UNIVERSITY OF SOUTHAMPTON

# PURIFICATION OF WHEAT LEAF PLASMA MEMBRANES AND CHARACTERIZATION OF PLASMA MEMBRANE ATPASE ACTIVITY AND PHYTCHROME BINDING

by

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To my parents

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ABSTRACT

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# PURIFICATION OF WHEAT LEAF PLASMA MEMBRANES AND CHARACTERIZATION OF PLASMA MEMBRANE ATPASE ACTIVITY AND PHYTOCHROME BINDING

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The aim of this project was to examine the mechanism of phytochrome action at the plasma membrane of wheat leaves. Both the proposed phytochrome-mediated regulation of the plasma membrane ATPase and the nature of the association of phytochrome with purified plasma membranes were investigated. In initial experiments, microsomal membrane preparations were shown to contain an ATPase that is highly active in the absence of a divalent cation and is more dependent on $Ca^{2+}$ ions than $Mg^{2+}$ ions, with a specific activity of 245 $\mu$mol $P_i$/mg protein/h in the presence of 2 mol $m^{-3}$ $CaCl_2$ at pH 6.0. Activity is high with all nucleotides tested, with ADP the preferred substrate, and is inhibited by vanadate and KCl. Preliminary proton transport experiments indicated that this is not a proton translocating enzyme.

Attempts to purify plasma membranes using a range of discontinuous sucrose density gradients were unsuccessful because of contamination by this non-specific ATPase activity. This problem was overcome by using an aqueous polymer two-phase system. The plasma membrane preparation isolated by this method was examined using the marker enzymes cytochrome c oxidase, glucan synthetase II, latent IDPase and NADH-cytochrome c reductase and was shown to be very pure. The properties of the plasma membrane ATPase were characterized and it was demonstrated, for the first time, that wheat contains a plasma membrane ATPase activity that is similar to that found in other species (Terry *et al* 1989, *J. Plant Physiol.* 134, 756-761). These properties include a dependence on $Mg^{2+}$ ions, stimulation by $K^+$ ions, sensitivity to vanadate and a high substrate specificity for ATP. The ATPase has a pH optimum of 6.0 and a $K_m$ for Mg:ATP of 0.54 mol $m^{-3}$ in the presence of $K^+$ ions with a specific activity of 98 $\mu$mol $P_i$/mg protein/h at pH 6.0. The characterization also included a study of the action of the plasma membrane $H^+$-ATPase inhibitors vanadate, erythrosin B, SW26 and $Ca^{2+}$ ions. The proposed light regulation of the plasma membrane ATPase was also investigated. These experiments showed that there were no consistent effects of light treatments given *in vivo* or *in vitro* on the properties of the ATPase assayed *in vitro*. This was also true when partially purified wheat phytochrome or purified oat phytochrome was added to the plasma membrane preparation prior to the *in vitro* light treatments.

An ELISA was used to accurately quantitate the amount of phytochrome that was bound to isolated plasma membranes. This demonstrated that a small proportion of the total cell phytochrome (~0.1%) is associated with the plasma membranes under conditions where a non-specific electrostatic association would not be expected. The bound phytochrome has an apparent molecular mass of 124 kDa (equal to that of the soluble pool) and appears to be bound predominantly by hydrophobic interactions. The amount of phytochrome bound (1-2 ng phytochrome /$\mu$g membrane protein) is not light regulated *in vivo*, a result that is in direct contrast to previous reports where heterogeneous membrane preparations were used. The membrane-bound and total type I phytochrome pools exhibit different kinetics for both destruction and synthesis, with plasma membrane-bound $P_{fr}$ stable for more than 1h in continuous R while the soluble pool is reduced by 50%.

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## ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine 5'–diphosphate |
| AMP | adenosine 5'–monophosphate |
| ATP | adenosine 5'–triphosphate |
| BSA | bovine serum albumin |
| BTP | bis–tris–propane |
| CTP | cytidine 5'–triphosphate |
| DCCD | N,N'–dicyclohexylcarbodiimide |
| DMSO | dimethyl sulphoxide |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol–bis($\beta$–aminoethyl ether)N,N,N',N'–tetraacetic acid |
| ELISA | enzyme–linked immunosorbent assay |
| FR | far–red light |
| GTP | guanosine 5'–triphosphate |
| Hepes | N–[2–hydroxyethyl]piperazine–N'–[2–ethanesulphonic acid] |
| HRP | horse radish peroxidase |
| IDP | inosine 5'–diphosphate |
| ITP | inosine 5'–triphosphate |
| kDa | kilodalton |
| Mes | 2–[N–morpholino]ethanesulphonic acid |
| NADH | nicotinamide adenine dinucleotide (reduced) |
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol 3350 |
| $P_{fr}$ | phytochrome (far–red absorbing form) |
| PMSF | phenylmethylsulphonyl fluoride |
| $PP_i$ | pyrophosphate |
| $P_r$ | phytochrome (red absorbing form) |
| PVP | polyvinylpyrrolidone |
| R | red light |
| RT | room temperature |
| SDS | sodium dodecyl sulphate |
| SED | standard error of the difference |
| SW26 | 2,2,2–trichloroethyl 3,4–dichlorocarbanilate |
| TBS | Tris buffered saline |
| TEMED | N,N,N',N'–tetramethylethylenediamine |
| Tris | tris(hydroxymethyl)aminomethane |

