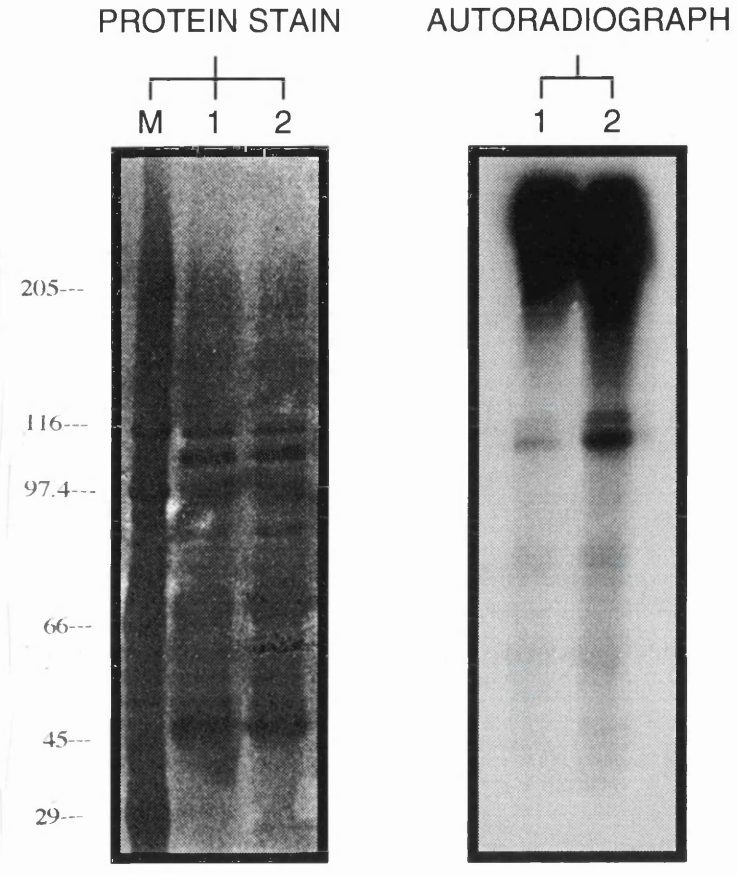

PROTEIN STAIN
AUTORADIOGRAPH
M 1 2
1 2
205---
116---
97.4---
66---
45---
29---

Figure 3.9 Time-course of phosphorylation of phosphoenolpyruvate carboxlase

Guard cell protoplasts were prepared and purified as described in Methods section 2.2.2, and incubated with [^{32}P] orthophosphate for 3h in the dark as described in Methods section 2.7.2 At time zero, the 1ml suspension of guard cell protoplasts was diluted to 2ml with 300mM mannitol, 20mM Mes/KOH pH 6.2, 50mM K_2SO_4, giving a final K^+ concentration of 50mM. The protoplast suspension, in a clear Pyrex tube, was then placed in the light. A water filter was used to prevent the protoplasts from being heated. Final incident light intensity was 200μE m^{-2} s^{-1}. The protoplasts were kept in suspension throughout the experiment by regular agitation. Samples of 350μl volume were removed at various times and extracted. Immunoprecipitation of phosphoenolpyruvate carboxylase was carried out as described in Methods section 2.6.1. The immunoprecipitate pellets were then denatured and subjected to electrophoresis on an 8% SDS polyacrylamide gel as described in Methods section 2.8.1. The tracks on the protein stained gel (left panel) and the autoradiograph (right panel) are as follows:

M - molecular weight markers (see Methods section 2.6.1)

numbered tracks are immunoprecipitate pellets from the following times;

1- 0min

2- 60min

3- 100min

4- 140min

5- 180min.

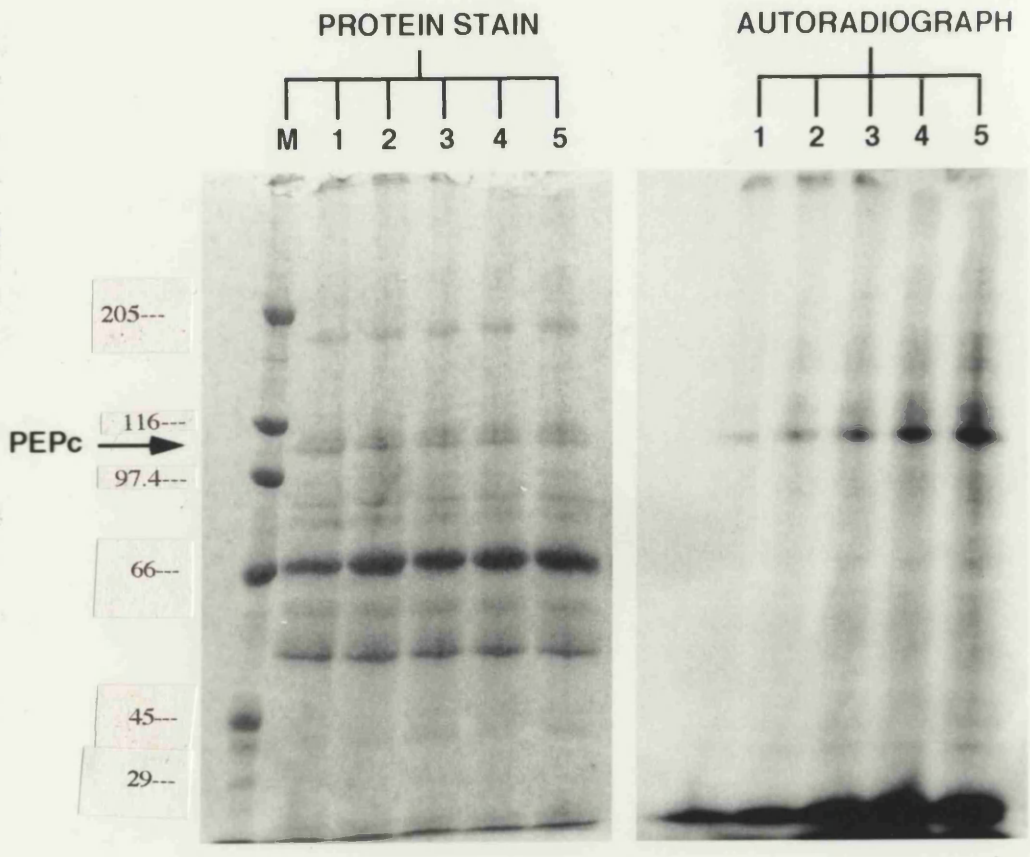

PROTEIN STAIN
AUTORADIOGRAPH
M 1 2 3 4 5
1 2 3 4 5
205---
116---
PEPc
97.4---
66---
45---
29---

Figure 3.10 Time-course of phosphorylation of phosphoenolpyruvate carboxylase in guard cell protoplasts incubated in the light and dark

A number of phosphorylation time course experiments were carried out using the same methods described in Figure 3.6 with the following changes:

Experiment 1: This is the experiment treated in Figure 3.6.

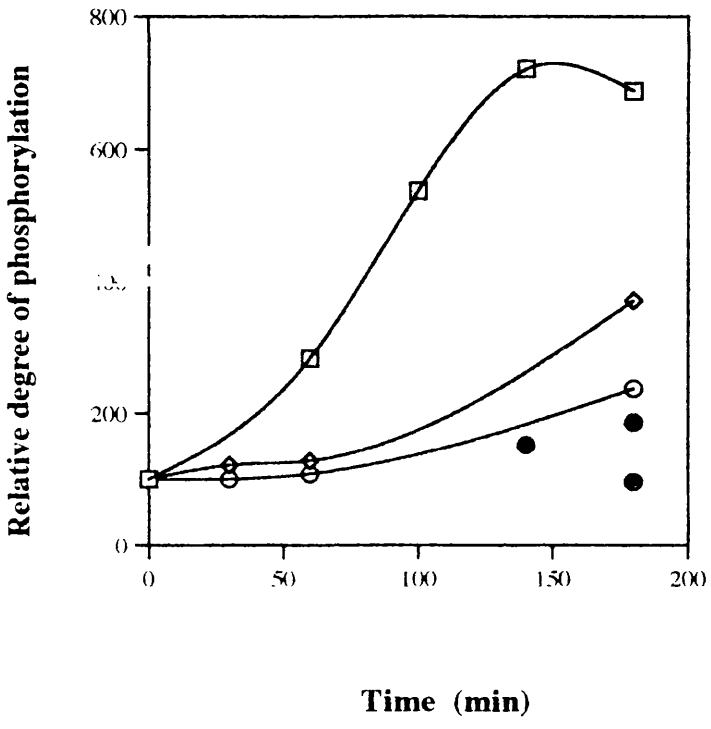
Experiment 2: As Figure 3.6, but sampling times were 30minutes, 60 minutes and 180 minutes.

Experiment 3: As experiment 2, but an additional incubation was carried out with guard cell protoplasts maintained in the dark throughout.

Experiment 4: Guard cell protoplasts were mainained in darkness throughout the time course, and samples were taken at 0minutes, 140minutes and 180minutes.

After gel electrophoresis and autoradiography as described in Figure 3.6, each of the autoradiographs was scanned as described in Methods section 2.6.4. The peak area data calculated by the scanner for the zero minutes time point in each case was assigned the value 100%. The rest of the data for each autoradiograph were then converted to percentage values by taking ratios with the zero time value, since each zero time point sample had been treated identically in all cases (see Figure 3.6). The conversion to percentage values was carried out so that comparisons could be made between the different experiments, as the actual peak area data from different experiments varied because exposure times of autoradiographs were not the same.

The graph shows phosphorylation state of phosphoenolpyruvate carboxylase as a percentage of the zero time value plotted against time for the following samples: experiment 1, open squares; experiment 2, open diamonds; experiment 3 (illuminated samples), open circles; experiment 4 and experiment 3 darkened sample, closed circles.


Relative degree of phosphorylation
800
600
200
0
0
50
100
150
200
Time (min)

incubated in the light, the level of phosphorylation of phosphoenolpyruvate carboxylase increased, whereas the level of phosphorylation of the dark controls remained at the same low starting level throughout. It can be clearly seen that the fold increase in phosphorylation state of phosphoenolpyruvate carboxylase varies greatly between the three experiments in which guard cell protoplasts had been illuminated. The largest increase in phosphorylation after 3h was 7 fold, the smallest about 2 fold. Apart from the different sampling times, all three experiments were carried out identically. Reasons for the variation are suggested in Discussion section 4.4.

In all three experiments carried out in the light, there appears to be an initial period of between 30 and 60 minutes before the rate of phosphorylation of the enzyme increases. This suggests that either induction of the kinase activity takes some time after the initial light stimulus is applied, or that light is not the stimulus at all. A change in cytosolic concentration of some metabolite or osmoticum (such as K^+) or a change in cytosolic pH may cause activation of the kinase, and it may take some time before an activating threshold is achieved.

3.3.6 The reversibility of phosphorylation of guard cell phosphoenolpyruvate carboxylase

If guard cell phosphoenolpyruvate carboxylase activity is controlled by phosphorylation, this phosphorylation must be reversible (see Introduction section 3.1), i.e. the phosphoenolpyruvate carboxylase must be dephosphorylated when any opening stimulus is removed.

Guard cell protoplasts which had been incubated for 3h in the dark with [^{32}P] orthophosphate were then placed in the light for 3h, and then placed back in the dark for 3h as described in Figure 3.11. A light stimulated increase in the phosphorylation state of phosphoenolpyruvate carboxylase was observed. The phosphorylation state of the enzyme then decreased after incubation in the dark.

