PHOSPHORYLATION OF TONOPLAST PROTEINS

IN THE

CAM PLANT *KALANCHOË DAIGREMONTIANA*

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JULY 1991

ACKNOWLEDGEMENTS

I would like to thank my supervisor , Dr. Andrew Smith for his expertise and encouragement throughout my time in Edinburgh. In addition, thanks to the staff and research students in the department who provided advice and practical help.

CONTENTS

ABBREVIATIONS

AMPS	- ammonium persulphate
ATBS	- 20 mM Tris/SO_4^{2-} (pH 7.5) and 0.5 M NaCl
ATP	- adenosine 5' -triphosphate
BSA	- bovine serum albumin
CaM	- calmodulin
CAM	- crassulacean acid metabolism
dH_2O	- distilled H_20
DTT	- DL-dithiothreitol
EDTA	- ethylenediaminetetraacetic acid
EGTA	- ethylene glycol-bis(β-aminoethylether) *N, N, N', N'*-tetraacetic acid
Hepes	- *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulphonic acid
PEPCase	- phospho*enol*pyruvate carboxylase
PMSF	- phenylmethanesulphonyl fluoride
PVP-40	- polyvinylpyrrolidone - 40
SDS-PAGE	- sodium dodecyl-sulphate polyacrylamide gel electrophoresis
TEMED	- *N, N, N', N'*-tetramethylethylenediamine
Tricine	- *N*-tris(hydroxymethyl)methylglycine
Tris	- 2-amino-2-(hydroxymethyl)-1,3-propanediol

ABSTRACT

A method for isolation of pure tonoplast vesicles was developed to obtain suitable yields of tonoplast from leaf homogenates of *Kalanchoë daigremontiana*. An improved method for isolation of a tonoplast fraction from mesophyll tissue was developed based on differential and density-gradient centrifugation in glycerol-containing media. This improved the average specific activity of the nitrate-sensitive ATPase (a specific tonoplast marker) from 0.19 to 0.43 μmol min^{-1} mg^{-1} protein. The effectiveness of this isolation protocol was studied by analysis of the polypeptide composition of this fraction by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. The results preliminary show that the new method yielded a tonoplast fraction substantially enriched in the $\underline{M}_r$ 68 000 and 56 000 subunits of the tonoplast H^+-ATPase and less contaminated with the cytosolic enzyme phospho*enol*pyruvate carboxylase (PEPCase).

The presence of protein kinase activity associated with the tonoplast was identified by following the incorporation of ^{32}P from [γ-^{32}P]ATP into TCA-precipitable proteins and by performing autoradiography. The major phosphorylated polypeptides labelled had $\underline{M}_r$ values of approximately 112 000, 105 000, 91 000, 60 000 and 55/56 000. The protein kinase activity was stimulated in the presence of approximately 1.15 μM Ca^{2+}. This increased phosphorylation of polypeptide $\underline{M}_r$ 55 000 and two further polypeptides became phosphorylated of $\underline{M}_r$ 37 000 and 10/11 000.

The possible significance of these results has been discussed and comparisons made with other studies concerning protein kinase activity at the tonoplast. Further experiments have been suggested.

CHAPTER 1

INTRODUCTION

1.0 Introduction

Important advances in our understanding of the mechanism of transport processes at the vacuolar membrane, the tonoplast, have resulted from the use of membrane vesicles in the investigation of these processes (Sze, 1985). Various transport processes operate across the tonoplast, and proton pumps play a central role in energisation of this membrane (Rea and Sanders, 1987). None of these transport processes is fully understood and very little is known concerning the way in which ion transport across the tonoplast is controlled under cellular conditions. Phosphorylation as a control mechanism has been implicated for ion transport across various cell membranes, e.g. for the nicotinic acetylcholine receptor (Browning et al., 1990), as well as the voltage-dependent Na^+ channel and voltage-dependent Ca^{2+} channels (Catterall, 1989). The work described here was concerned with protein kinase activity associated with the tonoplast and the possible relevance of phosphorylation of proteins in that membrane.

1.1 The Vacuole and its Functions in the Cell

The vacuole constitutes a major component of the plant cell. This compartment is filled with cell sap and is surrounded by a membrane called the tonoplast. In the immature plant cell there are numerous small vacuoles but in the mature cell about 90% of the cell volume is taken up by one or a few large vacuoles. In extreme cases, as in plants that exhibit crassulacean acid metabolism (CAM), up to 97% of the cell volume may be occupied by the vacuole (Steudle et al., 1980 ; Lüttge et al., 1982).

The vacuole has various roles to play in the plant cell (Boller and Wiemken, 1986 ; Marin, 1987 ; Raven, 1977).

1. It is a very "inexpensive" storage organelle, serving for example in the storage of NaCl in halophytes or nocturnal storage of malate in CAM plants.

2. It is involved in osmoregulation and turgor generation and maintains tissue rigidity.

3. It is important in detoxification processes, for example, by removing secondary products such as polyphenols and alkaloids from the cytoplasm.

As a consequence of these roles, transport of solutes across the tonoplast into the vacuole is of fundamental importance in cell compartmentation and cytoplasmic homeostasis. Figure 1 shows examples of the various types of transport process believed to operate across the tonoplast.

Pumps

An ATPase and an inorganic pyrophosphatase (PP_iase) exist in the tonoplast, and both enzymes can catalyse electrogenic H^+ translocation. These enzymes generate the proton-motive force required to drive secondary active transport of solutes across the tonoplast (Poole et al., 1987 ; Rea and Sanders, 1987).

Channels

Channels are molecules that form size-selective pores in the membrane to allow ion flow (Hille, 1984 ; Jan and Jan, 1989). The electrophysiological technique for the recording of ion currents from biological membranes - the patch-clamp technique - has allowed rapid progress in the study of ion transport at the molecular level for plant cells (Hedrich and Schroeder, 1989). Studies using this method have demonstrated the presence of at least two types of ion channel (differing in their sensitivity to Ca^{2+}) in the tonoplast (Hedrich and Neher, 1987). A channel specific for malate has been recently shown to exist in the tonoplast of the CAM plant *Kalanchoë daigremontiana* (J.A.C. Smith and A.J. Pennington, personal communication).

Carriers

Carriers at the tonoplast appear to be H^+-linked transporters that can be divided into two categories (Poole et al., 1987 ; Rea and Sanders, 1987) :

1. antiport systems, in which protons are exchanged for sodium ions, calcium ions or sucrose; and

2. symport systems, where negatively charged substances such as chloride (and possibly other ions such as nitrate or malate) are transported along with protons into or out of the vacuole.

However, very little is known at the biochemical or molecular level about the membrane proteins that form channels in the tonoplast.

1.2 Crassulacean Acid Metabolism

Solute transport at the vacuolar membrane has been the subject of much research in plants that exhibit crassulacean acid metabolism (CAM). These plants show large day-night changes in the malate content of their photosynthetic tissue. During the night, stomata are open and offer a low resistance to CO_2 diffusion into the leaves. The CO_2 enters the cell and is fixed as HCO_3^- *via* phospho*enol*pyruvate carboxylase (PEPCase) in the dark to form oxaloacetate (OAA), which is then reduced to malate *via* malate dehydrogenase (MDH).

$$HCO_3^- + PEP^- \xrightarrow[\searrow P_i]{PEPCase} OAA^{2-} + NADH + H^+ \xrightarrow{MDH} Malate^{2-} + NAD^+$$

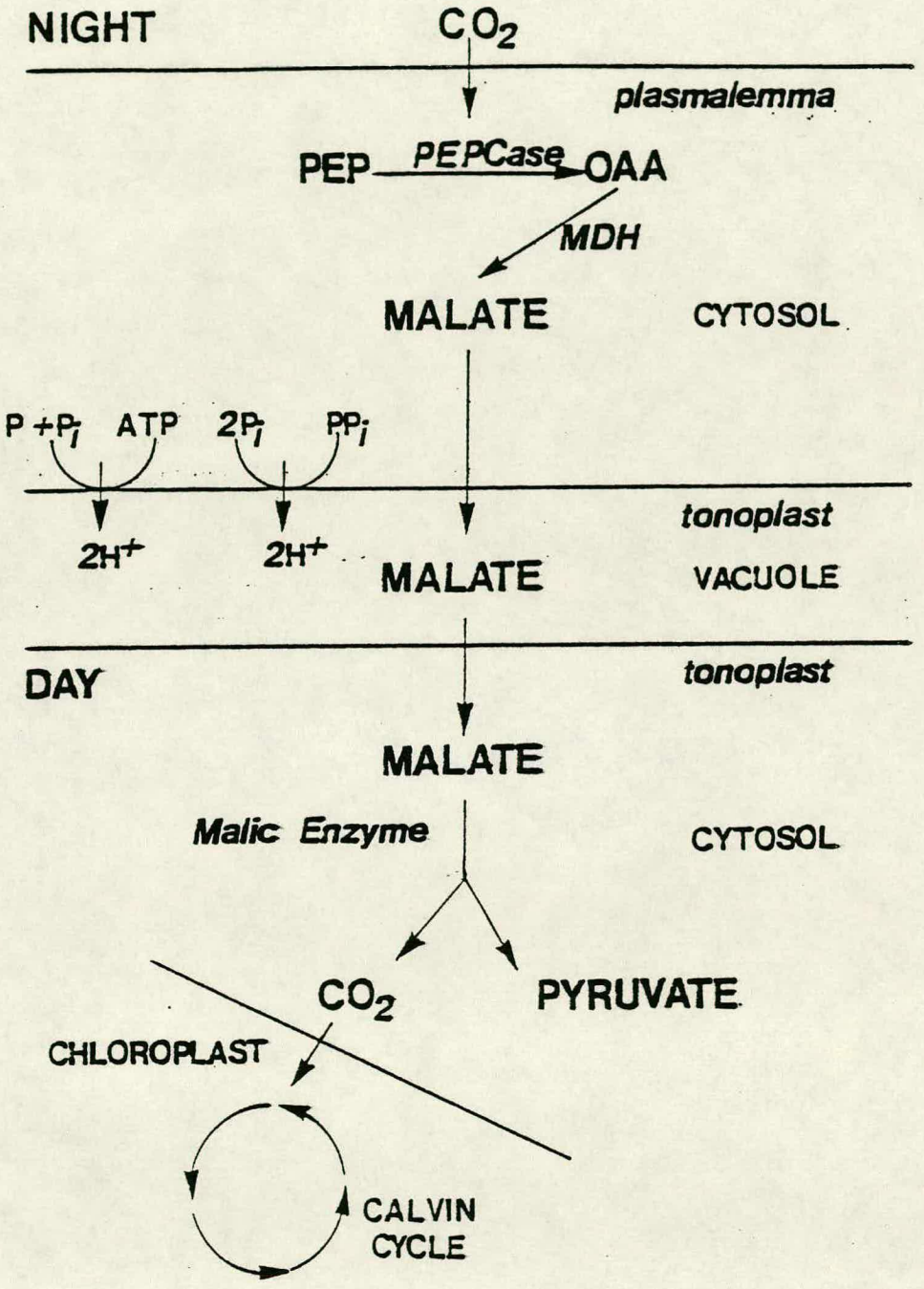
Accompanying the day-night changes in malic acid content of the vacuole are reciprocal fluctuations of storage carbohydrates. These provide the carbon substrate for nocturnal acid synthesis and are then resynthesised during the deacidification phase in the daytime. Therefore plants acquire carbon at low water cost by fixing carbon dioxide during the coolest part of the day-night cycle.

Malate is stored in the vacuole in CAM plants for release during the following light period when stomata are closed. During the light period, malate is released from the vacuole into the cytoplasm where it is decarboxylated either *via* NAD- (or NADP-) malic enzyme or *via* NAD malate dehydrogenase and PEP carboxykinase. The CO_2 released then enters the chloroplast where it is reduced in the Calvin cycle (see Osmond, 1978; Winter, 1985 ; Lüttge and Smith, 1988 for a general overview on CAM, and Figure 2). This form of photosynthesis is important for plants living in arid and semi-arid environments because water loss during the day is minimised. CAM occurs in an estimated 15,000 to 20,000 species of higher plants belonging to at least 25 families from both monocots and dicots. The experimental species used in the present work was a dicotyledonous species from the family Crassulaceae, *Kalanchoë daigremontiana*. This species has been used in several previous investigations concerning plant membrane transport (e.g. Jochem and Lüttge, 1987; Smith, 1987; White and Smith, 1989).