# CHAPTER ONE

# INTRODUCTION

The molecular mechanism by which the plant photoreceptor phytochrome acts is currently unknown. One possibility is that it initiates a biochemical signal by interacting directly with a membrane component. The purpose of this project is to examine the possible role of phytochrome at the plasma membrane. This chapter is intended to introduce topics relevant to this investigation such as methods used for membrane purification, the nature of the primary plasma membrane transporter, the $H^+$–ATPase, and the phytochrome molecule itself. More specific introductions covering previous results in a particular area of research are included, where neccessary, in the individual results chapters.

## 1.1. MEMBRANE PURIFICATION.

The importance of using purified, rather than mixed, membrane preparations has not always been fully recognized. However, it has become apparent in recent years that the use of purified membranes is critical for the investigation of complex physiological and biochemical phenomena. The first part of this chapter introduces the methods used in cell fractionation and membrane purification in plants, with particular emphasis on the techniques used to purify plasma membranes in this investigation.

### 1.1.1. Cell fractionation.

The development of membrane purification techniques for plant cells has, in the past, lagged behind that of animal systems. This is primarily due to the added complexity of plant cells which differ from animal cells in three important respects. They are surrounded by a cellulosic wall, contain plastids and also have a large fluid–filled vacuole. These three features all pose problems that hinder the effective isolation of homogeneous membrane preparations.

Homogenization conditions severe enough to break the cell wall can prove very destructive to the cell contents. This is a particular problem if intact organelles are required but, in general, harsh homogenization reduces the differences in size and structure of membranes and organelles, making separation more difficult. The cell walls themselves do not contaminate membrane preparations as they are removed during filtration and/or low speed centrifugation.

However, plasma membranes may be sedimented with cell walls and so reduce the yield of the subsequent purification procedures.

A further problem is caused by the rupturing of the tonoplast during homogenization which releases the contents of the vacuole. For this reason the homogenization medium is usually well buffered and contains a number of protectants. For example, polyvinylpyrrolidone (PVP) absorbs polyphenols and quinones, dithiothreitol (DTT) protects the sulphydryl groups of proteins, and chelating agents are used to remove heavy metals. Protease inhibitors, such as PMSF, may also be included or BSA can be added as a competing protease substrate.

Differential centrifugation has been widely used as a simple method of fractionating the membranes and cell components of this homogenate (Price 1983 for review). The method works on the principle that different particles have a different size and mass and will therefore respond differently to an applied centrifugal force. Larger particles (with high sedimentation coefficients) such as intact cells or organelles can be pelleted at low speeds ($\leq$6,000g) while smaller, less dense particles like membrane vesicles require greater force (100,000g is commonly used). The pellet resulting from this final centrifugation comprises all types of membrane vesicles, but not intact organelles or cell walls, and is termed the microsomal membrane fraction or microsomes. This microsomal fraction is used as the starting material for the purification of specific membranes by both density gradient centrifugation or phase partition techniques.

The proportion of each membrane type in microsomal fractions from plant cells is lower than in animal preparations making separation more difficult. In addition to the presence of the tonoplast, a membrane that is essentially equal in size to the plasma membrane, plant microsomal preparations also contain plastid membranes in an amount equal to or exceeding that of mitochondria (Morre *et al* 1987). Plasma membranes, for example, comprise only 13.1% of the total membrane within cells of etiolated soybean hypocotyls as determined by morphometry (Morre and Buckhout 1979).

### 1.1.2. Plasma membrane purification.

#### 1.1.2.1. Purification by density gradient centrifugation.

Plant plasma membranes have been predominantly purified either by using density gradients (Hall 1983) or by aqueous polymer two-phase partition (Larsson 1985). Density gradient centrifugation is the older of the two methods and was first used for plasma membrane purification by Hodges *et al* (1972). The

technique relies on membranes from different cellular origins having different densities. These membranes will then band at the appropriate position when they are centrifuged through a density gradient, usually of sucrose.