The reduction in the incorporation of ^{32}P in guard cell phosphoenolpyruvate carboxylase was observed when the protoplasts had been back in the dark for 3h, but not after 1h. Many reasons could be suggested for the delay. One is that the dark closing stimulus may not be a very strong one for guard cell protoplasts in suspension in 300mM mannitol with 50mM K^+ ions present. Another possibility is that the dephosphorylation of phosphoenolpyruvate carboxylase simply does not

Figure 3.11 The reversibility of light-stimulated phosphorylation of phosphoenolpyruvate carboxylase.

A 1ml suspension of guard cell protoplasts was prepared as described in Methods section 2.2.2, and was incubated with [^{32}P] orthophosphate for 3h as described in Methods section 2.7.2. The protoplast suspension was then diluted 1:2 by the addition of 1ml of 300mM mannitol, 20mM Mes/KOH pH 6.2, 50mM K_2SO_4. At this point one 450µl sample was taken, and the remainder was placed in the light using the apparatus described in Methods section 2.7.2. After 3h, another 450µl sample was removed, and the remaining guard cell protoplast suspension was placed in darkness. Two further samples were removed after 1 and 3h in darkness. The guard cell protoplasts were kept in suspension throughout the experiment by regular agitation. In each case, immediately after a sample was removed, the guard cell protoplasts were centrifuged and extracted as described in Methods section 2.4.2. The phosphoenolpyruvate carboxylase was immunoprecipitated from each extract as described in Methods section 2.8.1, and the pellets denatured and run on an 8% SDS polyacrylamide gel. After staining with Coomassie blue, the gel was dried and autoradiography carried out as described in Methods section 2.6.3. The tracks are :

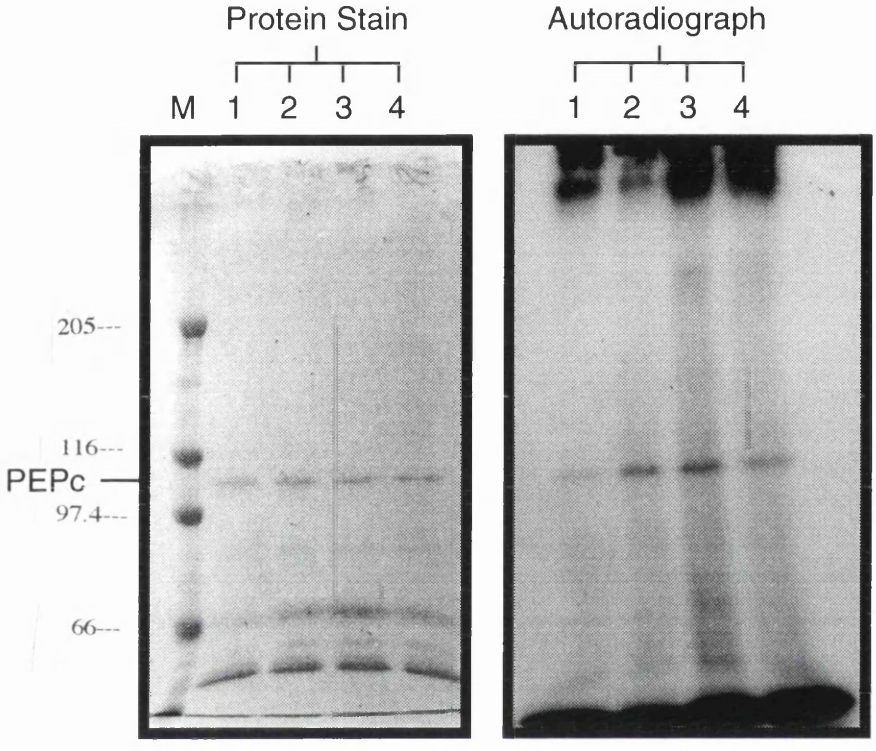
M- molecular weight markers

1- Time zero sample

2- Sample taken after 3h in the light

3- Sample taken 1h after returning to the dark

4- Sample taken 3h after returning to the dark.


Protein Stain
Autoradiograph
M 1 2 3 4
1 2 3 4
205---
116---
PEPc
97.4---
66---

occur very quickly in guard cells - this process presumably requires either the destruction or inactivation of the protein kinase, and if darkness was transitory (as for example with a cloud passing across the sun), the kinase would have to be resynthesised or activated. It would make sense, therefore, to have the dephosphorylation of phosphoenolpyruvate carboxylase delayed somewhat after the onset of a closing stimulus.

The dephosphorylation of phosphoenolpyruvate carboxylase in CAM and C4 systems is thought to occur via protein phosphatase 2A (PP2A) (Carter *et al.*,1990; McNaughton *et al.*, 1991). Phosphocasein phosphatase activity was therefore assayed in extracts of guard cell protoplasts. Extracts were diluted 5 and 10 fold to dissociate regulatory subunits, and phosphocasein phosphatase activity of 0.58mU/ml was detected (Table 3.4). This is nearly 3 times higher than the activity found in crude extracts of the CAM plant *Bryophyllum fedtschenkoi*, although extraction procedures were somewhat different (Carter *et al.*, 1990). By adding the inhibitor okadaic acid to the assay, 75% and 87% inhibition of the activity was obtained at 1nM and 10nM concentrations of the inhibitor respectively. This result indicates that the majority of the phosphocasein phosphatase activity in guard cells is type PP2A. In order to show unequivocally that PP2A is the phosphatase responsible for dephosphorylation of phosphoenolpyruvate carboxylase, it would be necessary to block the dark induced dephosphorylation by incubating the guard cell protoplasts in okadaic acid, which was not done.

3.3.7. Stomatal opening was delayed and phosphoenolpyruvate carboxylase phosphorylation was prevented by the protein synthesis inhibitor cycloheximide

The appearance of phosphoenolpyruvate carboxylase protein kinase in C4 and CAM plants is blocked by inhibitors of protein synthesis such as cycloheximide and puromycin (Carter *et al.*, 1991; Jiao *et al.*, 1991), suggesting that either it is synthesised *de novo* or that some protein must be synthesised for it to be activated. In order to determine whether protein synthesis is required for the phosphorylation of phosphoenolpyruvate carboxylase in guard cells, the effects of the protein synthesis inhibitor cycloheximide on fusicoccin stimulated phosphorylation of the enzyme and on light and fusicoccin stimulated stomatal opening were observed.

Table 3.4 Phosphocasein phosphatase activity in guard cell extract.

Guard cell protoplasts were prepared as described in Methods section 2.2.2, and extracted in 200µl of extraction buffer as described in Methods section 2.4.2, which had no okadaic acid included. Phosphocasein phosphatase activity was measured as described in Methods section 2.5.2. The extract was assayed undiluted and at 1:5 and 1:10 dilutions, with different concentrations of okadaic acid present.

Total phosphocasein phosphatase activity in extract (200µl) of approx 10^6 guard cell protoplasts at 1:10 dilution was 0.116 mU.

Activities (at 1/5 dilution)	**mU/ml**	**% of uninhibited activity**
Uninhibited activity	0.355	100
Activity at 1nM okadaic acid	0.089	25
Activity at 10nM okadaic acid	0.046	13

Cycloheximide at a concentration of 0.1mM was found to completely block the fusicoccin stimulated phosphorylation of guard cell phosphoenolpyruvate carboxylase (Figure 3.12). Moreover, the same concentration of cycloheximide was found to delay the opening of stomata in intact epidermis when stimulated to open either by light or by fusicoccin (Figures 3.13 and 3.14).

Cycloheximide is known to have effects other than the inhibition of protein synthesis. The compound has been shown to interfere with membrane transport processes (Evans and Smith, 1971; Parthier, 1974; Reilly *et al.*, 1970) and with respiration (Dheidah and Black, 1976). In order to ensure that non-specific effects did not account for the reduction in phosphorylation of phosphoenolpyruvate carboxylase when treated with cycloheximide, the viability of guard cells treated with the inhibitor was studied by vital staining using fluoresceine diacetate as described in Methods section 2.8.2. The viability of guard cells was checked at important stages during preparation and experimental manipulation. Once the guard cell protoplasts were prepared and purified, cycloheximide was added to 0.1mM concentration, and the cells were observed again 2h later. This is the same total length of time that the guard cell protoplasts spend in cycloheximide during experiments studying the effect of the compound on phosphoenolpyruvate carboxylase phosphorylation. The results of this study are shown in Figure 3.15. Clearly the guard cell protoplasts were gradually dying with time, and those treated with cycloheximide did appear to be dying slightly faster than those without. The difference in the number of viable cells after 2h with cycloheximide, however, is not large enough to account for the total lack of phosphorylation of guard cell phosphoenolpyruvate carboxylase in protoplasts treated with the inhibitor. This result does not rule out the possibility that other properties of cycloheximide, such as interference with membrane properties, could be causing the effect. It would be necessary to carry out the same experiments using different protein synthesis inhibitors in order to determine this.

3.3.8. The effects of okadaic acid and abscisic acid on the phosphorylation of guard cell phosphoenolpyruvate carboxylase

The responses of guard cell phosphoenolpyruvate carboxylase phosphorylation to okadaic acid and abscisic acid were studied (Figure

Figure 3.12 The effect of cycloheximide on fusicoccin-stimulated phosphorylation of phosphoenolpyruvate carboxylase.

A 700µl suspension of guard cell protoplasts was prepared as described in Methods section 2.2.2, and these protoplasts were incubated with [^{32}P] orthophosphate as described in Methods section 2.7.2. At the end of this incubation, 250µl of the suspension were removed and had cycloheximide added to 0.1mM concentration. All of the protoplasts were then incubated for one further hour in the dark. The suspension without cycloheximide was then split in half. One half of this had fusicoccin added to 10nM, the other had only a control amount of ethanol added. The cycloheximide treated sample had 10nM fusicoccin added. All three samples were then diluted 1:1 with protoplast suspension buffer containing 50mM K_2SO_4 and then incubated for one further hour in the dark. At the end of this incubation, 200µl of each suspension was removed and the guard cell protoplasts spun down and extracted as described in Methods section 2.4.2. phosphoenolpyruvate carboxylase was immunoprecipitated from all three samples and run on an 8% gel. After drying, autoradiography was carried out. The tracks on the gel and corresponding autoradiograph are:

M- molecular weight markers

1- minus fusicoccin minus cycloheximide treatment

2- plus fusicoccin minus cycloheximide treatment

3- plus fusicoccin plus cycloheximide treatment.

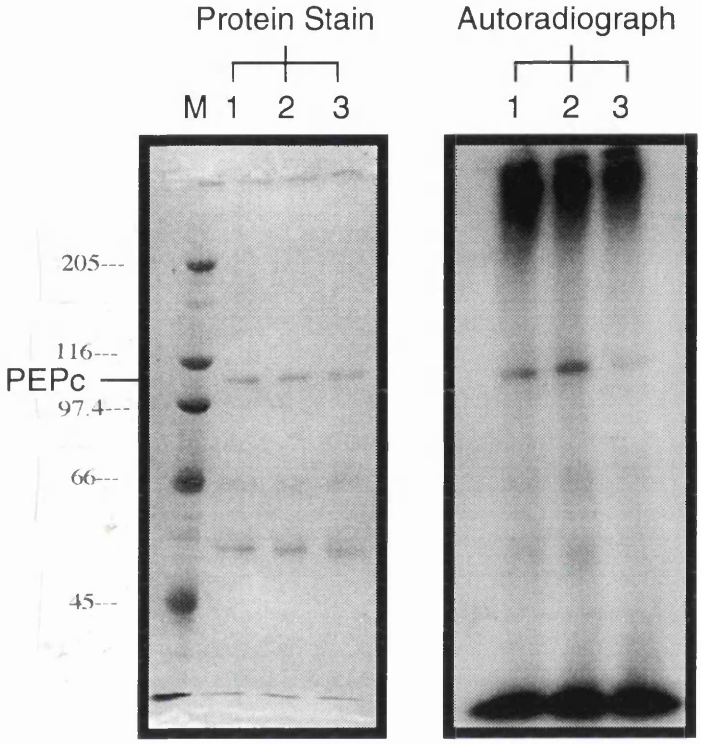

Protein Stain
Autoradiograph
M 1 2 3
1 2 3
205---
116---
PEPc
97.4---
66---
45---

Figure 3.13 The effect of cycloheximide on light-stimulated stomatal opening.