1.3 The Malate Transporter

The vacuole of CAM plants typically constitutes more than 95% of the cell volume (Lüttge et al., 1982) and therefore appears to be the main storage site for many solutes.

Figure 2.



Outline of crassulacean acid metabolism.

At night, malate anions are thought to be electro-phoretically transported across the tonoplast into the vacuole in response to an inside-positive membrane potential, $\Delta\Psi$, generated by the two proton pumps (the H^+-ATPase and H^+-PP_iase). Evidence for this comes from the fact that malate is able to dissipate an inside-positive $\Delta\Psi$ across the membrane in isolated tonoplast vesicles (Jochem and Lüttge, 1987; White and Smith, 1989). Fluorescence methods have been used as an indirect means of monitoring anion transport in isolated membrane vesicles (Pope and Leigh, 1988; White and Smith, 1989). The rates of vesicle acidification driven by the proton pumps can be used as a measure of the relative permeability of the tonoplast to different anions, since proton transport is constrained by the transport of charge-compensating anions. Using this system, it has been shown that the tonoplast in CAM plants is highly permeable to malate, presumably reflecting the presence of an anion transporter. This finding is of particular interest since the tonoplast of non-CAM species appears to be relatively impermeable to malate (Pope and Leigh, 1988; White and Smith, 1989), suggesting that CAM plants possess a specific malate transporter. It has also been inferred from such experiments that a *trans* carboxyl configuration is essential for transport of the dicarboxylate anion, since the order of effectiveness of malate analogues in supporting vesicle acidification is fumarate >> malate $\simeq$ succinate > oxaloacetate $\simeq$ tartrate (White and Smith, 1989).

1.4(i) The H^+-ATPase

Little is known at the biochemical level of the molecular components of the tonoplast. Bremberger et al. (1988) have separated and partially purified the tonoplast ATPase from *Kalanchoë daigremontiana* and shown that it is

composed of at least six subunits with relative molecular masses (M_r) of 72 000, 56 000, 48 000, 42 000, 28 000 and 16 000. Recent studies of M. Warren, D.K. Apps and J.A.C. Smith (unpublished results) have shown similar M_r values of 72 000, 56 000, 42/41 000, 33/34 000, 31 000 and 16 000 for the same enzyme purified by a different technique. Parry et al., however, have purified the vacuolar-type ATPase from red beet (Beta vulgaris L.), the subunits of which have slightly different M_r values of 100 000, 67 000, 55 000, 52 000, 44 000, 32 000 and 16 000, with minor components at 42 000 and 29 000. In chromaffin granules in animals there also exists an ATPase with subunit relative M_r of 115 000, 72 000, 57 000, 41 000, 39 000, 34 000, 20 000 and 16 000 (Percy et al., 1985; Percy and Apps, 1986). It has been shown that the catalytic subunits of the enzymes from plant and fungal vacuolar membranes and chromaffin granules display immunological crossreactivity (Manolson et al., 1987). All of these enzymes apparently belong to a large class of V-type ATPases, and are characteristically inhibited by nitrate (NO_3^-).

1.4(ii) The PP_iase

Another tonoplast membrane protein recently identified is the H^+-translocating inorganic pyrophosphatase H^+-PP_iase from mung bean (Maeshima, 1990) and red beet (Sarafin and Poole, 1989; Britten et al., 1989), which consists of a single polypeptide of M_r 73 000 (Maeshima, 1990). The $MgPP_i$-binding subunit (M_r 64 000) of the PP_iase of *Beta* tonoplast has been identified by substrate-protectable affinity-labelling and protein purification (Britten et al., 1989).

As yet, however, no secondary solute transporters or ion channel proteins have been identified in the tonoplast at the molecular level.

1.5 Phosphorylation : Introduction

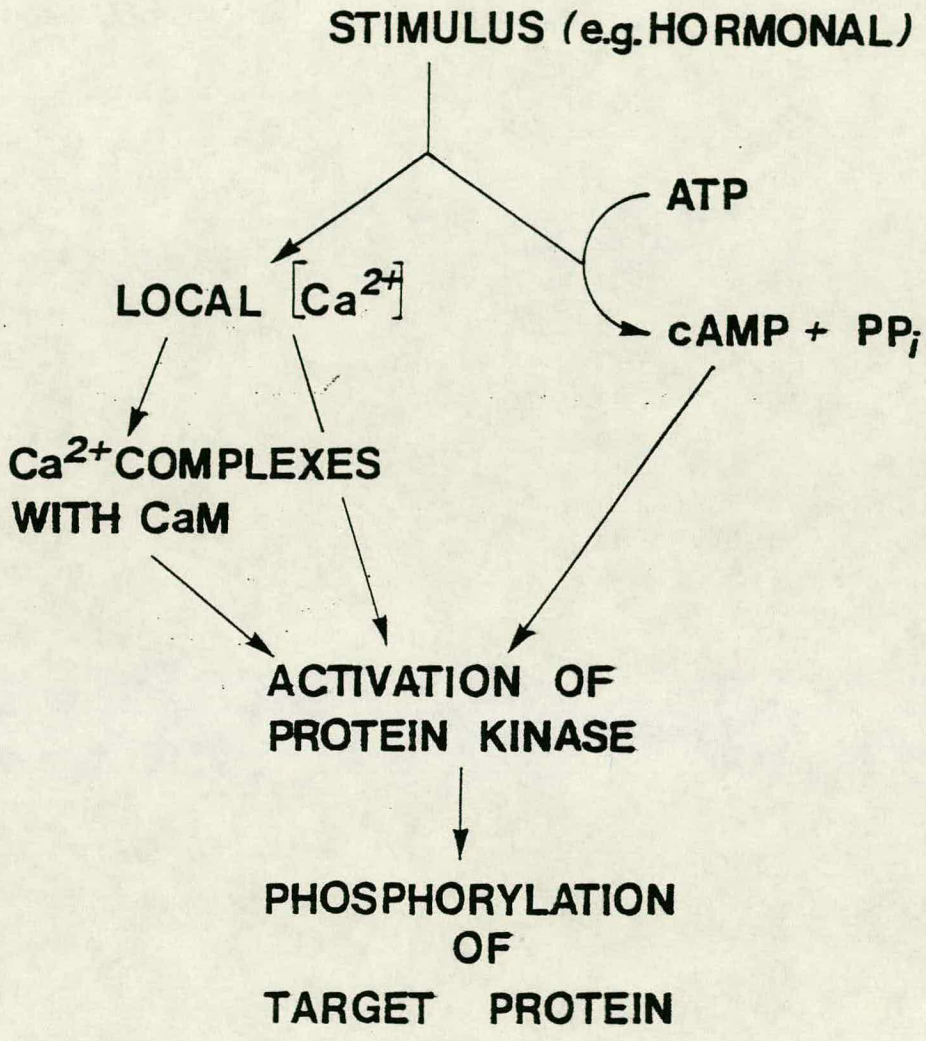
Phosphorylation is one of the most-studied post-translational regulatory modifications affecting proteins and is now recognised to be the major general mechanism by which intracellular events in mammalian tissues are controlled by external physiological stimuli (Cohen, 1982). The presence of phosphate in proteins has been known for over one hundred years. However, its regulatory importance was only realised from 1955 onwards when Krebs, Fischer and Larner discovered that the neural and hormonal control of glycogen metabolism was mediated by changes in the phosphorylation states of glycogen phosphorylase, phosphorylase kinase and glycogen synthase (Krebs and Fischer, 1956; Freidman and Larner, 1963).

The enzyme performing the phosphorylation reaction is called the protein kinase (PK).

$$\text{protein} + n\text{ATP} \xrightarrow{\text{PK}} \text{protein-P}_n + n\text{ADP}$$

Phosphate is covalently transferred from nucleoside triphosphates (e.g. ATP or GTP) to certain amino acids in the substrate proteins (Krebs and Beavo, 1987). The phosphorylation of proteins is considered a means for the conversion of internal and/or external stimuli into biochemical responses (Cohen, 1982; Ranjeva et al., 1984). This stimulus (normally a hormone) acts *via* a second messenger which activates a protein kinase (Fig. 3.). Two examples of second messengers are cyclic 3', 5' - adenosine monophosphate (cAMP) and Ca^{2+}/ calmodulin (CaM).

Figure 3



Outline of protein kinase activation.

1.5(i) Phosphorylation : Ion Channels / Transporters

Phosphorylation as a control mechanism has been implicated for ion transport across cell membranes, for example, for the nicotinic acetylcholine receptor, voltage-dependent Na^+ channel and voltage-dependent Ca^{2+} channel (Huganir, 1987; Catterall, 1989). There are multiple classes of Ca^{2+} channels in most excitable cells (Byerly and Hagiwara, 1988). In each class the transmembrane α_1 and β subunits are the substrates for phosphorylation by cAMP-dependent protein kinase (Leung et al., 1987; Takahashi et al., 1987) and are the sites of regulation of the Ca^{2+} channel ion conductance activity by this enzyme. These channels have not been shown directly to be phosphorylated in intact cells and the sites of phosphorylation within their amino acid sequence are not known. However, phosphorylation by cAMP-dependent protein kinase increases the initial rate of $^{45}Ca^{2+}$ influx 8-fold in reconstituted phospholipid vesicles containing purified Ca^{2+} channels from skeletal muscle and increases the number of purified Ca^{2+} channels that are active (Catterall, 1989; Nunoki et al., 1989). In contrast, studies of Na^+ channels have defined their sites of phosphorylation in situ but the functional significance is less clear.

Glucose entry into most cells occurs by facilitated diffusion. The regulation of glucose transport in skeletal muscle and adipose tissue is critical in maintaining glucose homeostasis. A glucose transporter in rat adipocytes is known to become phosphorylated, which may be involved in regulating the intrinsic activity of the transporter although this is still conjectural (Lawrence et al., 1990).

The role of protein phosphorylation has also been studied in the regulation of epithelial Cl^- channels (Landry et al., 1987). Studies have shown that these channels are critically involved in absorption and secretion and are regulated by a number of second messengers. This regulation has recently been identified as a central event in two important diseases; cystic fibrosis (Riordan et al., 1989), where the decrease in Cl^- conductance in epithelial cells is probably due to a failure of an outwardly rectifying anion channel to respond to phosphorylation by cAMP-dependent PKA or PKC. This Cl^- channel can also be activated in normal and cystic-fibrosis-derived airway epithelial cells by multifunctional Ca^{2+}/calmodulin-dependent protein kinase (Wagner et al., 1991). With the other disease, cholera, there is an increase in the Cl^- conductance (Landry et al., 1987). It has also been shown that cAMP - dependent PK modulates the glycine receptor Cl^- channels in spinal trigeminal neurons (Song and Huang, 1990). Transport of Cl^- and the involvment of phosphorylation in modulation of the process may be significant in relation to the Cl^- channel /transporter in the tonoplast.

1.5(ii) Phosphorylation : Plant Membranes

Although the concept of second messengers and protein kinases has been well established in animal biochemistry, less is known about their functioning in plants. It is, however, becoming more apparent that Ca^{2+} and Ca^{2+}/calmodulin regulated protein kinases are present in plant tissues and may have a regulatory role in metabolism (Ranjeva and Boudet, 1987; Blowers and Trewavas, 1989a). Various calcium-activated protein kinases have been identified associated with plant membranes, for example, in zucchini (*Cucurbita pepo* L.) hypocotyls (Salimath and Marme, 1983; Martiny-Baron and Scherer, 1989), rye grass (Polya et al., 1987), corn (Veluthambi and

Poovaiah, 1984), *Acer pseudoplatanus* cells (Basso et al., 1989), maize coleoptiles (Battey, 1990) and the peribacteroid membrane of soybean root nodules (Weaver et al., 1991). *In vitro* phosphorylation of plasma membrane proteins has also been shown in response to a proteinase inhibitor factor in tomato leaf (Farmer et al., 1989),and a Ca^{2+}-simulated protein kinase activity in pea has been well documented (Hetherington and Trewavas, 1982, 1984; Blowers et al., 1985). This latter kinase has been associated with a specific protein of relative molecular mass of 18 000 (Blowers and Trewavas, 1989b) although it is suspected that this may only be a proteolytic fragment of a larger polypeptide. Other examples of partially purified protein kinases associated with plant membranes are from wheat embryo (Polya and Davies, 1982; Polya and Micucci, 1984) and soybean (Putnam-Evans et al., 1990).