There are two types of density gradients, continuous and discontinuous. Discontinuous gradients have the advantage of being easy to prepare and of giving a reproducible source of membranes with substantial yield and purity. In addition, discontinuous gradients need not be centrifuged to equilibrium to obtain a good separation. Continuous gradients are perhaps more suited to analytical than preparative separations.

One of the major problems of plasma membrane purification using density gradients is contamination by mitochondrial membranes as they have a density of 1.18–1.22 g/cm$^3$, only slightly higher than that of plasma membrane vesicles (1.14–1.17 g/cm$^3$). This is also true of broken plastids (Quail 1979). In addition, there are problems associated with using sucrose. The high concentrations of sucrose used can cause shrinkage of the vesicles and it has also been reported that sucrose stimulates $\beta$–glucan synthetase activity (Ephritikhine *et al* 1980) and may displace the surface labelling agent concanavalin A from the plasma membrane (Boss and Ruesink 1979). Some alternatives have been tried, for example, glycerol (Scherer 1984), Ficoll (Ray 1979) and Renografin (Boss and Ruesink 1979), but these have not proved to be superior.

### 1.1.2.2. Purification by aqueous polymer two–phase partition.

Rather than separating particles by their size and density this technique relies on differences in surface properties. These include surface charge or the presence of special groups such as covalently bound carbohydrate moities. Phase partition was first used to purify plasma membranes from plant cells in 1981 (Widell and Larsson 1981, Lundborg *et al* 1981) but had previously been used for the isolation of a variety of particles, for example, mitochondria, chloroplasts, inside–out and right–side–out vesicles from chloroplast thylakoids and right–side–out plasma membrane vesicles from animal cells (Albertsson 1971, Albertsson *et al* 1982 and Larsson 1985 for reviews).

The phase system is comprised of the polymers polyethylene glycol 3350 (PEG) and Dextran T500 in aqueous solution, which are mixed to give a PEG–rich upper phase and a Dextran–rich lower phase. Separation of particles is achieved because different particles have different affinities for the two phases. As stated above, this affinity is dependant on surface properties. Plasma membranes (and protoplasts) partition into the upper, PEG–rich phase while intracellular membranes remain in the lower phase (Widell 1987). This separation is not an

absolute one; some plasma membranes will partition into the lower phase and similarly some intracellular membranes will be found in the upper phase. It is worth pointing out that particles actually separate to three locations in the phase system but for most purposes the interface between the phases is treated as part of the lower phase.

The effectiveness of this technique can be improved in two ways. Firstly, the partitioning can be repeated a number of times. With each partition the purity of the plasma membranes increase as more intracellular membranes are removed, but the yield falls as plasma membranes are also lost at each stage. Yields can be improved by re-extracting plasma membranes from the lower phases by using fresh upper phase. In most cases a batch procedure is used in which the upper phase is re-partitioned twice; a scheme for this procedure is shown in Chapter 2, Figure 2.1.

Separation can also be improved by changing the composition of the phase system. The most important factor is the concentration of the polymers used. The higher the polymer concentration the more material is excluded from the upper phase; intracellular membranes are preferentially excluded (Larsson 1985, Hodges and Mills 1986) but plasma membranes are also lost. For plant plasma membranes the effective polymer concentration range is 6.2-6.5% (w/w) and in some tissues selection of the correct polymer concentration may be crucial. An added complication is that commercially produced Dextran T500 contains some water. The amount varies between batches and needs to be determined by measuring the optical rotation of a Dextran solution.

The concentrations of ions in the phase system are also important. As different ions also have a different affinity for the two phases a potential difference between the two phases is created. This will affect the distribution of particles because of the surface charge they carry. A phosphate buffer is usually included in the phase system. $HPO_4^{2-}$ ions have a stronger affinity for the lower phase causing the upper phase to become 'positive'. This is generally true of all di- and trivalent anions. Monovalent anions, however, have greater affinity for the upper phase. With the addition of KCl, for example, $Cl^-$ ions can titrate the positive charge in the upper phase. As the concentration of KCl is increased fewer particles partition into the upper phase. Again intracellular membranes appear to be preferentially excluded, and the addition of salts to the phase system can be considered to be the 'fine tuning' of this separation technique. As for the number of partitions, the composition of the phase system is a balance between yield and purity and may require considerable time to perfect.