Strips of epidermis (5mm x 5mm) were pre-incubated (with agitation) at 22°C for 1h with 0.1mM $Ca(NO_3)_2$ containing 0.1% ethanol or 0.1mM cycloheximide (chx) in ethanol. Strips were then transferred to 25mM K_2SO_4 with either ethanol or cycloheximide. The strips were then illuminated and CO_2 free air was bubbled through the media as descrbed in Methods section 2.7.1. Apertures are means of 36 measurements from 3 strips. Units of aperture are eyepiece units (epu).

This graph shows the change in aperture of stomata in epidermis which was pre-incubated with (diamonds) or without (squares) 0.1mM cycloheximide.

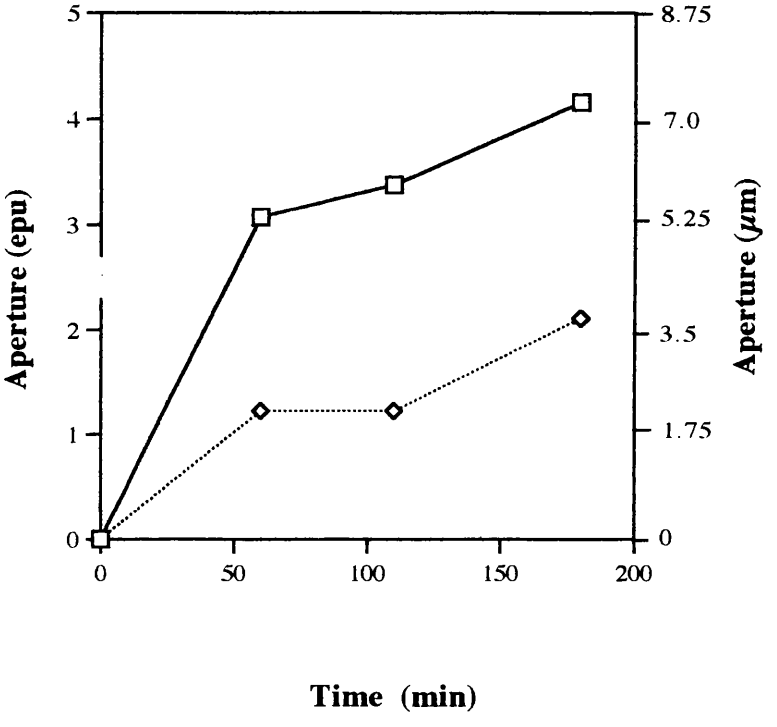

5
4
3
2
1
0
8.75
7.0
5.25
3.5
1.75
0
0
50
100
150
200
Aperture (epu)
Aperture (μm)
Time (min)

Figure 3.14 The effect of cycloheximide on fusicoccin-stimulated stomatal opening.

Strips of epidermis (5mm x 5mm) were pre-incubated with agitation for 1h on 0.1 mM $Ca(NO_3)_2$ containing 0.1% EtOH or cycloheximide in ethanol. Strips were then transferred to 25mM K_2SO_4, 10nM fusicoccin with either EtOH or cycloheximide. Incubation (with agitation) was at 22°C. Apertures (measured as described in Methods section 2.7.1) are means of 36 measurements from 3 strips. Units of aperture are eyepiece uints (epu).

This graph shows the change in aperture of stomata in epidermis incubated with 0.1mM cycloheximide (closed diamonds), 1.0mM cycloheximide (closed squares) or no cycloheximide (open squares).

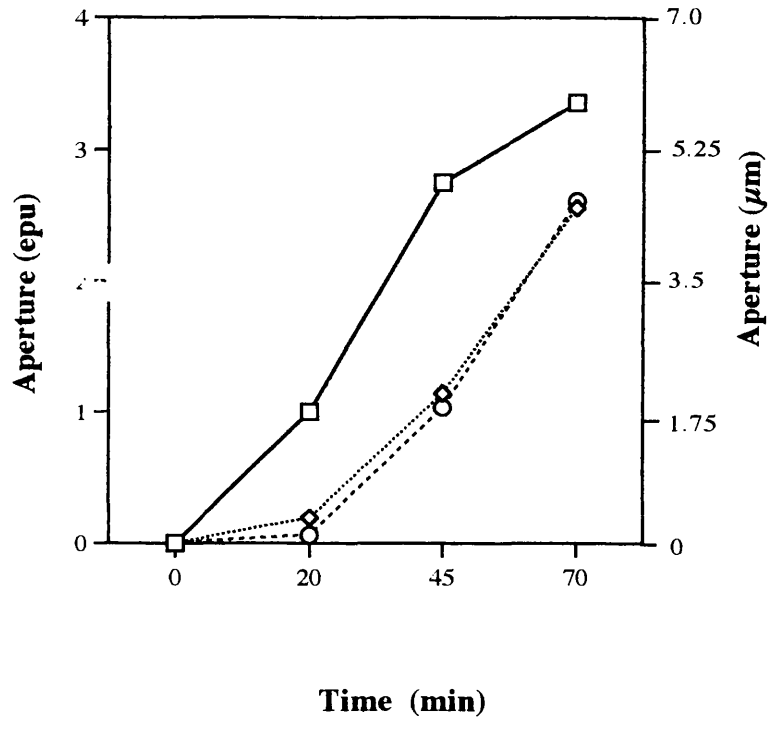
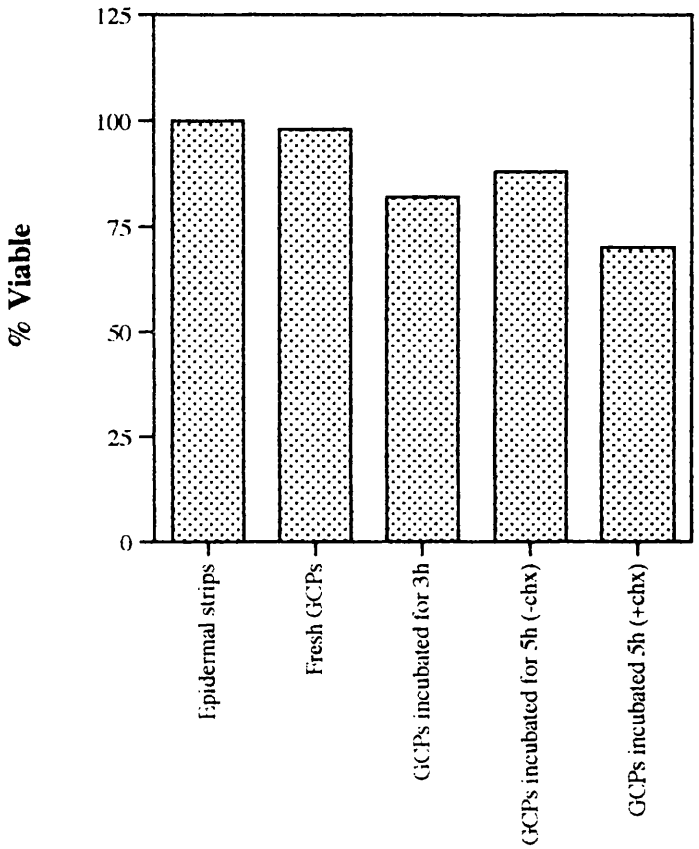

4
3
1
0
7.0
5.25
3.5
1.75
0
0
20
45
70
Aperture (epu)
Aperture (μm)
Time (min)

Figure 3.15 The viability of guard cells during production of guard cell protoplasts and their subsequent incubation with cycloheximide.

Fluorescein diacetate was mixed with protoplast suspension buffer as described in Methods section 2.8.2. At each stage studied, a sample of the tissue (either a few small pieces of epidermis or a few drops of protoplast suspension) were taken. The epidermis was floated on top of the buffer containing fluorescein diacetate, whilst the protoplast suspension was mixed 1:1 with the fluorescein diacetate buffer. After 5min in fluorescein diacetate, the samples were mounted onto a glass slide and observed under medium power under the microscope. All of the guard cells or guard cell protoplasts in the viewing field were counted under white light. The white light source was then turned off and the same field viewed under blue light. Guard cells or guard cell protoplasts fluorescing yellow/green were then counted. This fluorescence originates from fluorescein, released from the fluorescein diacetate by an esterase in the cytosol. Non living cells cannot take up fluorescein diacetate, so only viable cells will fluoresce. A total of four fields were observed for each sample. The stages at which samples were taken were;

1. Epidermis was sampled immediately after it had been stripped from the plants for protoplast preparation.
2. Guard cell protoplasts were sampled immediately after their preparation and purification.
3. Guard cell protoplasts were then incubated, in protoplast suspension buffer (Methods section 2.2.2), for 3h to mimic the time they would normally spend in incubation with [^{32}P] orthophosphate. At the end of this time, another sample was taken.
4. At this stage, the guard cell protoplast preparation was split into two. One part had 0.1mM cycloheximide (chx) added, the other did not. Two hours later, both the plus and minus cycloheximide guard cell protoplast suspensions were sampled.

The bar chart shows the percentage of guard cells which were found to be viable, using fluorescein diacetate fluorescence as an indicator, at various stages during guard cell protoplast preparation and incubation.



Cells tested for viability.

3.16). The effect of okadaic acid on the enzyme is of interest since, if in the dark this compound produces increased phosphorylation, there must be kinase present, albeit in amounts which cannot phosphorylate the enzyme more quickly than the phosphatase can dephosphorylate it. This result would also indicate that PP2A is the enzyme responsible for dephosphorylating guard cell phosphoenolpyruvate carboxylase. However, okadaic acid did not, in fact, increase the level of phosphorylation of guard cell phosphoenolpyruvate carboxylase (Figure 3.16), and this is difficult to interpret because;

1. We do not know if okadaic acid can cross the guard cell plasmalemma.
2. We do not know if PP2A is responsible for dephosphorylating guard cell phosphoenolpyruvate carboxylase.
3. In the darkened state, there may not be any phosphoenolpyruvate carboxylase kinase present, in which case stopping dephosphorylation would not lead to any increase in phosphorylation.

The result does not, therefore, rule out the possibility that guard cell phosphoenolpyruvate carboxylase is dephosphorylated by PP2A.

Abscisic acid is a strong closing stimulus for guard cells, and would not be expected to stimulate phosphorylation of phosphoenolpyruvate carboxylase. It is clear from Figure 3.16 that the response to abscisic acid is as expected. The response of the sample of the same guard cell protoplast preparation to fusicoccin shows clearly that they are capable of responding when an appropriate stimulus is applied. It is interesting to note that in all cases when a stimulus is applied to guard cell protoplasts, the level of phosphorylation of phosphoenolpyruvate carboxylase changes in the expected direction.