1.5(iii) Phosphorylation : The Tonoplast

Although there have been several protein kinases identified associated with plant membranes, there has been relatively little consideration given to phosphorylation of tonoplast proteins. To date, the presence of Ca^{2+}-regulated protein kinase activity has been shown to exist in tonoplast fractions from *Acer pseudoplatanus* cells (Teulieres et al., 1985) and from corn (*Zea mays* L.) roots (Ladror and Zielinski, 1989). These studies gave conflicting results as to the polypeptides that appear to be phosphorylated, and Ladror and Zielinski (1989) have even suggested that the kinase activity in their tonoplast fraction may be a contaminant from the plasmalemma. Another group has also suggested that the slight phosphorylation found in their tonoplast fraction from barley roots was a plasmalemma contaminant (Garbarino and DuPont, 1989). Thus, the significance of phosphorylation of

tonoplast proteins for control of solute transport into and out of the vacuole remains to be clarified.

1.5(iv) Phosphorylation : PEPCase

One enzyme known to be subject to control by phosphorylation and dephosphorylation in CAM plants is PEPCase. This homotetramer of M_r 400 000 catalyses the β-carboxylation of PEP, producing OAA and P_i. PEPCase is unusual in that it is the only non-biotin-dependent carboxylase that uses HCO_3^- as substrate (González and Andreo, 1989). In CAM plants, PEPcase exists in two physiological forms:

1. active night form

2. less active day form

The day form is very sensitive to inhibition by malate, whereas the night form is much less sensitive, allowing continued malate synthesis at night as malate concentrations increase. It is thought that the interconversion of these two forms of PEPCase is due to reversible phosphorylation (Kluge et al., 1988). Thus phosphorylation leads to a reduction in the sensitivity of the enzyme to inhibition by the allosteric effector L-malate at night, and *vice versa* during the daytime (Nimmo et al., 1984, 1987; Brulfert et al., 1986; Kluge et al., 1988).

The protein kinase that phosphorylates PEPCase, which is not dependent on Ca^{2+} for activity, has been partially purified. Because phosphorylation is an important regulatory process at this point in the CAM cycle it is conceivable that a protein kinase might also act on the tonoplast malate transporter to

provide a coordinated control of rates of malate synthesis and accumulation in the vacuole. Whether this could be the property of a single kinase or not has yet to be discovered.

1.6 Experimental Outline

Section 1

A method for isolation of pure tonoplast vesicles was developed to obtain suitable yields of tonoplast from leaf homogenates of *Kalanchoë daigremontiana*.

Section 2

Phosphorylation of tonoplast proteins and some of the characteristics of the protein kinase activity associated with the membrane were investigated.

CHAPTER 2

MATERIAL AND METHODS

Chemicals

[γ-^{32}P]-Adenosine 5'-triphosphate ([γ-^{32}P]-ATP) was obtained from Amersham International plc (Amersham, Bucks., UK) as the stabilised aqueous triethylammonium salt (specific activity was >185 Bq $mmol^{-1}$ equivalent to 370 MBq ml^{-1}).

All other chemicals were obtained as analytical grade reagents from either Sigma Chemical Co. (Poole, Dorset, U.K.) or BDH Chemical Co. (Poole, Dorset, U.K.).

Plant Material

Plants of *Kalanchoë daigremontiana* Hamet et Perrier de la Bathie were propagated vegetatively from leaf bulbils in a heated glasshouse. A few days prior to experimentation the plants were transferred to a controlled environment room with a reversed light/dark cycle. During the light period, plants were illuminated with a combination of metal-halide fluorescent lamps (Thorn 400 W MBFI/BU) and tungsten lamps (GEC PAR 38 150 W Flood) for 13 h. Air temperature was maintained at 25 °C during this period and at 15 °C during the dark period, with a relative humidity of approximately 35% and 70%, respectively, in the two phases.

Tonoplast Isolation

Mature leaves from *Kalanchoë daigremontiana* were harvested approximately 1 h into the dark phase from the controlled-environment plants aged between 7 and 9 months. A tonoplast fraction from the mesophyll tissue was isolated by a modification of the method of Bremberger et al. (1988) and Haschke et al. (1989) - **Method 1.** Approximately 100 g of mesophyll tissue were blended in 250 ml buffer containing 450 mM mannitol, 3.0 mM $MgSO_4$, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA), 0.5% (w/v) polyvinylpyrrolidone-40 (PVP-40), 10 mM *DL*-dithiothreitol (DTT) and 100 mM *N*-tris(hydroxymethyl)methylglycine (Tricine) buffered to pH 8.0 with 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris). The homogenate was filtered through two layers of cheesecloth and centrifuged at 13 000 *g* for 15 min in a Sorvall RC5B refrigerated superspeed centrifuge (DuPont Instruments) using an SS34 rotor. The resulting supernatant was layered onto a 25% (w/v) sucrose cushion containing 2 mM DTT and 5 mM Tricine buffered to pH 8.0 with Tris, and centrifuged in an AH629 swing-out rotor in a Sorvall ultracentrifuge OTD 65B (DuPont) at 100 000 *g* for 70 min. The band that resulted at the top of the sucrose cushion was removed using a pasteur pipette and diluted 1:1 (v/v) with buffer consisting of 150 mM mannitol, 2.0 mM DTT, 111 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulphonic acid (Hepes), adjusted to pH 7.0 with NaOH, and centrifuged in an MSE Superspeed 65 ultracentrifuge (10 x 10 rotor) at 100 000 *g* for 30 min. The pellet was resuspended in a medium containing 22.2 mM $MgCl_2$, 2.0 mM EGTA, 111 mM Hepes (pH 7.0 with NaOH). All steps were performed at 4 °C and a fresh tonoplast preparation was used in each experiment.

A second procedure (**Method 2**) was employed for tonoplast isolation using a modification of the method of Pope and Leigh (1988). This involved various additional steps and solutions to Method 1. The resulting supernatant from the first spin at 13 000 *g* was centrifuged in the AH629 swing-out rotor in the OTD 65D centrifuge at 80 000 *g* for 40 min. The microsomal pellet produced was resuspended in approximately 300-500 µl "glycerol (gly) medium" containing 1.1 M glycerol, 1.0 mM EDTA, 2.0 mM DTT, 10 mM Tricine, and buffered to pH to 7.4 with Tris. This was then diluted with gly medium to fill the tube and recentrifuged at 80 000 *g* and the pellet resuspended and diluted as before. This solution was then layered onto a 23% (w/v) sucrose solution in gly medium at pH 7.4 containing 2.0 mM DTT and centrifuged in the AH629 swing-out rotor in the OTD 65B centrifuge at 100 000 *g* for 70 min. The resulting band was removed and diluted with gly medium and spun at 100 000 *g* in the MSE superspeed 65 ultracentrifuge and resuspended as in Method 1.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method described by Laemmli (1970). Samples of membrane were pipetted into the required volume of sample buffer containing 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.35% (w/v) SDS, 0.014% (w/v) bromophenol blue and 62.5 mM Tris (adjusted to pH 6.8 with Tricine) and heated at 100°C for 2 min. The same process was performed for labelled samples after incubation in [γ-^{32}P]-ATP medium.

An exponential running gel (7.5%-15% (w/v)) was prepared using a gradient maker to produce concentrations from 7.5% (w/v) acrylamide and 0.2% (w/v) bisacrylamide to 15% (w/v) acrylamide and 0.4% (w/v) bisacrylamide

with 0.1% (w/v) SDS, 375 mM Tris/HCl (pH 8.8) and 0.013% (w/v) ammonium persulphate (AMPS) plus 0.05% (w/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED) to polymerise. The stacking gel was 5% (w/v) acrylamide, 0.13% (w/v) bisacrylamide, 0.1% (w/v) SDS, 125 mM Tris/HCl (pH 6.8) and 0.04% (w/v) AMPS plus 0.12% (v/v) TEMED to polymerise. Electrode buffer was used which contained 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS. The dimensions of the running gel were 15 cm x 15cm x 0.1 cm; the apparatus was constructed in the Botany Department workshop, Edinburgh University. Gels were run at a constant voltage of 150 V overnight at 4°C. Photographs were taken using Technical Pan 2415 or T-Max film.

Silver Staining

Gels were silver stained using the Sigma silver staining kit. Gels were fixed for 3 x 20 min. in 30% (v/v) ethanol/10% (v/v) glacial acetic acid solution. Equilibration was performed in a solution of 0.005% (v/v) silver concentrate solution. Staining bands were developed using the development mix. Between stages, washes were done with distilled H_2O (dH_2O) as required (Sigma silver staining kit, technical bulletin, 1987).

Mesurement of Protein Kinase Activity

Protein kinase activity was assayed by the method of Hetherington and Trewavas (1982) with modifications as follows. All solutions were made up in deionised H_2O. Assays were done at 20 °C in a medium of 111 mM Hepes, 5.0 mM EGTA, 22.2 mM $MgCl_2$ and varying concentrations of $CaCl_2$ to give the desired free Ca^{2+} concentrations as calculated using the computer

program SOLCON (Dr D.C.S. White, Univesity of York) and adjusted to pH 7.0 with HCl. After addition of tonoplast membrane (constituting approximately one-third of the total volume), the reaction was initiated by the addition of 9.2 μM (final concentration) [γ-^{32}P]-ATP .

Samples of between 7 μl and 13 μl were taken at appropriate time intervals and pipetted onto cellulose filter discs (pore size 3 μm) that had been pretreated with 100 μl 20% (w/v) trichloroacetic acid (TCA)/20 mM tetra-sodium pyrophosphate/10 mM EDTA (TCA mix). The discs were obtained from Whatman International Ltd. (Maidstone, U.K.). The filters were then dropped into 500 ml TCA mix and left overnight at room temperature. The filters were subsequently washed with fresh TCA mix for approximately 1 h or until the blank filter gave a reading of between 5 c.p.s. and 10 c.p.s. and then rinsed for 30 s with a solution of acetone : 1 M HCl in a ratio of 49 : 1 and air dried.

Incorporation of ^{32}P was determined by Cerenkov counting of each disc in 5ml dH_2O using a scintillation counter (Kontron Instruments, Scotland).

Autoradiography

X-Ray film (Hyperfilm MP) was obtained from Amersham International plc. Intensifying screens (Cronex Xtrlife- lightening plus) were obtained from DuPont (Herts., U.K.). Pre-flashed film was exposed directly to the dried labelled gel. A fluorescent screen (used to enhance the image) was placed on top of the film. The film was then exposed in the cassette at -80 °C for a defined amount of time (Laskey, 1980; Blowers, 1986). Photographs were taken using Technical Pan 2415 or T-Max film.

Protein Analysis

Protein concentrations were determined by the method of Bradford (1976) with absorbance measurements at 595 nm with bovine serum albumin (BSA) as standard. The colour reagent consisted of a filtered solution of 100 mg Coomassie brilliant blue G-250, 50 ml ethanol and 100 ml orthophosphoric acid in 1 l dH_2O. The assay mix consisted of 1 ml Bradford's reagent plus 100 µl of suitably diluted preparation or bovine serum albumin (BSA) standard in the range 1 to 10 µg final amount.

H^+-ATPase Hydrolytic Assay

H^+-ATPase hydrolytic activity was assayed by a modification of Smith et al. (1984b) by following the release of P_i from ATP. The standard reaction medium contained 3.0 mM $MgSO_4$, 50 mM KCl, 1.0 mM sodium molybdate, 50 mM Tricine (pH 8.0 with Tris), 3.0 mM ATP (added as the Na^+ salt), 0.5 mM sodium azide, or 0.5 mM sodium orthovanadate, where specified and 10% (of the total assay volume) membrane solution with or without 50 mM (final concentration) KNO_3. Blanks also contained 1 % (w/v) SDS. The reaction was started by the addition of ATP and samples were incubated at 37 °C in a shaking water bath for 30 min. The reaction was stopped by the addition of 1 % (w/v) SDS to the reaction tubes. P_i release was determined essentially as described in Leigh and Walker (1980) and Smith et al. (1984b) by adding 0.45% total final volume of an assay mix containing 6 parts of 0.42% (w/v) ammonium heptamolybdate in 0.5 M H_2SO_4 and 1 part of 10% (w/v) ascorbic acid. The samples were incubated as before at 37 °C for 45 min. As controls, the P_i assay was performed without membrane vesicles present and the volume made up with appropriate buffer. The absorbance of ~~all samples was~~

all samples was measured at 720 nm in a Beckman DU-64 spectrophotometer (Beckman Instruments Inc., Palo Alto, California, U.S.A.).