### 1.1.2.3. A comparison of plasma membrane purification techniques.

There are a number of advantages of using phase partition over using sucrose density gradients. The membranes can be kept in conditions more suited to biological material throughout the phase partition procedure and the technique is also more easily scaled up for bulk preparative procedures. More importantly, perhaps, plasma membranes prepared by phase partition are considered to be more homogeneous. Larsson (1985) claimed that plasma membrane preparations from a wide variety of tissues and species were probably greater than 90% pure and possibly near to 100%. In a direct comparison of the two purification methods using oat roots, Hodges and Mills (1986) concluded that, by most criteria tested, phase partition preparations were more pure. A similar conclusion was reached by Berczi and Moller (1986) in a limited study using wheat roots.

A major difference between the two techniques is in the properties of the isolated plasma membrane vesicles. Vesicles prepared by phase partition are predominantly right–side–out as determined by the latency of $K^+$,$Mg^{2+}$–ATPase activity (Larsson *et al* 1984). This evidence is supported by the observation that protoplasts also have a high affinity for the upper phase (Hallberg and Larsson 1981). In contrast, plasma membrane vesicles from density gradients appear to be more equally orientated (Randall and Ruesink 1983, Berczi and Moller 1986). Right–side–out vesicles are unsuitable for studies on ATP–driven ion transport and, in this respect, plasma membrane vesicles purified by density gradient centrifugation are preferable. Recently, however, two new methods of obtaining inside–out vesicles from phase partitioned plasma membrane fractions have been reported. The first is to subject these vesicles to a freeze/thaw cycle before separating the inside–out vesicles from the right–side–out ones by further phase partition steps (Larsson *et al* 1988). The second is to use free–flow electrophoresis (Canut *et al* 1988). Interestingly, in some species there are sufficient inside–out plasma membrane vesicles in the upper phase for transport experiments. For example, Graf and Weiler (1989) used these for investigating ATP–driven $Ca^{2+}$ transport in *Commelina communis*.

Another advantage of using density gradients is that vesicles from more than one cellular origin can be purified in a single separation. This is also an advantage of preparative free–flow electrophoresis which has been used successfully to purify plasma membranes and tonoplast from soybean hypocotyls (Sandelius *et al* 1986b).

### 1.1.3. Determination of plasma membrane purity.

Enzymes predominantly situated on one type of membrane can be used as markers for that membrane. Their presence can often be predicted by the function of that membrane and evidence for their location is obtained initially by correlation with characteristic morphological membrane features (*e.g.* membrane thickness or organelle shape) or with other previously characterized biochemical markers.

The morphological marker most commonly used for the identification of plasma membranes is staining by phosphotungstic acid–chromic acid (PTA). This enables plasma membranes and tonoplast to be resolved. They are both characteristically thick membranes but, at low pH, PTA is thought to specifically stain plasma membranes but not tonoplast (Roland 1978). However, the absolute validity of using the PTA stain as a plasma membrane marker is still in question as plasma membrane staining can be inconsistent and other structures may also be stained (Hall 1983).

#### 1.1.3.1. Biochemical markers for the plasma membrane.

The two most commonly used enzymic markers for the plasma membrane are the vanadate–sensitive $K^+$,$Mg^{2+}$–ATPase activity (the plasma membrane $H^+$–ATPase, see later) and glucan synthetase II which is involved in cell wall synthesis. Unfortunately neither of these markers are absolute for the plasma membrane and this has made assessment of plasma membrane purity difficult.

The vanadate–sensitive, $K^+$,$Mg^{2+}$–ATPase has the advantage of being easy to assay and has, in recent years, been unequivocally assigned to a plasma membrane location (Sze 1985, Serrano 1989). However, this is not to say that this enzyme is the ideal plasma membrane marker. There is evidence for a $K^+$–stimulated $Mg^{2+}$–ATPase at other locations, for example, the KCl–stimulated $Mg^{2+}$–ATPase found on Golgi membranes (Chanson *et al* 1984) while non–specific phosphatase activity which is also inhibited by vanadate (Gallagher and Leonard 1982) can cause problems. A further problem is that in some tissues, such as sugar beet (Briskin and Thornley 1985), the $K^+$–stimulated component is absent although in such cases vanadate sensitivity can be used alone.