3.4 Studies on the kinetic properties of guard cell phosphoenolpyruvate carboxylase

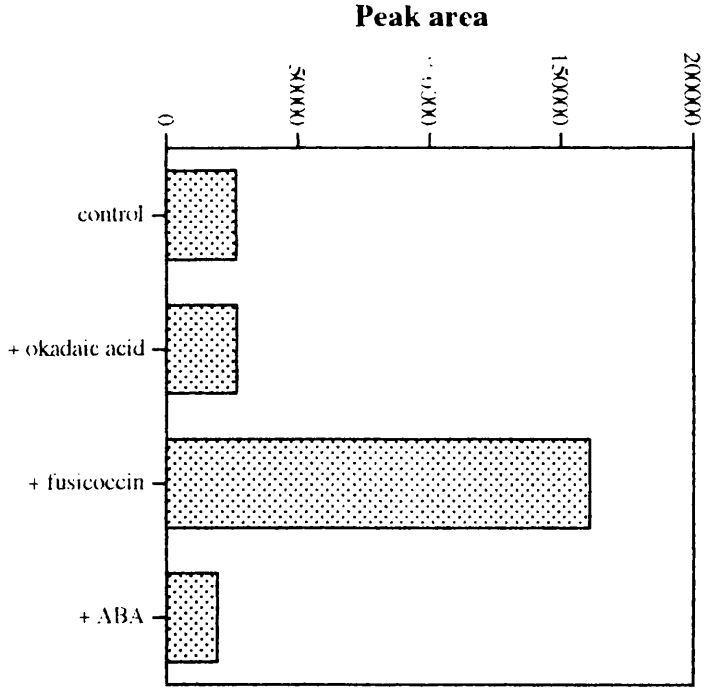
The kinetic properties of guard cell phosphoenolpyruvate carboxylase have been widely studied, but the question of whether the activity of the enzyme changes during opening and closure of stomata remains controversial (see Introduction section 1.2). Experiments were carried out in which both intact guard cell protoplasts and guard cell extracts were manipulated to alter the presumed phosphorylation state of phosphoenolpyruvate carboxylase. Kinetic parameters of the enzyme in various states were then measured.

Figure 3.16 The effect of okadaic acid and ABA on phosphorylation of guard cell phosphoenolpyruvate carboxylase in the dark.

A 1ml suspension of guard cell protoplasts was prepared as described in Methods section 2.2.2, and incubated with [^{32}P] orthophosphate as described in Methods section 2.7.2. The suspension was then diluted 1:1 with protoplast suspension buffer containing 50mM K_2SO_4. This was then divided into four equal parts, and the following additions made:

Incubation	**Additions**
control	0.2% EtOH, 0.1% DMS.
+ okadaic acid	0.2% EtOH, 0.1% DMS, 100nM okadaic acid.
+fusicoccin	0.2% EtOH, 0.1% DMS, 10nM fusicoccin.
+ABA	0.2% EtOH, 0.1% DMS, 100μM ABA.

All samples were incubated in the dark at room temperature for 1h before extraction as described in Methods section 2.4.2. PEP carboxylase was immunoprecipitated from all samples and run on an 8% gel. After drying and autoradiography, the bands on the autorad corresponding to phosphoenolpyruvate carboxylase were scanned using a Shimadzu flying spot laser densitometer as described in Methods section 2.6.4. Data are peak areas generated from the scans.


Peak area
0
150000
200000
control
+ okadaic acid
+ fusicoccin
+ ABA

The assay conditions (phosphoenolpyruvate carboxylase assay buffer A, Methods section 2.5.1) first used to measure phosphoenolpyruvate carboxylase activity in extracts of guard cell protoplasts and epidermal strips were those which had been used previously by Nimmo *et al.* (1986,1987) to measure the activity of the enzyme from maize and *Bryophyllum*. This assay was found to give easily measurable and reproducible rates of phosphoenolpyruvate carboxylase activity with low background when used to assay the enzyme in crude extracts.

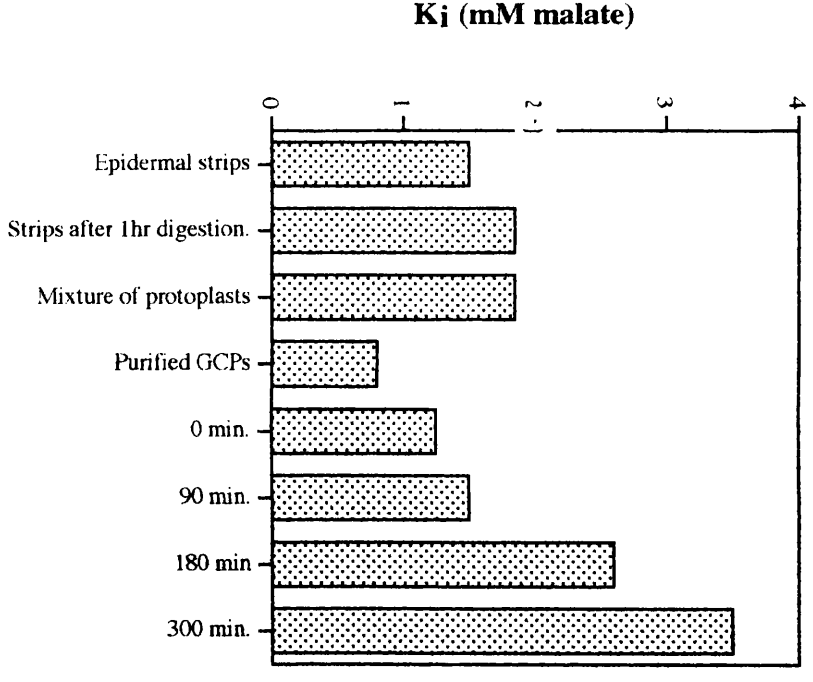
3.4.1 The stability of phosphoenolpyruvate carboxylase activity during preparation of guard cell protoplasts and in extracts

It is known that phosphoenolpyruvate carboxylase from maize and *Bryophyllum* is susceptible to proteolytic cleavage of a small peptide which results in loss of sensitivity to inhibition by malate (Nimmo *et al.*, 1986; McNaughton *et al.*, 1989). The possibility that guard cell phosphoenolpyruvate carboxylase becomes proteolysed in the same way, either during preparation of guard cell protoplasts or after preparation of crude extracts, was investigated (Figure 3.17). At a number of stages during the preparation of guard cell protoplasts, samples were removed and extracted and the K_i for malate was determined. An extract of epidermis was also prepared and allowed to stand on ice. Samples of this were also taken and the K_i of the enzyme was measured. The K_i of phosphoenolpyruvate carboxylase did not change during preparation of guard cell protoplasts until after the final stage, at which point it was reduced from 1.85mM to 0.8mM. This final stage in the procedure is the separation of guard cells from all other cell types, so the change in K_i may be due to a change in the population of phosphoenolpyruvate carboxylase in the assay, rather than to proteolysis. In an extract of epidermis, however, the sensitivity of phosphoenolpyruvate carboxylase to inhibition by malate decreased with time. There was only a small increase in K_i for malate during the first 90min of this incubation, but in the following 90min the K_i doubled. For this reason, all subsequent assays were carried out immediately after extraction unless indicated otherwise.

Figure 3.17 Variation of K_i for malate of epidermal and guard cell phosphoenolpyruvate carboxylase during preparation of guard cell protoplasts and incubation of epidermal extract

The procedure for preparation of guard cell protoplasts was as described in Methods section 2.2.2. At a number of stages during the preparation, samples of tissue were removed and immediately homogenised in extraction buffer as described in Methods sections 2.4.1 and 2.4.2. These extracts were then immediately assayed for phosphoenolpyruvate carboxylase activity and sensitivity to malate as described in Methods section 2.5.1. The following tissue samples were removed; epidermal strips after preparation and floating on 150mM mannitol, 10mM Mes/KOH pH6.2; epidermal strips removed after 1h incubation on cell wall digestion medium; mixed population of protoplasts centrifuged and washed after the complete digestion of the epidermis; finally, purified guard cell protoplasts were isolated at the end of the procedure. An extract was also made of epidermal strips, which was then incubated on ice. Samples of this were removed and assayed at the following times; immediately after homogenisation (0min); 90min; 180 min; 300min.

The bar chart shows the K_i for malate of phosphoenolpyruvate carboxylase from the tissues taken at various stages of the protoplast preparation procedure, and at various time points after extraction.


Ki (mM malate)
0
1
2
3
4
Epidermal strips
Strips after 1hr digestion.
Mixture of protoplasts
Purified GCPs
0 min.
90 min.
180 min
300 min.

3.4.2 Some kinetic parameters of guard cell phosphoenolpyruvate carboxylase

The pH profiles of phosphoenolpyruvate carboxylase activity and K_i for malate were determined (Figure 3.18). Both activity and K_i increase as pH increases, and these results are in reasonable agreement with those of Tarzcynski and Outlaw (1990). The large difference in K_i between that measured at pH 7.8 and that measured at pH 8.0 suggests that the enzyme loses sensitivity to inhibition by malate at higher pH values. Since at pH 7.8 the uninhibited activity was relatively high, but K_i for malate much lower, it was decided that this pH was the best to use to continue the investigation.

The sensitivity of the enzyme to inhibition by malate decreases as the concentration of PEP increases (Figure 3.19), suggesting that malate is acting as a competitive inhibitor. When K_m (PEP) and V_{max} were determined for phosphoenolpyruvate carboxylase assayed at different malate concentrations, however, both parameters were found to increase as the concentration of malate increased, and at 5mM malate they cannot be calculated (Table 3.5). The Enzpack program (Methods section 2.5.1) - which was used to calculate kinetic parameters from experimental results - involves the assumption that the kinetics of the enzyme concerned are hyperbolic in nature, i.e. that the enzyme has Michaelis-Menten type kinetics. An enzyme exhibiting sigmoidal properties cannot be characterised by this program. In the absence of malate, guard cell phosphoenolpyruvate carboxylase fits the Michaelis-Menten model well, whilst at 5mM malate, it does not fit the model at all, suggesting a transition from hyperbolic to non-hyperbolic kinetics with increasing malate concentration. Phosphoenolpyruvate carboxylase from maize has previously been shown to exhibit transitions between hyperbolic and sigmoidal kinetic forms under certain circumstances (O'Leary, 1982).

3.4.3 Attempts to alter the kinetic properties of guard cell phosphoenolpyruvate carboxylase by incubating cells and extracts in conditions likely to cause a change in phosphorylation state of the enzyme

Phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi* undergoes a ten-fold increase in K_i for malate (when assayed using assay buffer A) when it is fully phosphorylated. Initially, buffer A was also

Figure 3.18 Activity of phosphoenolpyruvate carboxylase and sensitivity to inhibition by malate at different pH values

Guard cell protoplasts were prepared as described in Methods section 2.2.2. The preparation was homogenised in extraction buffer (Methods section 2.4.2). Assays were carried out as described in Methods section 2.5.1 using assay buffer A with the following adjustments; assays at pH7.2 to 7.6 used Hepes buffer, adjusted to the appropriate pH, instead of Tris; assays at pH 7.8 and 8.0 were carried out using assay buffer A adjusted to the appropriate pH. Malate sensitivities were determined as described in section 2.5.1.

The graph shows phosphoenolpyruvate carboxylase activity (squares, solid line) and K_i (diamonds, dotted line) of guard cell phosphoenolpyruvate carboxylase assayed at different pH values.