Immunoblot Analysis

Imunoblotting was performed following a modification of Blowers (1986). The gel was equilibrated for 30 min in buffer containing 25 mM Tris, 192 mM glycine and 20 % (v/v) methanol (pH 8.3). Protein transfer onto nitrocellulose (pore size 0.2 μm obtained from Schleicher and Schuell (Dassel, FRG)) was done at room temperature overnight at 30 Volts and 0.1 Amps. All subsequent steps were performed at 37 °C unless otherwise stated. The nitrocellulose was then washed for 30 min in a solution containing 3 % (w/v) BSA in ATBS(20 mM Tris/SO_4^- (pH 7.5) and 0.5 M NaCl) then three more times in ATBS. Following this, first antibody was introduced in a solution of 1 % (w/v) gelatin in ATBS for 2 h. 30 min washings thereafter were in 0.05 % (v/v) TWEEN in ATBS. Second antibody in ATBS (goat anti-rabbit IgG horseradish peroxidase conjugate) was added for 1 h and three more subsequent 30 min washes were performed with ATBS. A colour development mixture was then added for approximately 1 min or until the Ab reaction was visible then stopped with dH_2O.

For second antibody horseradish peroxidase conjugates the colour development mixture used was as follows :- 60 mg horseradish peroxidase colour developoment in 20 ml ice cold pure methanol, mixed with 60 μl ice cold 30% (v/v) hydrogen peroxide (H_2O_2) in 100 ml ATBS, to give a final solution of 0.015% (v/v) H_2O_2. This solution was used at room temperature.

Staining of Protein Blots

AuroDye forte (colloidal gold reagent) obtained from Amersham was used for staining of protein blots. The staining procedure was as follows:- the blot was incubated in phosphate buffered saline (PBS) with 0.3 % (v/v) TWEEN-20 for 30 min at 30 °C followed by 3 x 5 min washes in PBS with 0.3 % (v/v) TWEEN-20 at room temperature. The blot was then incubated in AuroDye forte for 2 h and then washed with excess dH_2O for 15 min and dried between filter papers.

CHAPTER 3

RESULTS

3.1 Tonoplast Isolation Procedure

Initially, the procedure in Materials and Methods (Method 1) was developed to obtain suitable yields of tonoplast from leaf homogenates of *Kalanchoë daigremontiana*. Typically, the tonoplast yield was 0.80 ± 0.15 mg protein from 100 g fresh weight of mesophyll tissue. H^+-ATPase hydrolytic activity present in the tonoplast fraction was measured in the presence of 0.5 mM sodium orthovanadate, 0.5 mM sodium azide, or 50 mM KNO_3 (Table 1). Orthovanadate had little effect on the amount of activity present, signifying a very low contamination of the fraction with plasmalemma ATPase activity. On the other hand, azide (a potent inhibitor of mitochondrial ATPase activity) reduced the ATPase activity by approximately one quarter of the control value. Thus, all subsequent assays to measure NO_3^--sensitive ATPase activity also included 0.5 mM sodium azide in the reaction medium to exclude a contribution to the measured activity from the mitochondrial ATPase. The NO_3^--sensitive ATPase characteristic of the tonoplast contributed approximately 70 % of the total activity in the presence of azide. The NO_3^--sensitive H^+-ATPase hydrolytic activity in the presence of 50 mM KCl averaged 0.187 ± 0.089 μmol min^{-1} mg^{-1} (Table 2(A)).

Table 1.

Inhibitor Present in assay	Measured H^+-ATPase Hydrolytic Activity ($\mu mol\ min^{-1}\ mg^{-1}$)	Percentage of maximum ATPase Activity
None	0. 267 ± 0.113	100 %
0.5 mM sodium orthovanadate	0.254 ± 0.008	95 ± 3 %
0.5 mM sodium azide	0.203 ± 0.022	76 ± 8 %
50 mM KNO_3 (+ 0.5 mM sodium azide)	0.080 ± 0.013	30 ± 5 %

Comparison of the H^+-ATPase hydrolytic activities measured in the tonoplast fraction in the presence of different inhibitors. Percentages are expressed relative to the total hydrolytic activity of 100% (no inhibitor added), values are presented as means +/- S.D. from 5 experiments.

Table 2.

H^+-ATPase Hydrolytic Activity ($\mu mol\ min^{-1}\ mg^{-1}$)			
Method	Total ATPase activity	NO_3^--sensitive ATPase activity	% inhibition by NO_3^-
A	0.267 ± 0.113	0.187 ± 0.089	70
B	0.356 ± 0.100	0.244 ± 0.011	68
C	0.595 ± 0.220	0.431 ± 0.187	72

Measured H^+-ATPase hydrolytic activities after isolation of tonoplast vesicles by Method 1 (A), Method 1 with glycerol solutions (B) and Method 2 (C). Values for A are means +/- S.D. from the same 5 experiments as in Table 1. Values for B and C are means +/- S.D. from 3 separate experiments.

The polypeptide composition of the fraction isolated by Method 1 was investigated using SDS-PAGE (Fig. 4). This shows a typical developed banding pattern using the Sigma silver staining kit. There are approximately 20 to 25 visible polypeptide bands after silver staining, with maximally stained polypeptides at $\underline{M_r}$ values of 105 000, 67 000, 57 000, 54/55 000, 51 000, 47/48 000, 46 000, 38 000, 33 000 and 29/30 000.

To try to maximise the purity of the tonoplast fraction, this preparative method was compared with another procedure based on that of Pope and Leigh (1988), which itself is a modification of that of Rea and Poole (1985). Initially, all solutions excluding the homogenisation medium were replaced by a "glycerol medium" containing 1.1 M glycerol, 1.0 mM EDTA, 2.0 mM DTT, 10 mM Tricine (pH 7.4 with Tris)- (Method 1 with glycerol solutions). The NO_3^--sensitive ATPase activity increased by approximately 1.3-fold as a result of this change (Table 2). The 25 % (w/v) sucrose solution previously used was also changed to 23 % (w/v) sucrose in glycerol medium to give the solution a similar density.

The next adjustment to the tonoplast preparative method was to introduce an additional pelleting step at 80 000 *g* after the initial 13 000 *g* spin and before the sucrose density gradient spin. This resulted in an approximately 2.2-fold increase in total ATPase activity relative to Method 1 (Table 2). This modified method, Method 2, also gave similar protein yields to Method 1 of approximately 0.80 ± 0.20 mg, but with a higher NO_3^--sensitive specific ATPase activity of 0.431 ± 0.187 μmol min^{-1} mg^{-1} protein (Table 2).

Figure 4.

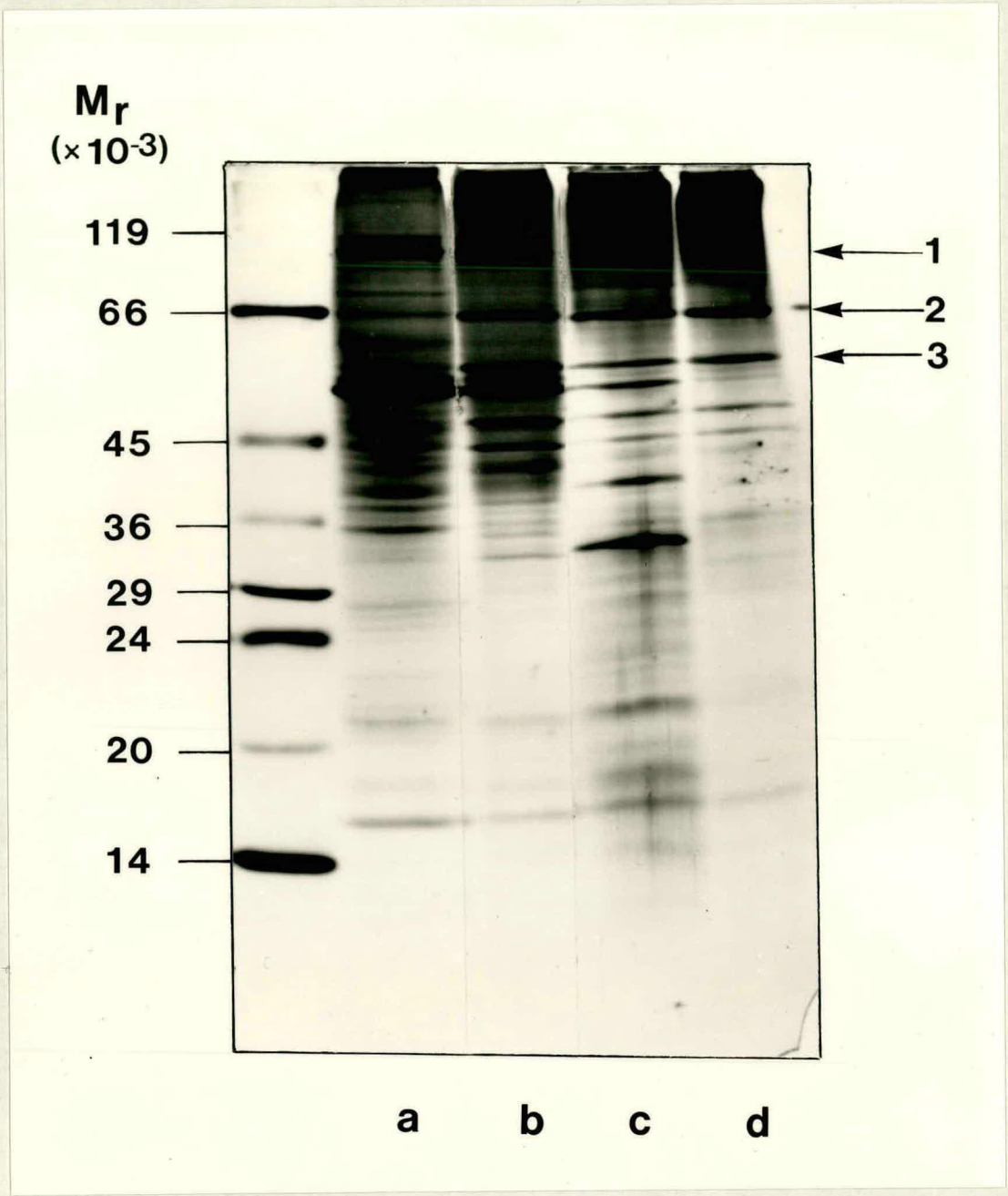
SDS-PAGE pattern of proteins in the tonoplast fraction of *Kalanchoë daigremontiana* prepared by Method 1. Samples of 4 µg protein were prepared and run on a gel as described in Chapter 2. Lane A is the molecular weight markers and lane B is the tonoplast fraction.

The polypeptide pattern of the tonoplast fraction prepared by Method 2 was compared with that of Method 1 (Fig. 5, lanes c & d). Fewer polypeptides were stained, suggesting the presence of contaminating cytosolic or other membrane proteins in the preparation from Method 1. These patterns were also analysed relative to the polypeptide compositions of the initial leaf homogenate, lane a (reflecting all the proteins present in the mesophyll tissue), and a microsomal fraction, lane b, isolated before the sucrose spin in Method 2 (Fig. 5). It should also be noted that the NO_3^--sensitive ATPase specific activities increased with increased purification of the tonoplast membrane thus signifying an increase in the tonoplast ATPase activity (Table 3).

Two heavily stained polypeptide bands at approximately M_r of 105 000 and 51/52 000 became less pronounced in the course of the purification. The former polypeptide was tentatively identified as the cytosolic enzyme PEPCase by immunoblotting using antisera raised in rabbits to purified PEPCase from *Kalanchoë daigremontiana*, kindly donated by P. Maier and M. Kluge, Technische Hochschule Darmstadt, Germany (Fig. 5, lane d, arrow 1, and Fig. 6). Two of the subunits of the tonoplast ATPase were also tentatively identified immunologically using anti-72 kDa and anti-57 kDa antisera raised in rabbits by the Department of Biochemistry, University of Edinburgh. These crossreacted with the polypeptide bands of M_r approximately 68 000 and 56 000 of the tonoplast H^+-ATPase, respectively (arrows 2 & 3, Fig. 5). The immunoblots have not been shown as the bands became faint and eventually disappeared before photography was able to take place, hence tentative rather than definite identification of these three polypep-tides has been discussed.

It was concluded that Method 2 gave the "purest" tonoplast fraction, and this was used as a preparative method for isolation of tonoplast membrane vesicles in all subsequent experiments.