Glucan synthetase II, assayed in the absence of $Mg^{2+}$ ions and with high concentrations of the substrate UDP–glucose, has been used as a marker for the plasma membrane (Ray 1979, Hall 1983). As with $K^+$,$Mg^{2+}$–ATPase activity there are problems assigning all glucan synthetase activity to the plasma

membrane. There is a similar enzyme, glucan synthetase I, which is thought to be located at the Golgi. This Golgi enzyme is normally assayed in the presence of 10 mol $m^{-3}$ $Mg^{2+}$ and low substrate concentrations but there is a possibility of some cross-reaction under the different assay conditions. It is also possible that either synthetase may be located on both membranes, further complicating matters.

There are a number of other putative plasma membrane markers available (Hall 1983 for review). These include naphthylphthalamic acid binding, surface labelling with a variety of substances such as concanavalin A or lanthanum and the determination of sterol content. The most important consideration, however, for determining plasma membrane purity is that a single marker is not used alone, but in conjunction with other plasma membrane markers and markers for potentially contaminating membranes.

### 1.1.3.2. Biochemical markers for intracellular membranes.

There are a wide variety of markers available for all the major intracellular membranes (Hall and Moore 1983, Morre *et al* 1987 for reviews) but only those used in this study will be briefly mentioned. Cytochrome c oxidase is an absolute marker for the inner mitochondrial membrane and has the advantage of being easy to assay. In addition, azide or oligomycin both inhibit mitochondrial ATPase activity and this can also be a useful indicator of mitochondrial contamination. NADH-cytochrome c reductase is used as a marker for the endoplasmic reticulum and is usually assayed in the presence of antimycin A which inhibits mitochondrial reductase activity. The Golgi marker most commonly used is latent IDPase activity although in some species (*e.g.* wheat or barley), where the ATPase activity that predominates has poor substrate specificity, this can lead to an overestimate of Golgi contamination. Similarly, when using $NO_3^-$-sensitive ATPase activity as a tonoplast marker, high levels of phosphatase activity may lead to an underestimate of the amount of tonoplast present.

## 1.2. $H^+$-TRANSPORTING ATPASES OF HIGHER PLANTS.

The primary active transport process in higher plants is the electrogenic transport of protons out of the cell across the plasma membrane and from the cytoplasm to the vacuole. This is achieved by two $H^+$-transporting ATPases ($H^+$-ATPase) on the plasma membrane and tonoplast respectively. In total there are three principle membrane-bound transporting ATPases, the other being the mitochondrial (and chloroplast) $H^+$-ATPase. The mitochondrial enzyme will be dealt with only very briefly in this introduction which will concentrate on the

properties of the plasma membrane $H^+$–ATPase. It is, perhaps, worth mentioning at this juncture that there are a number of excellent, recent reviews covering both the plasma membrane and tonoplast $H^+$–ATPases (Marre and Ballarin–Denti 1985, Sze 1985, Bowman and Bowman 1986, Poole 1988) and the plasma membrane $H^+$–ATPase alone (Serrano 1985, 1988, 1989, Sussman and Surowy 1987) and the reader is referred to these for a more detailed discussion.

### 1.2.1. The plasma membrane $H^+$–ATPase.

The plasma membrane $H^+$–ATPases of plants and fungi have been shown to be biochemically very similar (Serrano 1984). Together they form a novel group of the $E_1/E_2$ type ion–pumping ATPases and have been assigned the code number EC 3.6.1.35 by the Enzyme Nomenclature Committee of the International Union of Biochemistry (1984). The biochemical characterization of the fungal $H^+$–ATPases is in advance of that in higher plants. In particular, the yeast *Saccharomyces cerevisiae* has provided a model system for the study of this enzyme as the molecular genetics of this system are well developed (Serrano 1988). Consequently, information derived from investigations with these systems is important in any discussion of the structure and function of the plasma membrane $H^+$–ATPase of higher plants.

#### 1.2.1.1. Physiological role.

The plasma membrane $H^+$–ATPase is the primary active transporter in higher plants and it has been estimated that this single enzyme consumes 25–50% of the total cytoplasmic ATP (Sussman and Surowy 1987). It generates an electrochemical gradient across the plasma membrane and this is a source of potential energy for the cell which can be used as the driving force for the transport of solutes (Poole 1978) as predicted by the chemiosmotic theory (Mitchell 1976). This is termed secondary active transport and solutes transported by this mechanism across the plasma membrane include ions, amino acids, sugars and growth regulators. The utilization of the energy of the electrochemical gradient is accomplished *via* three types of transporters: antiports by which solutes are moved from the inside to the outside of the cell in exchange for protons; symports which cotransport solutes with protons moving down the electrochemical gradient; and uniports by which charged molecules can move down the membrane potential component of the electrochemical gradient.