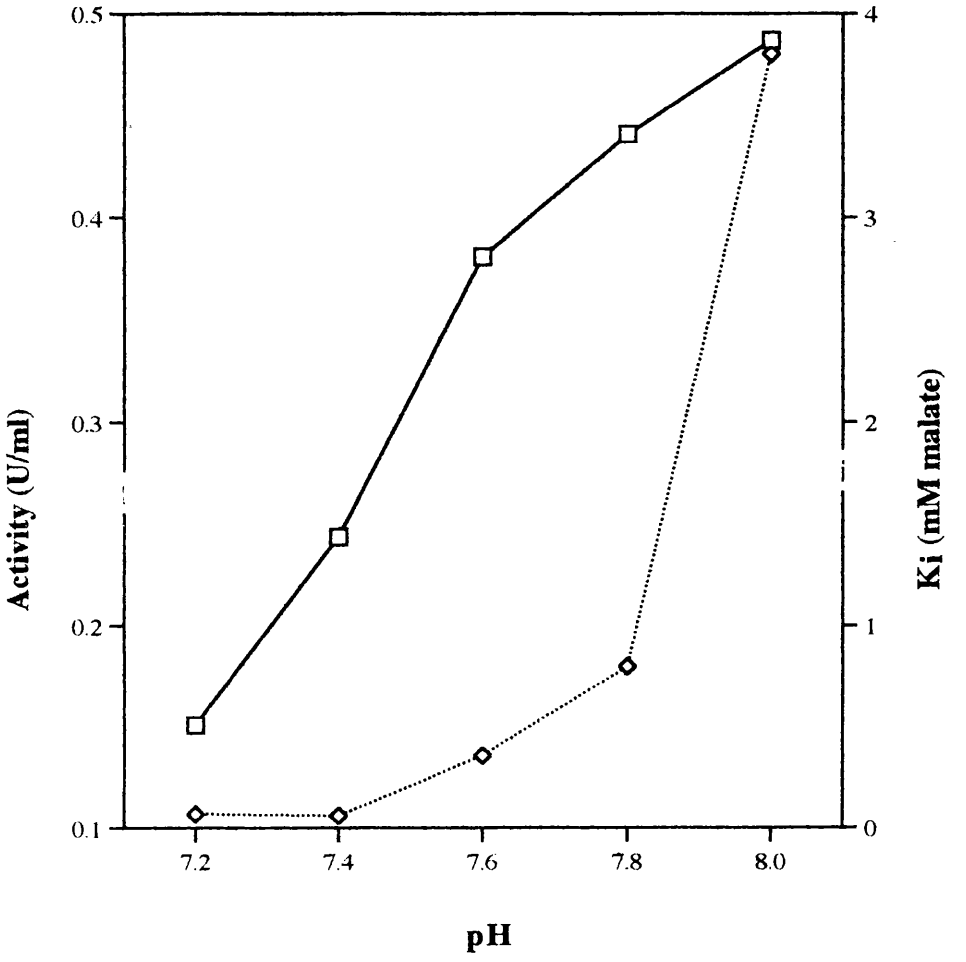

0.5
0.4
0.3
0.2
0.1
Activity (U/ml)
4
3
2
1
0
Ki (mM malate)
7.2
7.4
7.6
7.8
8.0
pH

Figure 3.19 Variation of K_i of phosphoenolpyruvate carboxylase at different concentrations of PEP

Guard cell protoplasts were prepared as described in Methods section 2.2.2, and homogenised in extraction buffer as described in Methods section 2.4.2. Assays were carried out at pH 7.8 as described in Methods section 2.5.1 using assay buffer A with different concentrations of PEP and malate. K_i values were calculated as described in Methods section 2.5.1.

The graph shows the variation in K_i for malate with increasing PEP concentration.

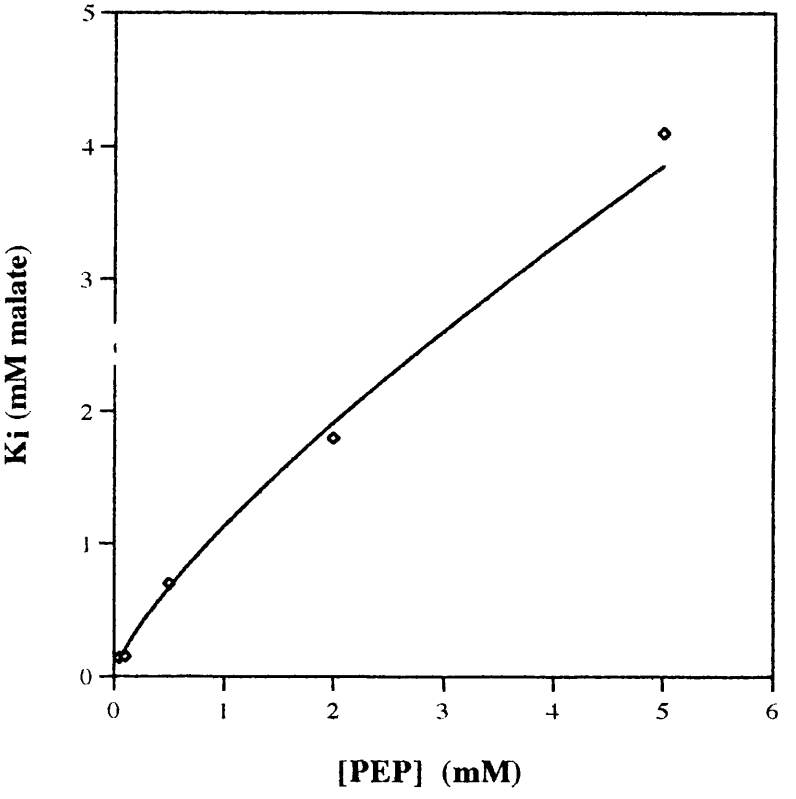

Ki (mM malate)
[PEP] (mM)
0
1
2
3
4
5
6

Table 3.5 Variation of K_m (PEP) and V_{max} of guard cell phosphoenolpyruvate carboxylase at different malate concentrations

Guard cell protoplasts were prepared as described in Methods section 2.2.2, and homogenised in 300µl of extraction buffer. K_m (PEP) values were determined at pH 7.8 as described in Methods section 2.5.1, using assay buffer A with different concentrations of malate. The malate concentrations used were 0.0mM, 1.0mM, 2.5mM and 5.0mM. K_m and V_{max} values were obtained by the direct linear method using the Enzpack program as described in section 2.5.1. The V_{max} values recorded are in arbitrary units, but are directly related to one another since the same volume of extract from the same homogenate was used in all assays.

Concentration of malate (mM)	K_m (PEP) mM	V_{max} (arbitrary units)
0.0	0.10	15.6
1.0	2.57	23.1
2.5	11.6	37.0
5.0	*	*

* These values could not be calculated by the Enzpack program, as the reaction rate varied with substrate concentration in a way which did not fit with Michaelis-Menten assumptions.

used to study the way in which guard cell phosphoenolpyruvate carboxylase responds to phosphorylation.

Table 3.6 shows the results of experiments in which attempts were made to alter the K_i (malate) of guard cell phosphoenolpyruvate carboxylase by incubating guard cell protoplasts in conditions which had been shown to alter the phosphorylation state of the enzyme. In the first set of experiments, in which guard cell protoplasts were incubated either in the light or in the dark in the presence of 50mM K^+ ions, no significant increase in K_i was observed. In a single experiment with fusicoccin as the phosphorylation stimulus, again no change in K_i was observed. It was not known if these incubations had brought about a large enough change in phosphorylation state to produce an observable change in K_i. Attempts to directly change the phosphorylation state of phosphoenolpyruvate carboxylase in crude extracts by incubating them with either protein kinases or protein phosphatases were carried out. Figure 3.20 shows how the K_i for malate of guard cell phosphoenolpyruvate carboxylase responded to incubation of guard cell protoplast extract with PP2A. K_i for malate did not change. When guard cell extracts were incubated with PP2A or PP1, again there was no change detected in K_i for malate (Figure 3.21). It is possible that all of the phosphoenolpyruvate carboxylase in these extracts was in a non-phosphorylated state, in which case incubation with phosphatase would not have any effect. Incubation of guard cell extracts with Mg ATP in the presence of protein kinase A (PKA) catalytic subunits or partially purified phosphoenolpyruvate carboxylase kinase did not bring about a significant change in K_i (Figure 3.22). In order to eliminate the possibility that protein phosphatase activity in the extracts rapidly reverses the phosphorylation of the phosphoenolpyruvate carboxylase, extracts were made either with or without the phosphatase inhibitor okadaic acid. Subsequent incubation of these extracts did not bring about a change in K_i for malate (Figure 3.23). It was concluded that none of the manipulations carried out - either with intact guard cell protoplasts or with extracts - had brought about a degree of phosphorylation sufficient to change the kinetic properties of guard cell phosphoenolpyruvate carboxylase in a way which could be detected using assay buffer A.

Table 3.6 K_i (malate) values measured using assay buffer A of phosphoenolpyruvate carboxylase from guard cells incubated under different conditions

Guard cell protoplasts, prepared as described in Methods section 2.2.2, were incubated in protoplast suspension buffer with 50mM K^+ ions (Methods section 2.7.2). Each preparation was split into two approximately equal parts and incubated either in darkness or with illumination at 200μE/m^2/ s (done three times) or in darkness +/- 10nM fusicoccin (one single experiment). These incubations were carried out as described in Methods section 2.7.2. After incubation, guard cell extracts were made and malate sensitivities determined using assay buffer A as described in Methods section 2.5.1. Means and standard deviations of the data from the darkened and illuminated guard cell protoplasts were determined, and a Student's t test used to determine the probability of identity of the two means.

	Dark		**Light**
K_i (mM malate)	1.3		1.9
Standard deviation	0.38		1.43
n	4		3
Probability of identity		> 47%	

	– Fusicoccin	**+ Fusicoccin**
K_i (mM malate)	1.3	1.5

Figure 3.20 Variation of K_i (malate) of guard cell phosphoenolpyruvate carboxylase during incubation of guard cell protoplast extract with PP2A

Guard cell protoplasts were prepared as described in Methods section 2.2.2, and extracted as described in Methods section 2.4.2. The K_i (malate) was then determined, using assay buffer A, as described in Methods section 2.5.1. Two 117µl aliquots of this extract were then taken. One aliquot had 3µl of PP2A (see Methods section 2.5.2) added (diamonds), the other (control) had 3µl of extraction buffer A added (squares). Both aliquots were incubated at 30°C and samples were removed at 20 and 45 minutes. K_i determinations were made immediately on removal of samples from incubation. The graph shows K_i (malate) values during the incubation.