Figure 5.



SDS-PAGE pattern of proteins in various fractions isolated from mesophyll tissue from *Kalanchoë daigremontiana*, where a = leaf homogenate, b = microsomal fraction, c = tonoplast fraction from Method 1 and d = tonoplast fraction from method 2. Samples of 4 μg protein were prepared and run on a gel as described in Chapter 2.

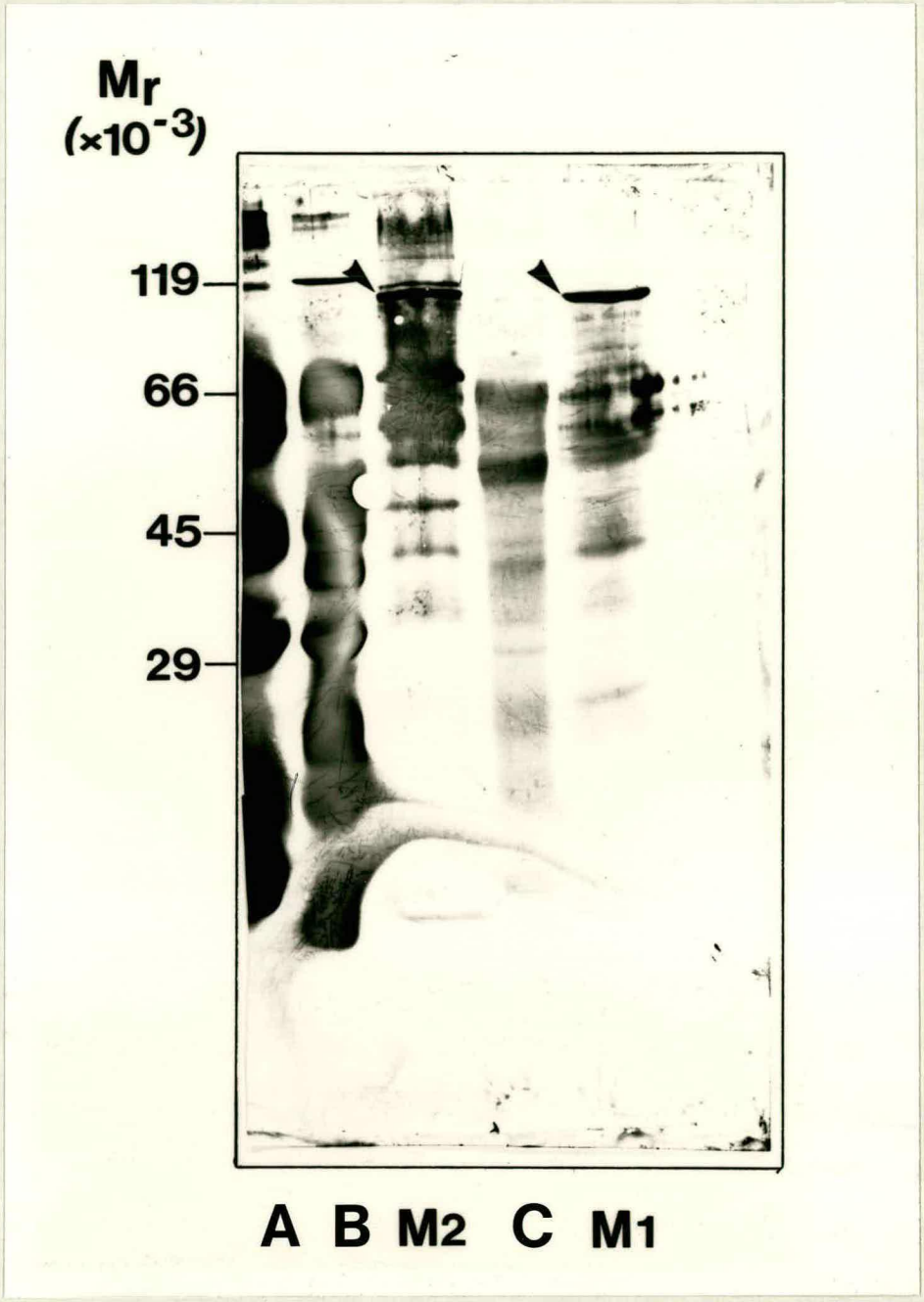
Table 3.

	**H^+-ATPase Hydrolytic Activity ($\mu mol\ min^{-1}\ mg^{-1}$)	
	TOTAL	NO_3^- SENSITIVE
a = leaf homogenate	0.049	0.024
b = microsomal	0.168	0.108
c = Method 1	0.255	0.158
d = Method 2	0.786	0.545

**Typical of one experiment of the type illustrated in Fig. 5.

H^+-ATPase hydrolytic activities associated with the various fractions isolated from the mesophyll tissue, Fig 5.

Figure 6.



Half of a protein blot stained with AuroDye forte. Arrows highlight polypeptides of Mr approximately 105 000 in lane M2 (tonoplast vesicles isolated by Method 2) and lane M1 (tonoplast vesicles isolated by Method 1). Lanes A and B show protein markers of Mr values as shown at the side. Lane C is a protoplast preparation which did not crossreact with anti-PEPCase antisera.
The other half of the blot (not shown due to fading) was treated as described on page 23 (Immunoblot Analysis), indicating the possibility that the polypeptide Mr 105 000 shown in M1 and M2 is PEPCase.

3.2 Phosphorylation of Tonoplast Proteins

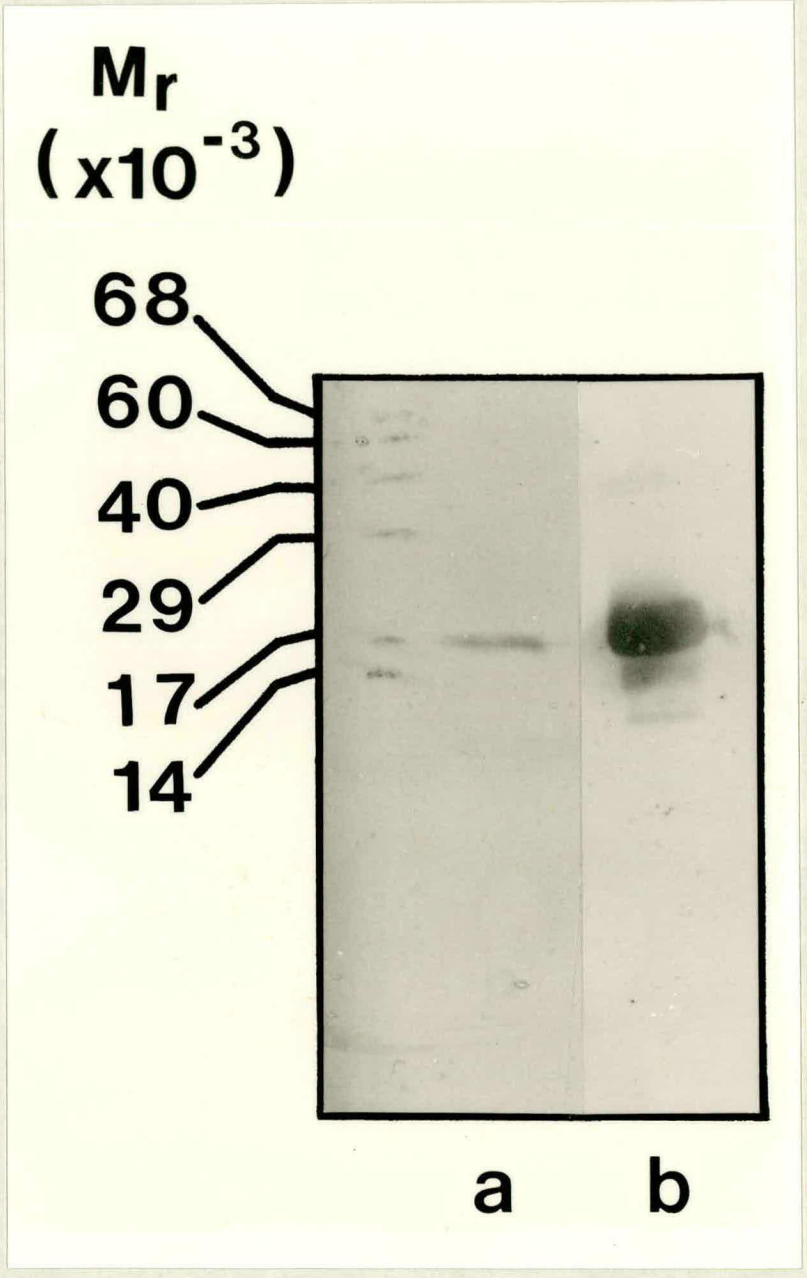
3.2(i) Preliminary Results

In preliminary experiments using vesicles isolated by Method 1, it was shown that the tonoplast fraction isolated from *Kalanchoë daigremontiana* contained protein kinase activity that was stimulated by micromolar concentrations of calcium (Graham et al., 1989). There appeared to be no significant effect of added exogenous calmodulin (CaM) on the kinase activity in the tonoplast fraction. However, the presence of endogenous calmodulin in this fraction was detected using immunoblot analysis with mouse anti-spinach cleared serum to CaM and goat anti-mouse horse-radish-peroxidase-linked whole IgG second antibody (Fig. 7). This might explain the lack of any effect of added exogenous CaM on protein kinase activity in this fraction.

Since these preliminary experiments were performed with a membrane fraction isolated by Method 1, which did not yield the purest tonoplast fraction (cf. Table 2), the protein kinase may have been a cytosolic contaminant or even a contaminant associated with another membrane system. Nonetheless, the presence of protein kinase activity in this fraction shows that phosphorylation of tonoplast polypeptides can occur. It was therefore of interest to investigate further the characteristics of this protein kinase activity.

Method 2 was established to obtain a purer fraction of tonoplast vesicles and the next step was to assess whether protein kinase activity detected in the preliminary experiments was present in this fraction.

Figure 7.



Immunoblot identifying the presence of CaM in the tonoplast fraction of (lane A)
Kalanchoë daigremontiana prepared by Method 1. Analysis was done using mouse anti-spinach cleared serum to CaM and goat anti-mouse horse-radish-peroxidase-linked whole IgG second antibody.

Lane B is the control reaction with CaM.

3.2(ii) Protein Kinase Activity associated with the Tonoplast

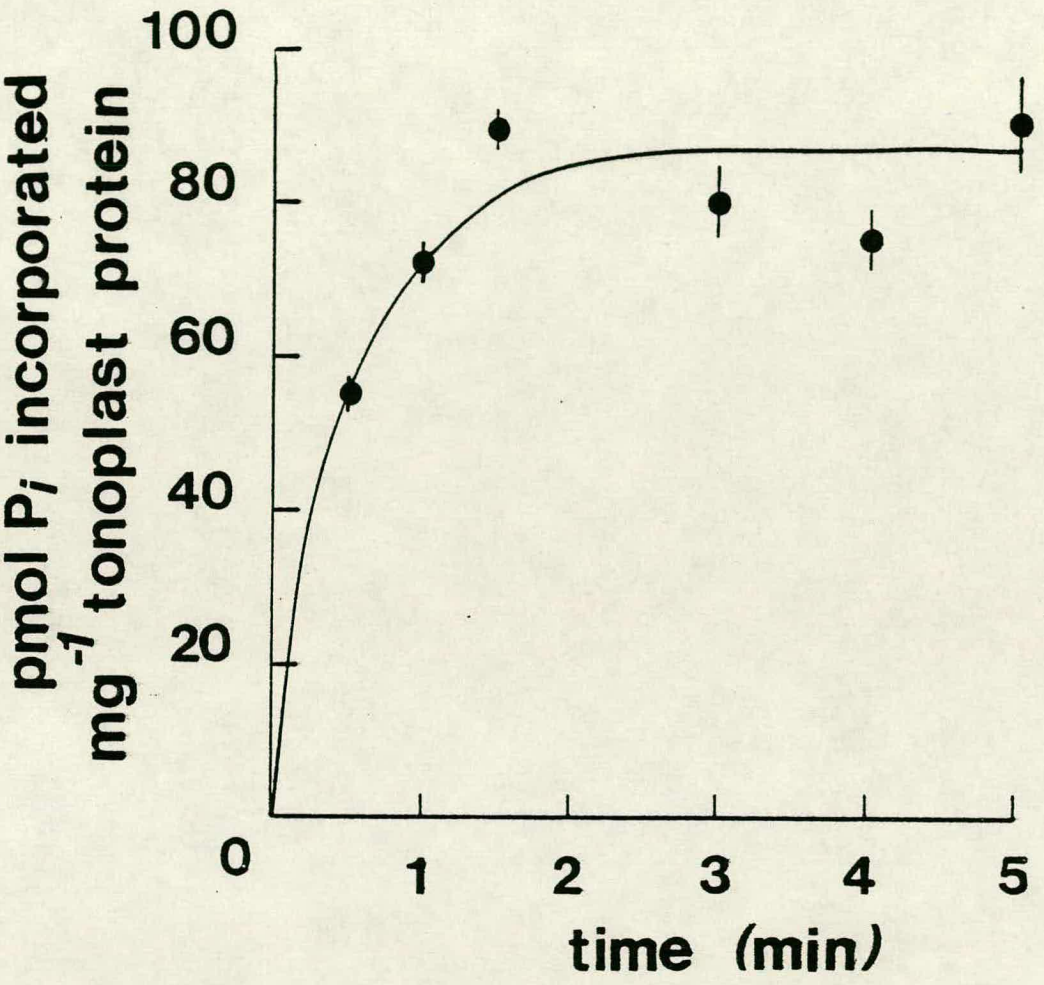
Protein kinase activity was estimated by measuring the incorporation of ^{32}P from [γ–^{32}P] ATP into TCA-precipitable proteins as described in Chapter 2.