This movement of nutrients at both the cellular and organ level is vital for plant growth. Cell turgor is also dependent on the electrochemical gradient as

it is mostly determined by the concentration of intracellular $K^+$ ions and other solutes. The maintenance of cell turgor is important for growth and in addition is thought to be responsible for stomata and pulvini movements (Serrano 1985). A third growth related function of the plasma membrane $H^+$–ATPase is that proposed by Rayle and Cleland (1970) who suggested that the acidification of the wall space stimulates cell wall loosening thereby permitting cell elongation. This is termed the acid growth theory. Other physiological roles for the plasma membrane $H^+$–ATPase are the short term regulation of intracellular pH (Felle 1988) and the development of polarity in growing cells and organs such as roots, root hairs and pollen tubes (Jaffe 1981).

These proposed physiological roles are based on *in vitro* experiments or correlations between physiological responses and proton transport and in most cases it has yet to be shown unequivocally that it is a cause–effect relationship. A genetic approach to the problem is required where specific mutations of the $H^+$–ATPase gene should demonstrate conclusively the role of this enzyme in the plant. Mutants of the proton pump in yeast have already been used to show that the plasma membrane $H^+$–ATPase is essential and rate–limiting for growth and that decreased proton pumping activity can be correlated to a decrease in amino acid transport (Serrano 1989). Three $H^+$–ATPase genes from *Arabidopsis thaliana* have recently been identified of which two have so far been sequenced (Harper *et al* 1989, Pardo and Serrano 1989) and it may not be long before a similar approach is possible in higher plants (Sussman and Harper 1989).

### 1.2.1.2. Techniques used in the study of the plasma membrane $H^+$–ATPase.

Early *in vivo* experiments on salt uptake in plants showed that cation uptake was linked to an efflux of protons and could be stimulated by high external pH (Hodges 1973). It was proposed by Pitman (1970) that this observed efflux was due to the outward transport of protons and not to increased respiration or sugar breakdown. Poole (1974) subsequently demonstrated that this was not a movement of $HCO_3^-$ ions. Further evidence for a proton pump at the plasma membrane were provided by experiments correlating an inducible proton efflux with a hyperpolarization across the plasma membrane. Both responses could be abolished by the addition of a protonophore. These experiments have been reviewed by Marre (1979) and Marre and Ballarin–Denti (1985). The energy source for the proton efflux was proposed to be ATP as inhibitors of respiration, such as azide or cyanide which reduce intracellular ATP levels, also inhibit electrogenic proton extrusion (Higinbotham and Anderson 1974). This conclusion is supported by the observation that proton efflux can be inhibited without altering

ATP levels (Cocucci *et al* 1980).

A second approach to the problem has been to look at ATPase activity *in vitro* by measuring the release of inorganic phosphate ($P_i$). Initial experiments only succeeded in demonstrating the ubiquity of membrane-associated ATPases in higher plants (Hodges 1976) and the first demonstration that plasma membranes contained a cation-stimulated ATPase activity was by Hodges *et al* (1972). Subsequent attempts to determine which ion(s) was being transported were inconclusive (Hodges 1976, Poole 1978, Spanswick 1981). However, Tazawa and Shimmen (1982) were able to show, using perfused cells of *Chara*, ATP-dependent electrogenic proton pumping at the plasma membrane. Unfortunately this technique was not applicable to most plant tissues.

Since 1980 the use of membrane vesicles as a model system has allowed major advances to be made in the study of transport processes in higher plants (Sze 1985). There are a number of advantages of using vesicles over intact cells. The vesicles do not contain any cytoplasm and metabolic activities are restricted to those on the plasma membrane. There is also no significant transport activity in the absence of added substrate. Chemical or electrical gradients can be imposed on the system and the importance of these and added chemical or physical parameters can be examined directly. Two further points are that vesicles from a particular subcellular location can be used, as can vesicles of different orientation.

The transport of protons is studied in isolated vesicles using radioactive weak acids or weak bases or, more commonly, fluorescent probes. One such probe is quinacrine, an amine which is lipid permeant in the neutral form but is trapped inside the vesicles by protonation. As protons are pumped into the vesicles by the $H^+$-ATPase, quinacrine accumulates and the fluorescent intensity becomes quenched. This is probably because at higher concentrations energy transfer between neighbouring quinacrine molecules reduces the quantum yield (Lee *et al* 1982). Quinacrine has been successfully used to investigate proton transport in both tonoplast and plasma membrane vesicles of higher plants (DuPont *et al* 1982, Bennett and Spanswick 1983, Giannini and Briskin 1987). Plasma membrane proton transport was shown to be vanadate sensitive and this result, in conjunction with the demonstration that the generation of a $Mg^{2+}$,ATP-dependent membrane potential was also vanadate sensitive, showed conclusively that the higher plant plasma membrane ATPase was an electrogenic proton pump (Sze 1985).