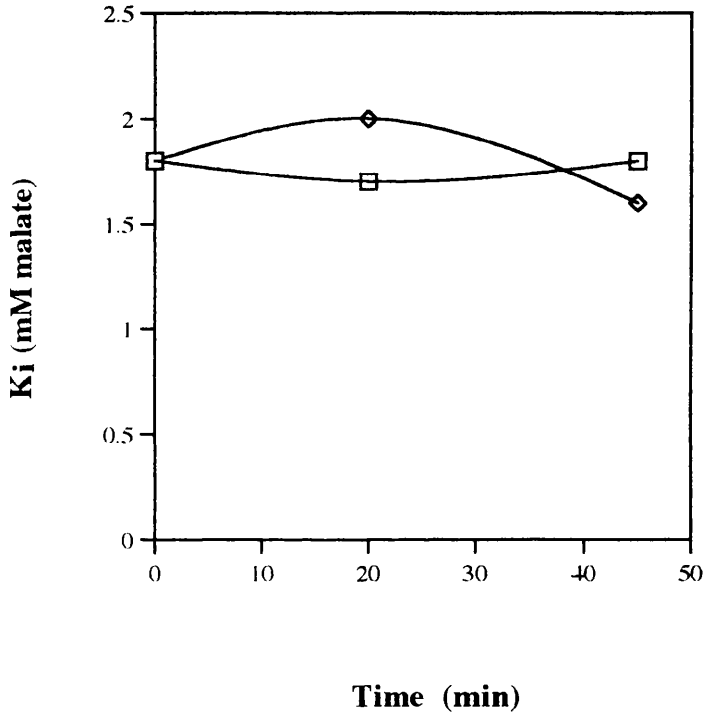

2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
Ki (mM malate)
Time (min)

Figure 3.21 The effect of incubation of extracts with PP2A and PP1 on the malate sensitivity of guard cell phosphoenolpyruvate carboxylase

Guard cell protoplasts were prepared as described in Methods section 2.2.2, and extracted in 300µl of extraction buffer as described in Methods section 2.4.2. Immediately after homogenisation, the K_i (malate) was determined as described in section 2.5.1. Three samples of extract of 90µl each were taken and numbered 1 to 3.

Sample 1 had 10µl of PP2A added.

Sample 2 had 10µl of PP1 added.

Sample 3 had 10µl of extraction buffer added.

All three were incubated for 1h at 30°C. At the end of these incubations, the K_i (malate) was determined for all three samples. The columns on the bar chart show the K_i (malate) values for the following samples:

column 1, extract immediately after preparation

column 2, sample 1 (+PP2A) after 1h

column 3, sample 2 (+PP1) after 1 h

column 4, sample 3 (control) after 1h

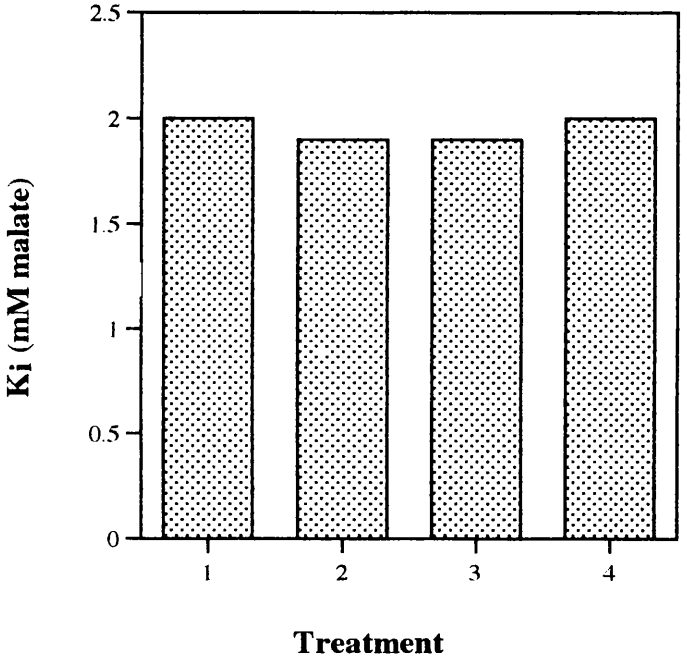

2.5
2
1.5
1
0.5
0
Ki (mM malate)
1
2
3
4
Treatment

Figure 3.22 The effect of protein kinases on the K_i (malate) of guard cell phosphoenolpyruvate carboxylase

Guard cell protoplasts were prepared as described in Methods section 2.2.2, and homogenised in 400µl of extraction buffer as described in Methods section 2.4.2. The K_i (malate) of this extract was immediately determined as described in section 2.5.1. Three 100µl aliquots of this extract were taken and the following additions made:

1 (control) 20 mM Mg^{2+}, 0.5mM ATP, 10µl extraction buffer.

2 (+PKA) 20mM Mg^{2+}, 0.5 mM ATP, 10µl PKA (Methods section 2.8.4)

3 (+PEP carboxylase kinase) 20mM Mg^{2+}, 0.5mM ATP, 10µl PEPc kinase (Methods section 2.8.4)

All three samples were incubated at 30°C. Samples were removed at 15min, 45min and 90min, and K_i (malate) was determined. The graph shows the K_i values for the control sample (squares, plain line), +PKA sample (diamonds, evenly dashed line) and the +PEPc kinase sample (circles, unevenly dashed line). Lines were fitted using the Cricket graph 3 program on Apple Macintosh.

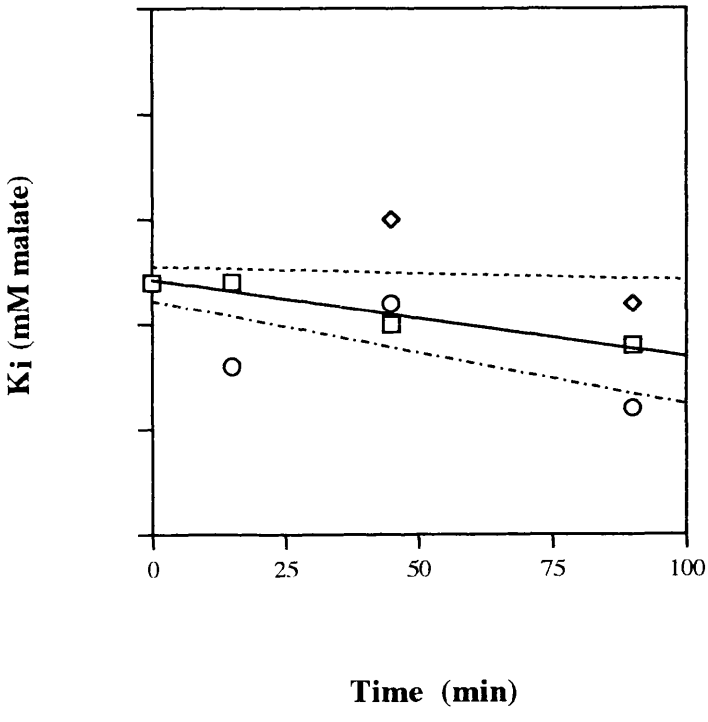
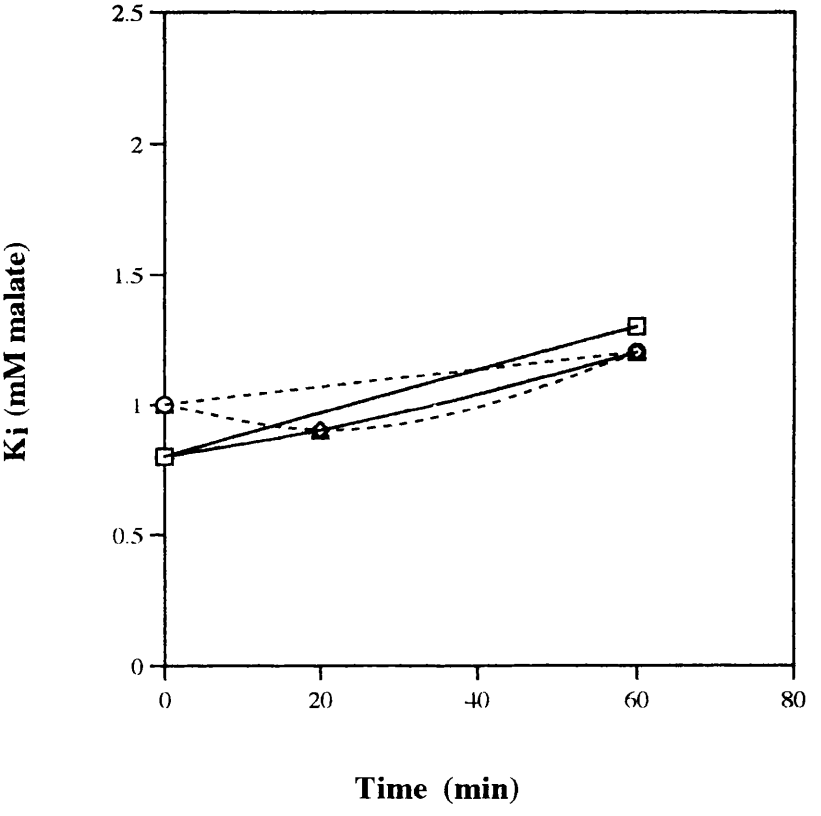

Ki (mM malate)
0
25
50
75
100
Time (min)

Figure 3.23 The effect of okadaic acid and protein kinase A catalytic subunits on K_i (malate) of guard cell phosphoenolpyruvate carboxylase

Guard cell protoplasts were prepared as described in Methods section 2.2.2. The preparation was split into two parts. One part (-OA) was homogenised in 200µl of extraction buffer . The other part (+OA) was homogenised in 200µl of extraction buffer with 10nM okadaic acid. Each of these extracts was then split into two parts, with the following additions being made:

1 -OA (control); 20mM Mg^{2+}, 0.5mM ATP, 10µl extraction buffer
2 -OA (+PKA); 20mM Mg^{2+}, 0.5mM ATP, 10µl PKA
3 +OA (control); 20 mM Mg^{2+}, 0.5mM ATP, 10µl extraction buffer
4 +OA (+PKA); 20mM Mg^{2+}, 0.5mM ATP, 10µl PKA

The Ki (malate) of samples 1 and 3 were determined immediately, and then all four were incubated at 30°C. Aliquots of samples 2 and 4 were removed at 20 and 60 minutes, and their K_i (malate) values determined. The K_i (malate) of samples 1 and 3 was also determined after 1h incubation. The graph shows the K_i values for each sample during the incubation timecourse: **sample 1**; squares, plain line: **sample 2**; diamonds, plain line: **sample 3**; circles, dashed line: **sample 4**; triangles, dashed line.


2.5
2
1.5
1
0.5
0
0
20
40
60
80
Ki (mM malate)
Time (min)

3.4.4 Significant changes in the kinetic properties of guard cell phosphoenolpyruvate carboxylase in response to environmental stimuli were observed using altered assay conditions

Nimmo *et al.* (1987) studying maize phosphoenolpyruvate carboxylase, used different assay conditions to detect a change in malate sensitivity in response to phosphorylation compared to those used to measure maximal activity. These assay conditions (assay buffer B, Methods section 2.5.1) were used to assay phosphoenolpyruvate carboxylase in extracts from guard cell protoplasts which had been incubated either in the light or dark (Table 3.7). The difference in K_i for malate between the two samples is not significant.

K_m (PEP) and V_{max} values were obtained, using assay buffer B conditions as described in Methods section 2.5.1, for phosphoenolpyruvate carboxylase in extracts made from guard cell protoplasts which had been incubated either in the dark, the light or in the dark + fusicoccin (Table 3.7). Using these conditions, significant changes in kinetic properties were observed. When guard cell protoplasts were stimulated either by light or fusicoccin, the K_m (PEP) was reduced by about 50%. In the case of stimulation by light, the probability of identity between the K_m (PEP) of phosphoenolpyruvate carboxylase from stimulated and unstimulated protoplasts was <7%. In the case of fusicoccin, this probability was <18%. Whilst a reduction in K_m (PEP) in response to a stimulus can be regarded as an activation of the enzyme, Table 3.7 also shows that in all cases V_{max} was reduced upon stimulation. This would be regarded as deactivation of the enzyme. As mentioned in Results section 3.4.2, it has been found that phosphoenolpyruvate carboxylase from various species can undergo a transition between hyperbolic and sigmoidal kinetics in various circumstances. It appears also that guard cell phosphoenolpyruvate carboxylase can undergo this same transition - manifested as an increase in both K_m (PEP) and V_{max}- as the concentration of the inhibitor malate increases in assays at pH 7.8. It is possible that phosphorylation brings about a similar change in the enzyme.