The purer tonoplast fraction isolated by Method 2 was again found to contain protein kinase activity. A time-course experiment was performed, which showed that there was rapid incorporation of ^{32}P during the first 60 seconds, but that net incorporation was saturated after about 2 min. (Fig. 8).

3.2(iii) Effect of Mg^{2+}

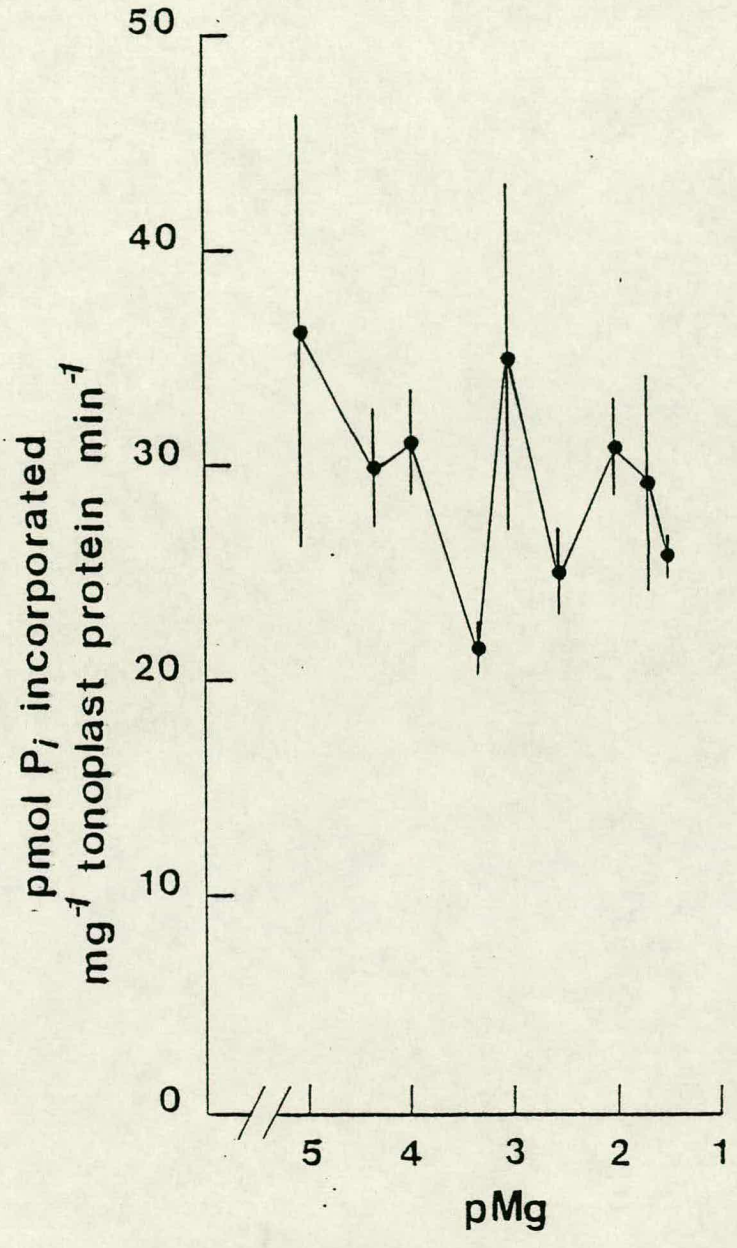
Various free Mg^{2+} concentrations worked out using the SOLCON computer program were employed to assess the concentration required for optimal protein kinase activity. Solutions containing 111 mM Hepes and 5.0 mM EGTA (purity of 96.4 %; Bers, 1982; Miller and Smith, 1984) were used with the addition of concentrations of $MgCl_2$ giving pMg values ranging from 1 to 5, i.e. 10 µM to 100 mM. There appeared to be no significant effect on protein kinase activity over this range (Fig. 9). For this reason, the original concentration of $MgCl_2$ of 22.2 mM (pMg= 1.65) was chosen to be included in all the necessary solutions. (Values are overall lower than Fig. 8 due to the time of year when the experiments were performed). This is in the observed range of the Mg^{2+}-optima for soluble wheat germ protein kinase of between 15 and 30 mM Mg^{2+} (Davies and Polya, 1983). However, there have been several other reports of sharp Mg^{2+}-optima between 3 and 10 mM (Murray et al., 1978; Yan and Tao, 1982; Salimath and Marmé, 1983; Ladror and Zielinski, 1989).

Figure 8.



Time-course of incorporation of ^{32}P from [γ-^{32}P]-ATP into polypeptides of the tonoplast fraction of *Kalanchoë daigremontiana* prepared by Method 2. Tonoplast membranes were incubated in a medium containing 111 mM Hepes (pH 7.0), 22.2 mM $MgCl_2$ and 5.0 mM EGTA. Results are the average of three separate experiments and are given as means +/- S.E..

Figure 9.



Mg^{2+}-concentration-dependence of the protein kinase activity associated with the tonoplast fraction of *Kalanchoë daigremontiana*. Samples were prepared and assayed as described (30 sec incubation) with the addition of varying concentrations of $MgCl_2$ to give the described free Mg^{2+} levels. Results are the average of two separate experiments and are given as means +/- S.E..

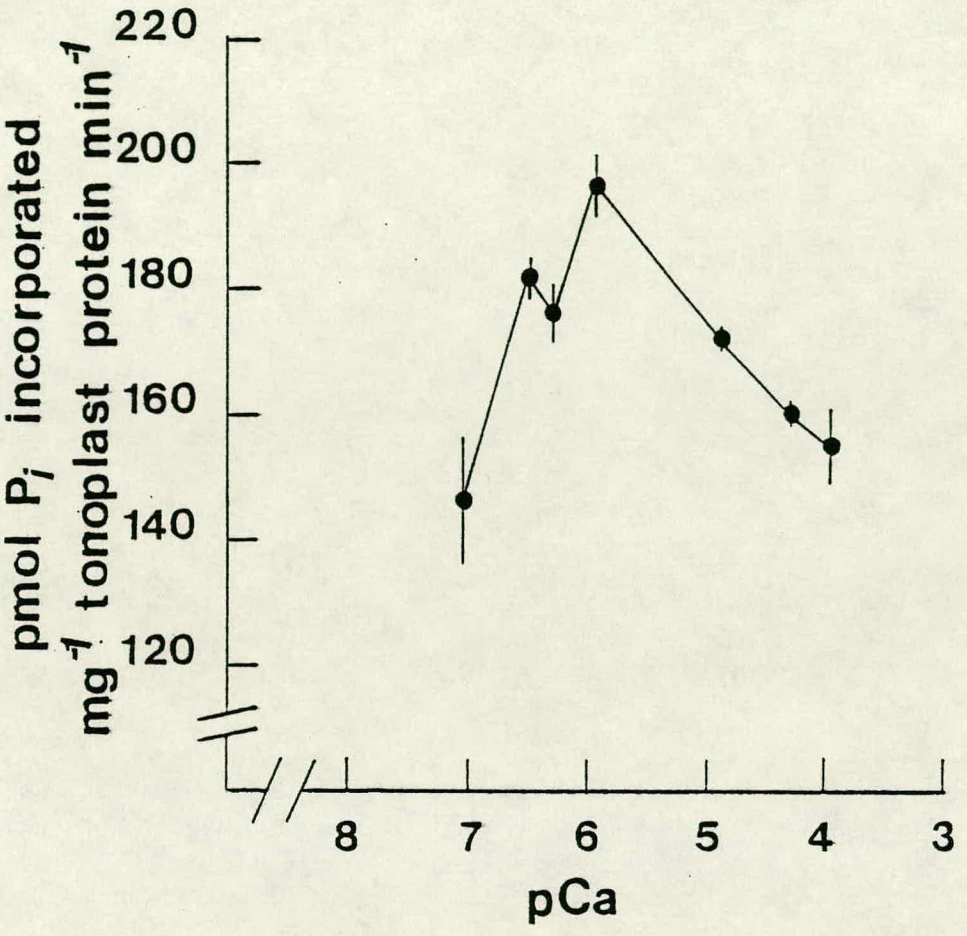
3.2(iv) Effect of Ca^{2+}

The Ca^{2+}-concentration-dependence of the protein kinase activity was also examined. Again, the computer program SOLCON was employed to calculate free Ca^{2+} concentrations present in various solutions containing 111 mM Hepes, 22.2 mM $MgCl_2$ and 5.0 mM EGTA and various concentrations of $CaCl_2$. Incorporation of ^{32}P was optimal when the free Ca^{2+} concentration was approximately 1.0 μM, i.e. pCa= 6 (Fig. 10).

Autoradiography was also performed to visualise the polypeptides in the tonoplast fraction that became phosphorylated in the reaction after 30 seconds in the presence of [γ-^{32}P]-ATP (Fig. 11 and Table 4). It can be seen that some phosphorylation takes place in the absence of Ca^{2+} (lane A, Ca^{2+} concentration < 10^{-8}). Five polypeptides with $\underline{M}_r$ values of approximately 112 000, 105 000, 91 000, 60 000 and 55/56 000 were phosphorylated in the 30-second reaction time in the absence of Ca^{2+}. Various changes can be seen in the phosphorylated polypeptide pattern with different free Ca^{2+} concentrations as compared with that seen in the absence of Ca^{2+}.