The use of purified membrane vesicles has enabled the plasma membrane $H^+$-ATPase of many species and tissues to be characterized both by conventional ATPase assays and proton transport experiments. These experiments have now been taken a stage further with the reconstitution of the $H^+$-ATPase

into phospholipid vesicles after detergent solubilization (O'Neill and Spanswick 1984a, Serrano 1984) and by partial purification (Vara and Serrano 1982, Serrano 1984). These advances have finally allowed many of the questions concerning the structure and mechanism of action of this enzyme to be answered.

### 1.2.1.3. Structure.

The minimal catalytic unit of the plasma membrane $H^+$–ATPase is a 100 kDa polypeptide. This has been shown by the enrichment of a 100 kDa band on SDS gels (Vara and Serrano 1982, Anthon and Spanswick 1986) and by the incorporation of $^{32}P$ after incubation with [$\gamma$–$^{32}P$] labelled ATP (Briskin and Poole 1983, Scalla *et al* 1983, Vara and Serrano 1983). It is thought that the enzyme exists *in vivo* as a dimer (Briskin *et al* 1985, Briskin and Reynolds–Niesman 1989) but there is some disagreement. Anthon and Spanswick (1986) proposed a trimeric structure for the tomato root $H^+$–ATPase and a dimeric (Bowman *et al* 1985) and a hexameric (Chadwick *et al* 1987) arrangement has been suggested in the fungi *Neurospora crassa*.

Currently, it is not thought that any additional polypeptide subunits are associated with the enzyme. In *Neurospora crassa* it has been clearly demonstrated that no other polypeptide co–purifies during reconstitution (Scarborough and Addison 1984) and that this monomer can catalyze proton transport (Goormaghtigh *et al* 1986). However, the $Ca^{2+}$–ATPase of the cardioplasmic reticulum which does have regulatory polypeptides (Katz 1981) can also function efficiently as a reconstituted monomer (Andersen *et al* 1982) and the possibility that there are additional subunits, particularly in higher plants, cannot be ruled out.

Figure 1.1 shows the proposed transmembrane structure and the functional domains of a monomer of an $E_1/E_2$ type ATPase. The figure is taken from Serrano (1989) and is based on the amino acid sequences of ten related enzymes from higher plants, mammals, fungi and bacteria (see 1.2.1.5.). There are six regions of conserved sequence (although III and IV are only present in eucaryotes) and these have been assigned functions as shown in the model. Reasons for these assignments are discussed fully by Serrano (1989).

Although only a single type of $H^+$–ATPase has so far been identified there appears to be at least three genes encoding plasma membrane $H^+$–ATPases in *Arabidopsis thaliana* (Harper *et al* 1989, Pardo and Serrano 1989). This is in contrast to yeast where only one gene has been identified (Serrano 1988) but is consistent with other higher eukaryotes. For example, the mammalian $Na^+$,$K^+$–ATPase has at least four separate genes (Shull and Lingrel 1987).