Table 3.7 Kinetic responses, measured using assay buffer B, of phosphoenolpyruvate carboxylase from guard cell protoplasts incubated in darkness, illumination or with fusicoccin

Guard cell protoplasts were prepared and incubated as described in the legend to Table 3.5. The number of times each incubation was carried out is shown in Table 3.6 After incubation, the protoplasts were extracted as described in Methods section 2.4.2, and either K_i (malate) or K_m (PEP) and Vmax were determined as described in Methods section 2.5.1. The means and standard deviations of the K_m (PEP) and K_i (malate) data were determined, and a Student's t test used to determine the probability of identity of these means from stimulated and unstimulated cells. In each experiment where V_{max} was determined, the ratio of V_{max} from the stimulated cells to V_{max} from the unstimulated cells was determined. The mean of these ratios was determined for each stimulus type, and these means are recorded in the Table.

	Dark		Light
K_i (μM malate)	23.3		19.7
n	3		3
Standard deviation	3.1		6.0
Probability of identity		> 40%	
K_m (PEP)	10.7		4.7
n	5		3
Standard deviation	4.3		2.0
Probability of identity		<7%	

	– Fusicoccin		+ Fusicoccin
K_m (PEP)mM	10.7		6.7
n	5		3
Standard deviation	4.3		0.6
Probability of identity		<18%	

Ratio of V_{max} (stimulated guard cell protoplasts) to V_{max} (unstimulated guard cell protoplasts):

Stimulation by light ratio = 0.54

Stimulation by fusicoccin ratio = 0.79

3.5 Conclusions

Guard cell phosphoenolpyruvate carboxylase can be phosphorylated both in intact protoplasts and by protein kinase A catalytic subunits in extracts. The phosphorylation state of the enzyme in intact protoplasts is increased by incubation of the protoplasts in the light or with fusicoccin, both of which are opening stimuli. Incubation in the dark or with abscisic acid, both of which are closing stimuli, does not stimulate phosphorylation. Additionally, the phosphorylation state of the enzyme in protoplasts which have been incubated in the light can be reduced by placing the protoplasts in the dark. Cycloheximide, an inhibitor of protein synthesis, prevents phosphorylation of the enzyme in intact protoplasts. Ca^{2+} does not appear to stimulate phosphorylation of the enzyme in extracts of protoplasts. Okadaic acid, an inhibitor of protein phosphatases, does not stimulate phosphorylation in intact protoplasts in the dark, suggesting that there is no kinase activity present until an opening stimulus is perceived.

After incubation of protoplasts in the light or with fusicoccin, the apparent K_m (PEP) of the enzyme decreases from approximately 11mM to approximately 5mM when assayed at pH 7.0 in the presence of glucose 6-phosphate. The apparent V_{max} also appears to decrease.

It is concluded that there is strong evidence to suggest that guard cell phosphoenolpyruvate carboxylase is controlled by reversible phosphorylation in a manner analagous to the control of this enzyme in CAM and C4 plants.

Chapter 4

General discussion

4.1 The control of guard cell phosphoenolpyruvate carboxylase by reversible phosphorylation

The main aim of this project was to investigate whether guard cell phosphoenolpyruvate carboxylase is controlled by reversible phosphorylation. The results indicate that phosphoenolpyruvate carboxylase in guard cell protoplasts is more phosphorylated in protoplasts incubated in conditions which promote stomatal opening than in those incubated in conditions which promote closure (Results section 3.3). Some kinetic properties of phosphoenolpyruvate carboxylase were also found to be different when guard cell protoplasts were incubated in different conditions (Results section 3.4.4), and so it would appear that the phosphorylation of phosphoenolpyruvate carboxylase and a change in its kinetic properties correlate with stomatal opening.

Further evidence for the correlation between stomatal opening and phosphoenolpyruvate carboxylase phosphorylation comes from a comparison of the time courses of these two processes. When stomata were stimulated to open by illumination at 400μE/m^2/s, a mean aperture of approximately 3.5 epu is achieved in 120min (Figure 3.13). Phosphoenolpyruvate carboxylase in guard cell protoplasts illuminated at 200μE/m^2/s became phosphorylated in one experiment (Figure 3.9) to a maximum of 7-fold higher than the non-stimulated level after 140min, although in other experiments the phosphorylation state of phosphoenolpyruvate carboxylase was lower and appeared to be still increasing after 180min (Figure 3.10). Fusicoccin, on the other hand, stimulates stomata to open more rapidly, an aperture of 3.5 epu being obtained after 60min (Figure 3.14). No time course of phosphorylation was carried out using fusicoccin as a stimulus; however, after 60min an approximately 6.5-fold increase in phosphorylation state was observed (Figure 3.16), although in other experiments the increase obtained was not as large (Figure 3.12). Whilst conditions used for opening stomata in the light (Methods section 2.7.1) are not identical to those used for stimulating guard cell protoplasts (Methods section 2.7.2), the time courses do show that when stomatal opening is rapid (as stimulated by fusicoccin), the rate of phosphorylation of phosphoenolpyruvate

carboxylase is high, and when the opening of stomata is slower (as stimulated by light), so too is the rate of phosphorylation of phosphoenolpyruvate carboxylase. This suggests that stomatal opening and phosphorylation are coordinated, and the implications of this are discussed in section 4.5. When illuminated guard cell protoplasts were placed in the dark, the phosphorylation state of the enzyme decreased, albeit not immediately (Figure 3.11), indicating that the effect of illumination on the phosphorylation state of guard cell phosphoenolpyruvate carboxylase is reversible.

The kinetic differences between phosphoenolpyruvate carboxylase from darkened and illuminated guard cell protoplasts do not immediately suggest that the form of the enzyme from illuminated protoplasts (the putative phosphorylated form) is necessarily more active than the darkened (putative non-phosphorylated) form. The illuminated form has both a lower apparent K_m and lower apparent V_{max} than the darkened form. A lower K_m suggests that the activity of the enzyme *in vivo* might be increased, whilst a lower V_{max} suggests the opposite. It is likely that the conditions under which the kinetic differences were observed are unlike cytosolic conditions in guard cells. A further investigation of assay conditions may allow more clearcut effects of phosphorylation on the enzyme kinetics to be observed. These in turn might then be more readily related to physiological effects. It should also be noted that the effect of malate on guard cell phosphoenolpyruvate carboxylase is to change both the apparent K_m (PEP) and V_{max} in the same direction (Table 3.5). It is possible to speculate, therefore, that the conformational change induced by phosphorylation of the enzyme is the same as that induced by malate. Whatever the fine detail, the important conclusion to come out of this study is that the enzyme in illuminated guard cells is kinetically different from that in darkened guard cells, indicating that it has become altered, possibly by phosphorylation.

Whilst the correlation of stomatal aperture, phosphorylation state of phosphoenolpyruvate carboxylase and the kinetic data strongly suggests a role for phosphorylation in the control of the enzyme in guard cells, an unequivocal proof would require further experiments. First, purified or partially purified guard cell phosphoenolpyruvate carboxylase should be shown to be phosphorylated *in vitro* by a kinase activity extracted from guard cells at the same site that becomes phosphorylated *in vivo*. Secondly, if incubation of the enzyme with a protein

phosphatase both reduced its phosphorylation state and reversed the kinetic changes, this would provide further strong evidence that the enzyme can be controlled by reversible phosphorylation.

4.2 The effects of cycloheximide on stomatal aperture and phosphorylation of phosphoenolpyruvate carboxylase

The protein synthesis inhibitor cycloheximide retards stomatal opening stimulated by either light or by fusicoccin (Figures 3.13 and 3.14) and prevents the phosphorylation of guard cell phosphoenolpyruvate carboxylase (Figure 3.12). The latter of these findings conforms with the observations that protein synthesis inhibitors block phosphorylation of phosphoenolpyruvate carboxylase in the CAM plant *Bryophyllum fedtschenkoi* (Carter *et al.*, 1991) and in the C4 plants maize (Jiao *et al.*, 1991) and *Sorghum* (Bakrim *et al.*, 1992). In these systems it is now generally accepted that protein synthesis is necessary for the appearance of the kinase activity which phosphorylates phosphoenolpyruvate carboxylase, but it is not known if the kinase itself or some activating protein is synthesised (Nimmo, 1993).

The observation that cycloheximide appears to retard stomatal opening in detached epidermis is in disagreement with the findings of Thimann and Tan (1988) and Lee and Walton (1990) who observed an inhibition of stomatal closure by cycloheximide. It is possible that protein synthesis is required for both opening and closure of stomata, although another possibility is that cycloheximide has effects on guard cell protoplasts other than inhibiting protein synthesis. Alterations to the plasma membranes, changing their ion transport characteristics, have been reported for many organisms during the use of cycloheximide (Evans and Smith, 1971; Parthier, 1974; Reilly, 1970). The viability data obtained for guard cell protoplasts incubated in cycloheximide (Figure 3.15) suggest that the great majority of guard cell protoplasts can survive for at least two hours in cycloheximide, so protoplast death during stomatal opening is unlikely to be the cause of the reduced opening rate. This does not, however, rule out possible ion transport effects. In order to show that the effect of cycloheximide is specifically due to inhibition of protein synthesis, it would be necessary to use a variety of different protein synthesis inhibitors with unrelated structures and different modes of action. This has been done in the *Bryophyllum fedtschenkoi* system, and all inhibitors of nuclear encoded protein synthesis used block the

phosphorylation of phosphoenolpyruvate carboxylase, whilst chloramphenicol, an inhibitor of organelle protein synthesis does not. The similarities between the phosphoenolpyruvate carboxylase phosphorylation systems of guard cells, C4 plants and CAM plants (see section 4.3, below) are so great that it is likely that, as with the other systems, the effects of cycloheximide are due to the inhibition of protein synthesis.

4.3 Similarities and differences of the phosphoenolpyruvate carboxylase phosphorylation systems of guard cells and the C4 and CAM systems

The fact that cycloheximide inhibits the phosphorylation of phosphoenolpyruvate carboxylase is common to guard cells, maize and *Sorghum* (both C4 plants) and *Bryophyllum fedtschenkoi* (a CAM plant). Also the enzyme from guard cells, maize and *Sorghum* tissues can be phosphorylated in extracts by protein kinase A catalytic subunits (this thesis, Figure 3.6; Terada *et al.*, 1990; Arrio-Dupont *et al.*, 1992). In maize and *Sorghum*, this *in vitro* phosphorylation has been shown to occur on the same residue which is phosphorylated *in vivo*. Protein kinases generally phosphorylate specific target motifs, and in the case of protein kinase A, this motif consists of two basic residues followed by a serine or threonine residue with one or two intervening residues (Martin, 1987). The phosphorylation site on phosphoenolpyruvate carboxylase from maize and *Sorghum* is not of this type, which explains why the enzyme is quite a poor substrate for protein kinase A. In addition, the N-terminal amino acid sequence of the phosphoenolpyruvate carboxylase from the CAM plant *Mesembryanthemum crystallinum* is very similar to that of both *Sorghum* and maize, in which the phosphorylated serine residue has been identified. All of this evidence stongly suggests that the phosphorylation site is likely to be very similar for the enzymes from guard cells, C4 and CAM plants.