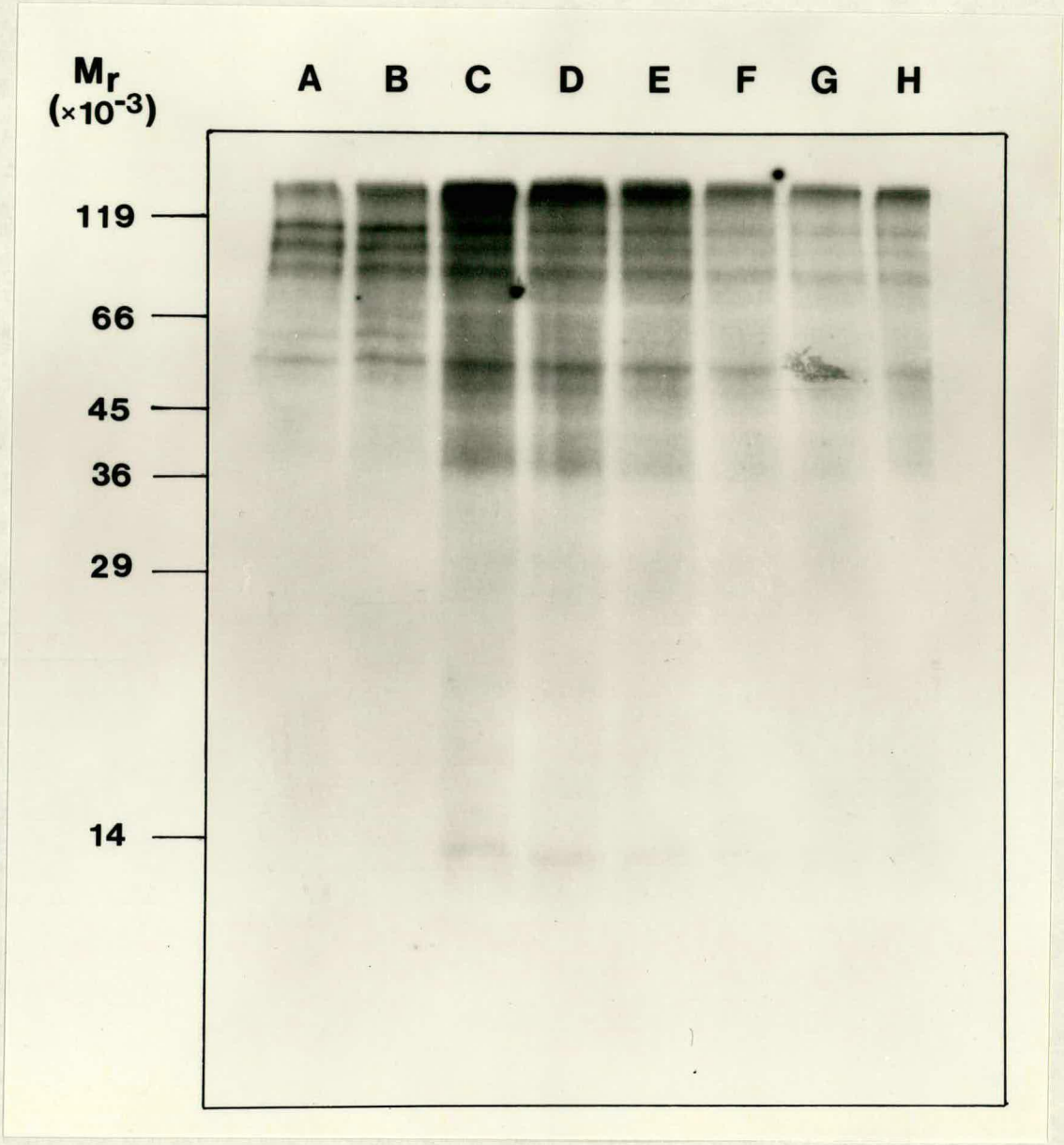
Lanes B to H (Fig. 11) show bands that are phosphorylated under different Ca^{2+} concentrations ranging from pCa 7.1 (0.08 μM) to pCa 3.96 (110 μM). The optimal Ca^{2+} concentration for phosphorylation appeared to be between pCa 6.50 to pCa 5.94, i.e. 0.31 to 1.15 μM free Ca^{2+} (C & D). Figure 10 backs up this result showing optimal incorporation at approximately pCa 5.9, i.e. approx. 1.15 μM. Phosphorylation has increased significantly in the tonoplast fraction in the presence of free Ca^{2+}. The most significant within this range, as compared with lane A (-Ca^{2+}), is an increase in labelling of polypeptides of $\underline{M}_r$ 55 000 and the appearance of phosphorylated bands at $\underline{M}_r$ values of

Figure 10.



Ca^{2+}-concentration-dependence of the protein kinase activity associated with the tonoplast fraction of *Kalanchoë daigremontiana*. Samples were prepared and assayed as described (30 sec incubation) with the addition of varying concentrations of $CaCl_2$ to give the desired free Ca^{2+} concentrations. Results are the average of three separate experiments and are given as means +/- S.E..

Figure 11.



Autoradiography of the polypeptides in the tonoplast fraction that became phosphorylated after incubation in 111 mM Hepes (pH 7.0), 22.2 mM $MgCl_2$, 5.0 mM EGTA and various free Ca^{2+} concentrations as specified in Table 4. Samples were prepared and SDS-PAGE performed as outlined in Chapter 2. Refer to Table 4 for the free Ca^{2+} concentrations A to H.

Table 4.

Lane	$[Ca^{2+}]$ (μM)	pCa
A	<0.01	—
B	0.08	7.10
C	0.31	6.50
D	0.49	6.30
E	1.15	5.94
F	13.86	4.86
G	48.78	4.31
H	110.58	3.96

List of free Ca^{2+} concentrations, pCa, as used in autoradiography of the tonoplast polypeptides (Fig 11).

approximately 37 000 and 10/11 000. With higher Ca^{2+} concentrations above approximately 1.15 μM there appeared to be a general inhibitory effect on the protein kinase activity with an overall decrease in labelling intensity of these bands. Comparing lanes E to H with C & D, as the free Ca^{2+} concentration increased there appeared to be a decrease in the phosphorylation of all the polypeptides up to the addition of 110 μM Ca^{2+} (lane H). The most pronounced decreases in polypeptide phosphorylation were associated with those of $\underline{M_r}$ 105 000 and 60 000. These two phosphorylated polypeptides decreased in intensity substantially to a level lower than lane A ($-Ca^{2+}$). The band at $\underline{M_r}$ 55 000 appeared to be similar in intensity in both lanes A and H. It is interesting to note that the polypeptides at 55 000 and 105 000 may be subunits of the tonoplast H^+-ATPase and of the cytosolic PEPcase, respectively.

3.2(v) Effect of Protease Inhibitors

To test for any effect on protein phosphorylation of proteases present in the tonoplast fraction, three protease inhibitors were added to the final resuspension medium in the tonoplast isolation procedure and throughout the filter assay. These inhibitors were leupeptin (2 μg/ml), pepstatin A (2 μg/ml) and phenylmethanesulphonylfluoride, PMSF: 2 mM, chosen to cover a wide range of specificities (Table 5). Their inclusion appeared to have no significant effect on the ^{32}P incorporation into the tonoplast polypeptides (data not shown; see Fig. 8 for time-dependence curve).

Table 5.

Protease Inhibitor	Specificity
Leupeptin	cathespin B, papain, plasmin,trypsin
Pepstatin A	carboxyl proteases
PMSF	serine proteases

List of protease inhibitors tested and their specificities.

3.2(vi) Protein Phosphatase Activity

The level of phosphorylation of any protein depends on the relative activities of both protein kinases and protein phosphatases (Cohen, 1988). Far less attention, however, has been given to protein phosphatases due to the difficulty in identifying their normal physiological substrate (Cohen and Cohen, 1989). Okadaic acid (OA) has improved the procedure for measuring protein phosphatase activity. OA is a complex polyketal fatty acid first isolated from marine sponges *Halichondria okadaii* and *Halichondria melanodocia*, and is a potent inhibitor of protein phosphatase activity of type 1 and type 2A phosphatases (Cohen et al., 1990). To assess whether phosphatase activity of these types could be affecting the protein phosphorylation associated with the tonoplast fraction, the incorporation of ^{32}P was measured in the presence and absence of 12.5 nM OA. There appeared to be no effect on the time-dependent incorporation of ^{32}P into tonoplast proteins with the addition of OA (data not shown; see Fig. 8 for time-dependence curve). Thus, it was concluded that there was little or no contaminating phosphatase activity present in the tonoplast fraction.

CHAPTER 4

DISCUSSION

4.1 Isolation of a Pure Tonoplast Fraction.

From the tonoplast isolation procedure, Method 2, enough material was obtained from the mesophyll tissue of the CAM plant, *Kalanchoë daigremontiana* for,

1. analysis of the polypeptide composition of the tonoplast by SDS-PAGE,

2. measurement of the tonoplast H^+-ATPase hydrolytic activity, and

3. investigation of protein kinase activity associated with the tonoplast fraction.

It has been shown that glycerol is an important ingredient in the isolation medium in this procedure. Glycerol (and other organic cosolutes) promote self-association of proteins, thus stabilising their structure (Stamatakis et al., 1988). The amount of inhibition by NO_3^- relative to the total amount of ATPase activity present remained the same (approx. 70%) comparing the two tonoplast methods. However, the results have shown that Method 2 produces tonoplast vesicles which possess a NO_3^--sensitive H^+-ATPase specific activity approximately 2.3-fold greater than that of the vesicles isolated following the procedure of Method 1 (Table 2). Pope and Leigh

(1988) noted that the glycerol behaved as a highly permeant osmoticum with tonoplast vesicles from higher plants, and it is possible that osmotically induced lysis of the vesicles caused by their transfer to glycerol-containing media assists in the removal of non-tonoplast proteins from this membrane fraction. Thus this increase in activity has arisen as a consequence of the combination of the addition of glycerol into the media and also the introduction of extra centrifugal stages in the isolation procedure.

SDS-PAGE has highlighted various differences in the polypeptide composition of the tonoplast vesicles isolated by Method 1 and Method 2 (Fig. 5, lane c & lane d, respectively). There appears to be a greater amount of protein stain at the top of lane d compared to that of lane c, and an equivalent decrease in protein stain towards the bottom of the gel. It should be noted, however, that staining patterns from gel to gel may vary (cf. Fig. 5., lane c and Fig. 4), and that separation may not resolve all polypeptides. It is also possible that groups of polypeptides may migrate as single bands.

NO_3^--sensitive H^+-ATPase activity was measured at two other stages in the procedure of Method 2 :

1. leaf homogenisation, and

2. microsomal preparation.

This was performed to monitor the enrichment of the tonoplast through the isolation procedure. The results indicated a decrease in the number and intensity of several stained polypeptides with increased tonoplast purity along with the enrichment of at least two polypeptides of $\underline{M}_r$ approximately

68 000 and 56 000. These two subunits have been tentatively identified immunologically as subunits of the H^+-ATPase (see Fig. 5, arrows 2 & 3). Therefore, with increased enrichment of tonoplast fewer contaminating polypeptides appeared to be associated with the fraction and both the amount and activity of tonoplast H^+-ATPase increased. There were still minor contaminants in the pure fraction such as the polypeptide with M_r approximately 105 000 that crossreacted with antisera to PEPCase (Fig. 5, arrow 1). However, this polypeptide appeared to constitute a relatively small proportion of the total protein present. Smith et al. (1984b) also noted that, of all the cytoplasmic marker enzymes they studied, PEP carboxylase was the one that was most difficult to dissociate from isolated vacuoles of *Kalanchoë daigremontiana*, possibly on account of the hydrophobicity of the protein.

It is now important to identify further polypeptides in the pure tonoplast fraction in order to be able to study and understand more about the biochemistry of these membrane proteins.

4.2 Kinase Activity at the Tonoplast

The presence of protein kinase activity in the tonoplast fraction from *Kalanchoë daigremontiana* suggests that protein phosphorylation may play an important role in regulation processes at the membrane. The kinase activity showed some similarities to the characteristics of the Ca^{2+}-CaM and Ca^{2+} activated protein kinase isolated from *Acer pseudoplatanus* cells (Teulieres et al., 1985) and corn (*Zea mays*) roots (Ladror and Zielinski, 1989) respectively. However, overall these studies have given conflicting results.

The activity in the tonoplast fraction isolated from *Kalanchoë daigremontiana* was stimulated optimally by approximately 1.0 μM Ca^{2+}. Preliminary experiments using Method 1 to isolate the tonoplast demonstrated the presence of endogenous CaM, which might also be involved in the Ca^{2+} stimulation. Further analysis, however, for the presence of CaM in the tonoplast fraction isolated by Method 2 is now necessary to assess the significance of CaM in the activation of the protein kinase. It is also interesting to note that, in sugar beet taproot cells, the presence of CaM at the tonoplast may play a role in regulation of the Ca^{2+}/nH^{+} antiporter *in vivo* (Andreev at al., 1990). These cells are known to contain proteins that are very similar to CaM in their properties. Therefore the presence of CaM in the tonoplast fraction from *Kalanchoë daigremontiana* may have several functions to play in regulation of cell metabolism.

Incorporation of P_i in the absence of Ca^{2+} is rapid and maximal rate of incorporation takes place after approximately 30-60 seconds, saturates at about 2 min and then appears linear for up to 5 min reaching absolute values

of approximately 90 pmol P_i incorporated mg^{-1} tonoplast protein after a 5 min reaction time. This is a lower activity than results of a previous study of Ladror and Zielinski (1989) who recorded absolute activities of approximately 1.2 nmol P_i. This significantly higher value may be attributable to an increased assay temperature of 30 °C as compared with 20 °C for the experiments with tonoplast vesicles isolated from *Kalanchoë daigremontiana*. On the other hand, Teulieres et al. showed significantly reduced protein kinase activity reaching only to a maximum of 15 pmol P_i incorporated mg^{-1} tonoplast protein in the absence of Ca^{2+}. In the presence of Ca^{2+}, however, protein kinase activities appeared to reach approximately 85 pmol P_i mg^{-1} tonoplast protein after 2 min. It should be noted, however, that absolute values varied depending on the time of year that the experiment was performed. During the winter (January to March), the plants appeared to go through a phase where it became difficult to isolate a good yield of tonoplast vesicles. The protein kinase activity during this period was significantly reduced. The reason for this may be due to the plant's seasonal rhythm, but this has not been proven experimentally.