In terms of the nature of the physiological stimuli, the phosphorylation of guard cell phosphoenolpyruvate carboxylase appears to be more similar to the C4 system than the CAM system in that in both *Sorghum* and maize, the phosphorylation is directly stimulated by light (Bakrim *et al.*, 1992; Nimmo *et al.*, 1987a; Jiao and Chollet, 1988), whilst in the CAM plant *Bryophyllum fedtschenkoi*, a circadian rhythm is responsible (Nimmo *et al.*, 1987b). The guard cell enzyme is also

phosphorylated in response to fusicoccin, and it is possible that other stimuli which cause stomata to open will have the same effect.

Phosphoenolpyruvate carboxylase from maize, *Sorghum* and *Bryophyllum fedtschenkoi* has a higher K_i (malate) in the phosphorylated form than in the nonphosphorylated form. Since the change in malate sensitivity in response to phosphorylation in maize is very much smaller than that in *Bryophyllum*, it is perhaps not unreasonable to suggest that any change in the guard cell enzyme is still smaller. From a physiological standpoint, it is likely that the change in malate sensitivity required to allow phosphoenolpyruvate carboxylase to function in a particular cell type depends on the extent to which the cytoplasmic malate concentration increases in that cell type. It is possible that there is actually little increase in guard cell cytoplasmic malate concentration during stomatal opening. It might be physiologically more relevant to reduce the enzyme's K_m for PEP during opening. Thus far, no change in the malate sensitivity of guard cell phosphoenolpyruvate carboxylase in response to factors which change the phosphorylation state of the enzyme has been detected, but it is possible that under certain assay conditions a K_i (malate) change will be detected.

In summary, the available experimental evidence suggests that the phosphorylation site in guard cell phosphoenolpyruvate carboxylase is very similar to that of the enzyme from other systems which have been studied. The phosphorylation also responds similarly to cycloheximide. The kinetic changes caused by phosphorylation seem to be different, but further work is required in this area, particularly attempts to obtain stoichiometric phosphorylation of the guard cell enzyme. One potential difference between the guard cell system and the others may be its ability to respond to a large number of stimuli, although further work is required here also.

4.4 The variability of data from experiments involving stomata and guard cells

During the course of this project, it was found that the responses of both intact stomata and guard cell protoplasts to various stimuli could be very variable. The degree of increase in phosphorylation in response to light was particularly variable, despite identical conditions being used on all occasions. Other workers have also found large variation in data from experiments using guard cells. Gilroy *et al.*

(1991), McAinsh *et al.* (1992) and Irving *et al.* (1992) all found that in experiments on responses to abscissic acid, increases in cytosolic Ca^{2+} occurred in less than 100% of the cases in which stomata closed. Mansfield *et al.* (1990) also noted that there is heterogeneity in the responses of stomata in a single leaf. Since stomata respond to such a variety of stimuli, it follows that even small variations in the cultivation of different batches of plants may cause variation in stomatal responses. In the present work, variations were greatest between different experiments, rather than within single experiments. Plant age varied from 5 to 8 weeks, although all of the plants in any one batch were of the same age. In almost all cases only plants from a single batch were used for a single experiment, and so it is possible that batch-to-batch variation in plant age and treatment could be responsible for the variability observed in the data. It is clear that, when working with stomata, it is necessary to keep all experimental and growth conditions uniform, and to use as large a sample number as possible for all experiments.

4.5 Possible signalling mechanisms involved in the phosphorylation/dephosphorylation of guard cell phosphoenolpyruvate carboxylase

In section 1.5 of the Introduction, mechanisms of signal transduction were discussed which suggested that the Ca^{2+}/phosphoinositide system could be an important signalling mechanism in guard cells. The experimental data obtained during this project allow some further suggestions to be made concerning signal transduction mechanisms operating in guard cells. Ca^{2+} does not stimulate phosphorylation of phosphoenolpyruvate carboxylase in guard cell extracts (Table 3.3), although the effect of Ca^{2+} in the incubation medium (analagous to the experiments carried out by Pierre et. al. (1992)) were not carried out, but protein synthesis appears to be vital for phosphorylation to occur in intact protoplasts. Since both light and fusicoccin can stimulate the phosphorylation of the enzyme, and since other stimuli (such as high concentrations of K^+ in the medium) can cause an increase in guard cell malate levels, it can be considered that phosphoenolpyruvate carboxylase lies at the downstream end of a converging signalling network, with possible inputs coming from various stimuli. Immediately upstream from phosphoenolpyruvate carboxylase lies the kinase responsible for phosphorylation. Further upstream from this lies the factor(s) responsible for protein synthesis leading to the appearance of the kinase activity. It is feasible that this factor - lying at the focus of this converging signalling network - is K^+ itself.

When stomata are stimulated to open by light, the rate of phosphorylation of phosphoenolpyruvate carboxylase is low compared to that stimulated by fusicoccin (Figures 3.8 and 3.9). The mechanisms by which these two stimuli work are extremely different. Light acts by stimulating either the photosynthetic apparatus or a specific receptor (Zeiger, 1983). Subsequently, H^+ pumping and K^+ transport at the plasma membrane, as well as other metabolic processes associated with stomatal opening, are stimulated. Some signal transduction mechanism must exist to produce the effects observed after the light stimulus is received. Fusicoccin, on the other hand, acts directly by stimulating the plasma membrane H^+ / ATPase (Marre,1979). However, despite the lack of a primary stimulus (for example light or a hormone), many of the processes associated with opening in response to a normal opening stimulus still occur; examples include malate accumulation (Snaith and Mansfield, 1984) and phosphorylation of phosphoenolpyruvate carboxylase (this thesis). There are two possible explainations for this:

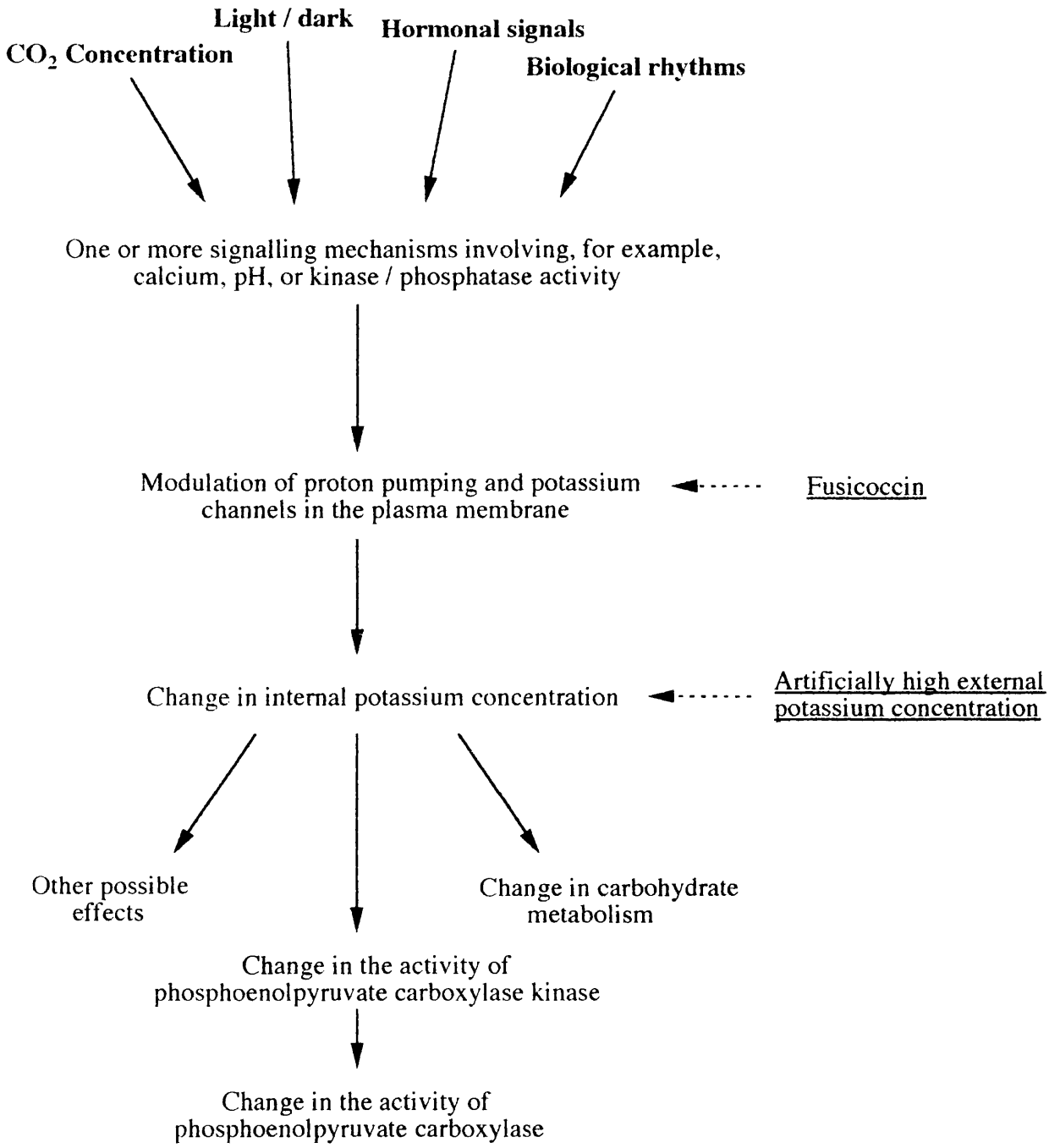
1. If all of the responses observed after application of an abnormal stimulus such as fusicoccin require modulation by the signalling pathway normally activated by the reception of a primary stimulus, then there must be some feedback mechanism which can activate that pathway. This feedback system may itself be triggered by a rise in cytosolic K^+, by a change in cytosolic pH or by detection of guard cell swelling.
2. The signalling pathway activated by primary stimuli may only modulate H^+ pumping and K^+ transport at the plasma membrane. All other responses could be modulated by cytosolic K^+ concentration, and this would allow all of the responses normally observed after stimulation by, for example, light to occur after stimulation by fusicoccin. A model for such a signalling network is given in Figure 4.1. If this signalling mechanism does indeed operate in guard cells, there is the implication that malate synthesis will not immediately balance K^+ uptake, as the cytoplasmic K^+ concentration would have to increase before malate production would be switched on. There have been suggestions in the literature that the constitution of the osmotic pool in guard cells changes with time, and that other osmotica, such as sugars, may make up a larger percentage of this during the earliest stages of opening, with malate levels rising later (Poffenroth *et al.*, 1992; MacRobbie, 1987).

The use of K^+ channel blockers and the incubation of guard cell protoplasts without K^+ present could show the importance of potassium

to responses far downstream from the primary stimulus. The responses of the plasma membrane H^+ / ATPase to cytosolic Ca^{2+} and pH change should also reveal much about how guard cells detect the various opening stimuli to which they are exposed. The way in which guard cells balance opening and closing stimuli that are received simultaneously (such as strong sunlight on a dry day) is likely to be complex, but it seems possible that total control over aperture could be achieved by controlling H^+ and K^+ transport alone, with all other responses dependent on cytosolic K^+.

This study into regulation of guard cell phosphoenolpyruvate carboxylase has added to previous findings that the control of a plant enzyme by phosphorylation is a protein synthesis dependent process. In addition, the results suggest a role for K^+ in signalling in guard cells. It is to be hoped that this work will be continued in order to help elucidate the complex system of signalling which allows plants to respond to their environment, the understanding of which will be of importance in the design of genetically engineered crops with "programmed" life cycles.

Figure 4.1 Model of possible signalling pathways in guard cells



KEY

Stimuli are underlined

Solid lines indicate processes occurring as a result of the operation of normal signal transduction mechanisms

Dashed lines indicate processes occurring because of abnormal stimuli

Chapter 5

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