Comparing initial rates of ^{32}P incorporation it was seen that within the first 2 min. of reaction, rates were not as fast in both *Zea mays* (Ladror and Zielinski, 1989) and *Acer pseudoplatanus* cells (Teulieres et al., 1985) than in tonoplast isolated from *Kalanchoë daigremontiana*. With the addition of approximately 1.0 µM Ca^{2+}, the incorporation of P_i into the tonoplast polypeptides after 30 sec. increased by approximately 40 % in *Kalanchoë daigremontiana* (see Fig. 8 and 10). Ladror and Zielinski (1989) demonstrated a similar Ca^{2+} stimulation of protein kinase activity of up to 30 % in corn root tonoplast (results based on a 4 min. reaction time).

The major phosphorylated polypeptides labelled in the absence of Ca^{2+} had M_r values of approximately 112 000, 105 000, 91 000, 60 000 and 55/56 000 ; and in the presence of 1 μM Ca^{2+}, incorporation of P_i was increased in the polypeptide M_r 55 000 and two further polypeptides became phosphorylated of M_r 37 000 and 10/11 000 (Fig. 11). Teulieres et al. (1985) showed that Ca^{2+} alone had a poor stimulatory effect on their protein kinase. However, the addition of CaM led to the rapid phosphorylation that takes place maximally after 2 minutes. In their studies, few polypeptides were significantly labelled by Ca^{2+} alone, but with the addition of CaM eight polypeptides in total were phosphorylated, with the most significant at M_r 30 000 and 40 000. This is notable given the labelled band at a similar molecular weight of 37 000 in *Kalanchoë daigremontiana,* to the latter of M_r, 40 000. Ladror and Zielinski (1989) found major phosphorylated bands in the tonoplast fraction from corn (*Zea mays*) roots with M_r of 102 000, 100 000, 98 000, 64 500, 58 000 and 50 000 in the absence of Ca^{2+}, and a further 3 polypeptides in the presence of excess Ca^{2+} of M_r 87 000, 35 000 and 30 000. Their results indicated that CaM-dependent protein kinase was not detectable, nor was Ca^{2+}-stimulation of the protein phosphorylation dependent on bound CaM. Their results suggested that the major ATPase subunits are not phosphorylated by an intrinsic protein kinase. It is interesting to note that the phosphorylated polypeptide in the tonoplast isolated from *Kalanchoë daigremontiana* of M_r 55 000 could be a subunit of the ATPase. However, this has still to be proven experimentally.

It is significant, however, that Ladror and Zielinski (1989) found the protein kinase activity and pattern of phosphorylated polypeptides (on SDS-PAGE) in both their tonoplast fraction and plasmalemma fraction were similar. They even suggested that the tonoplast protein kinase may only be a plasmalemma contaminant as a higher rate of activity was observed in the plasmalemma fraction than the tonoplast fraction. Garbarino and DuPont (1989) have also reported similarities in the pattern of phosphorylated polypeptides in both the tonoplast and plasmalemma fractions isolated from roots of intact barley (*Hordeum vulgare cv* CM72). The polypeptide pattern is significantly enriched in the plasmalemma fraction, suggesting that the tonoplast protein kinase activity may originate from plasmalemma contamination. None-the-less, there is also the possibility in both these sets of results that a soluble protein kinase is present which regulates polypeptide function in the tonoplast. Therefore, the physiological significance of the intrinsic protein kinase activity in the tonoplast membrane is as yet unclear.

The polypeptide of M_r 105 000 is of particular significance as it may be a subunit of the important CAM enzyme, PEPCase. This enzyme is known to be regulated by phosphorylation/dephosphorylation performed by a cytosolic protein kinase and an equivalent phosphatase. It is interesting to note therefore that the protein kinase activity present in the tonoplast fraction would phosphorylate PEPCase.

This may suggest that the protein kinase activity associated with the tonoplast fraction has a wide range of specificities. On the other hand, more than one protein kinase may be present that possess high specificities for different polypeptides present in the fraction including the protein kinase involved in the regulation of PEPCase. It would be interesting to perform protein kinase experiments with the addition of exogenous protein kinase specific for

PEPCase which has been partially purified by Nimmo et al., University of Glasgow (personal communication).

Comparing, then, the characteristics of the protein kinase activities described above, the activity present in the tonoplast isolated from the mesophyll cells of *Kalanchoë daigremontiana* appears to show more similarity to that described by Ladror and Zielinski (1989). The polypeptides phosphorylated in the absence of Ca^{2+} are close to the values found in corn roots. However in the presence of Ca^{2+}, only one of the phosphorylated polypeptides is comparable (M_r 35 000 compared to M_r approx. 37 000, (*Zea mays*)). The phosphorylated polypeptide of M_r approx. 40 000 (Teulieres et al., 1986) may also correspond to that of 35/37 000 as one can expect up to a 10 % error when employing SDS-PAGE.

Ladror and Zielinski's suggestion, however, that the protein kinase in their tonoplast fraction is a plasmalemma contamination has important implications for the protein kinase described for *Kalanchoë daigremontiana*. The indication that the subunit, M_r 55 000 of the tonoplast H^+-ATPase undergoes phosphorylation only in the tonoplast isolated from *Kalanchoë daigremontiana*, however, suggests a possible role in regulation of metabolism at the tonoplast *via* a protein kinase. Also, the rigorous isolation procedure to obtain a pure tonoplast fraction (Method 2) suggests that the protein kinase activity is a genuine component present associated with the tonoplast in *Kalanchoë daigremontiana*. Further studies to establish more of the characteristics of the protein kinase associated with the tonoplast are now necessary to establish a fuller understanding of its action at the membrane.

Experiments, also, to investigate the presence and effect of phosphatases in the reaction ought to be performed. Although okadaic acid appeared to have no effect on the phosphorylation reaction, this does not absolutely exclude the

presence of protein phosphatase activity associated with the tonoplast, because only a selection of phosphatases are sensitive to inhibition by this polyketol.

It would now be exciting to investigate the effect (if any) of phosphorylation/dephosphorylation on the transport of malate across the tonoplast. Patch-clamp studies have recently shown the presence of an ion channel in the tonoplast of *Kalanchoë daigremontiana* that is specific for malate (A.J. Pennington and J.A.C. Smith, personal communication). Also, in parallel, ^{14}C-malate studies are in progress to study transport across this membrane. These two techniques could eventually be employed to investigate effects of the addition of ^{32}P-ATP with or without the addition of exogenous protein kinase on the transport of malate.

It would be most benefecial to be able to identify and characterise the malate transporter / channel in the tonoplast of *Kalanchoë daigremontiana*. Selection of an affinity label to inhibit malate transport would be the first step. The affinity label eosin-5-maleimide has been used to identify the 2-oxoglutarate carrier in mitochondria (Zara and Palmieri, 1988). This dicarboxylate carrier mediates the facilitated exchange diffusion of malate and 2-oxoglutarate. Its substrate specificity shows remarkable similarity to that of the tonoplast malate transporter, i.e. recognising fumarate, malate, succinate and 2-oxoglutarate (cf. White and Smith, 1989). Therefore this seems a good label to initiate investigations to identify the malate transporter in the tonoplast of *Kalanchoë daigremontiana*.

It is hoped that such future research will further the quest for a greater biochemical understanding of this membrane system, the **tonoplast**.

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CALCIUM-DEPENDENT PHOSPHORYLATION OF TONOPLAST PROTEINS IN THE CAM PLANT *KALANCHOË DAIGREMONTIANA*

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INTRODUCTION

The possible physiological significance of protein-kinase-mediated phosphorylation of tonoplast proteins has as yet received relatively little attention. In animal cells, several membrane proteins associated with ion transport are known to be affected by phosphorylation (1). Thus far, there have been two conflicting reports concerning the presence of protein kinase activity in the tonoplast of plant cells. Teulieres *et al.* (2) detected the presence of Ca^{2+}-stimulated protein kinase activity in the tonoplast fraction from *Acer pseudoplatanus* suspension culture cells. However, Ladror and Zeilinski (3) suggested that the kinase activity detected in the tonoplast fraction from *Zea mays* roots was a contaminant from the plasmalemma. In the present work, we have been studying the phosphorylation of polypeptides in tonoplast preparations from the CAM plant *Kalanchoë daigremontiana* to assess its significance in the control of solute accumulation in vacuoles.

MATERIAL AND METHODS

A tonoplast fraction from leaf mesophyll tissue of *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie was isolated by a modification of the method of Bremberger *et al.* (4) as described by White and Smith (5). Polypeptides were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with an exponential running gel of 7.5 to 15 % (w/v) acrylamide.

RESULTS AND DISCUSSION

Analysis of the tonoplast fraction by SDS-PAGE showed that there are many polypeptides associated with the tonoplast in mesophyll cells of *Kalanchoë daigremontiana* (Fig. 1a). Six of these have been identified (3) with the tonoplast ATPase - those with relative molecular masses (M_r) of approximately 72 000, 56 000, 48 000, 42 000, 28 000 and 16 000. The tonoplast fraction also showed protein kinase activity, which was stimulated up to threefold by the presence of micromolar concentrations of Ca^{2+}. The precise dependence of protein kinase activity on Ca^{2+} concentration is currently under investigation.

Incubation of the tonoplast fraction with [γ-^{32}P]-ATP, followed by SDS-PAGE and autoradiography, demonstrated that there was a time-dependent, Ca^{2+}-stimulated incorporation of ^{32}P into several polypeptides, in particular those

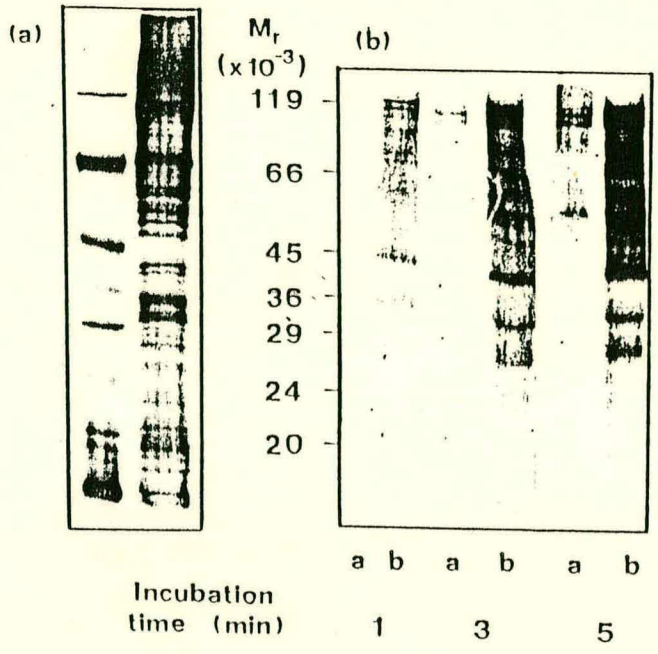

FIGURE 1. (a) SDS-PAGE analysis of polypeptides in the tonoplast fraction or *Kalanchoë daigremontiana*, with marker polypeptides of indicated M_r values in left-hand lane. (b) Autoradiograph of tonoplast polypeptides separated by SDS-PAGE after incubation for the times shown in the absence (*a*) and presence (*b*) of 100 μM Ca^{2+} in a medium containing 111 mM Hepes (pH 7.0), 22.2 mM $MgCl_2$, 0.89 mM EGTA, together with 9 μM ATP (specific activity 407 TBq mol^{-1}).

of approximate M_r 40 000, 32 000 and 28 000 (Fig. 1b). Addition of exogenous calmodulin had no effect on Ca^{2+}-dependent protein kinase activity. However, immunoblot analysis of the tonoplast fraction using anti-spinach calmodulin antibody revealed the presence of endogenous calmodulin, which may play a role in the protein-kinase-mediated phosphorylation of tonoplast proteins *in vivo*.

These results indicate that Ca^{2+}-calmodulin stimulated protein kinase activity may be important in the phosphorylation of tonoplast proteins and suggest that the role of phosphorylation in the control of solute transport accross this membrane should be further investigated.

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