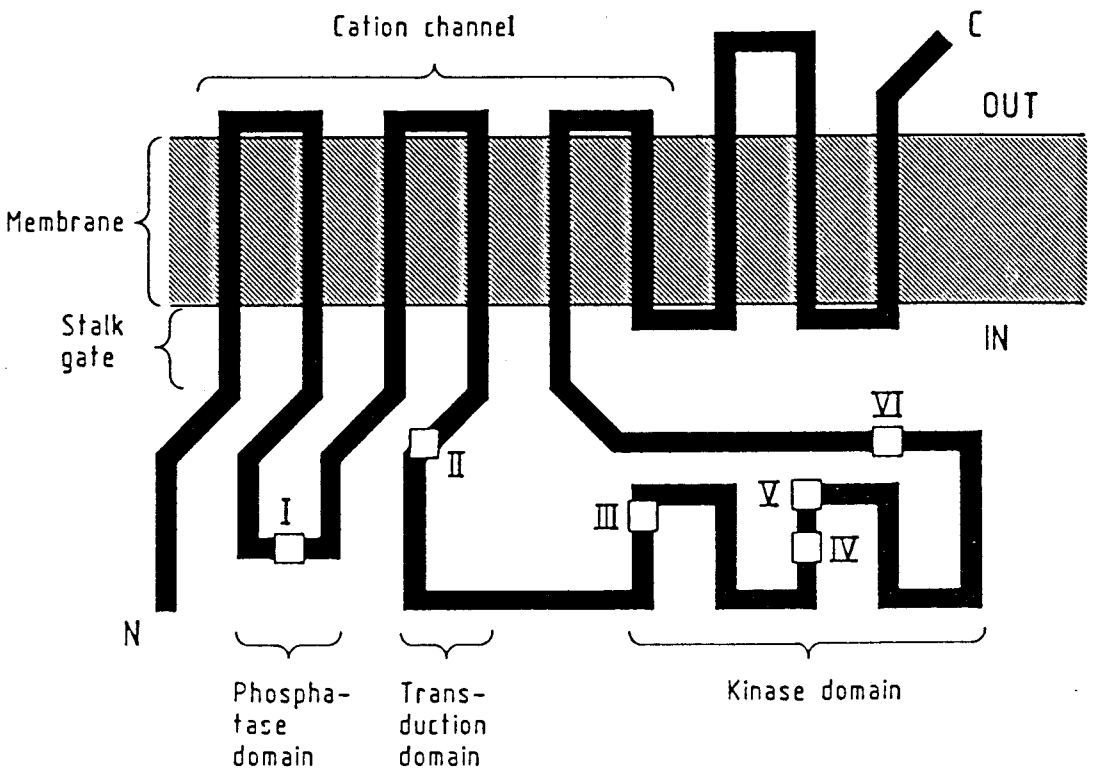


Figure 1.1: Proposed transmembrane structure and functional domains of $H^+$-ATPases. Motifs I to VI are conserved for $E_1/E_2$ type ATPases. From Serrano (1989).

### 1.2.1.4. Mechanism of Action.

The enzyme is thought to exist in two forms, $E_1$ and $E_2$ (Serrano 1989). $E_1$ is open to the cytoplasmic side of the membrane and has a high affinity for protons and $E_2$ is orientated to the outside of the cell and has a low affinity for protons. $E_1$ is able to bind ATP and form a phosphorylated intermediate (Briskin 1986) as in equations 1 and 2.

$$E_1 + ATP \longrightarrow E_1.ATP \quad (1)$$

$$E_1.ATP \longrightarrow E_1\text{-}P.ADP \quad (2)$$

$$E_1\text{-}P.ADP \longrightarrow E_2\text{-}P + ADP \quad (3)$$

$$E_2\text{-}P + H_2O \longrightarrow E_2 + P_i \quad (4)$$

The formation of the phosphorylated intermediate may be induced by the binding of the proton to be transported (Serrano 1989), *i.e.* the phosphorylation site (motif II, Figure 1.1) is brought nearer to the kinase domain. The formation of the phosphorylated intermediate itself would induce another conformational change resulting in the enzyme adopting the $E_2$ configuration and the release of ADP (equation 3). The enzyme is now able to release the bound proton. The release of the proton may bring the phosphorylation site into close proximity with the phosphatase domain and the phosphate is cleaved (equation 4). The exact nature of these proposed conformational changes is completely unknown but is currently an area of great interest for biochemists in this field.

The plasma membrane $H^+$–ATPase is dependent on $Mg^{2+}$ ions because the complex $MgATP^{2-}$ is the true substrate (Balke and Hodges 1975, Koland and Hammes 1986) and is therefore required for the formation of the phosphorylated intermediate. The stimulatory role of $K^+$ ions is more contentious. In ATPases such as the $Na^+,K^+$–ATPase of animal cells the stimulation of activity by $K^+$ reflects the direct transport of this ion (Cantley 1981). The similarity between the kinetics of $K^+$ uptake and the stimulation of $H^+$–ATPase activity suggest that this may also be the case in higher plants (Leonard 1984). However, the uptake of $K^+$ can be uncoupled from proton transport (Mengel and Schubert 1985, Kochian *et al* 1989) and $K^+$ transport may be *via* a separate carrier *i.e.* a uniport or a $K^+$–ATPase. $K^+$ carriers have recently been identified at the plasma membrane (Takeda *et al* 1985, Ketchum *et al* 1989).

Interestingly, Briskin (1986) has shown that $K^+$ ions can stimulate

ATPase activity by increasing the rate of breakdown of the phosphorylated intermediate. This finding is analagous to that observed for the $Na^+,K^+$–ATPase of animal cells which does transport $K^+$ ions directly (Cantley 1981). The question of whether the $H^+$–ATPase transports $K^+$ ions directly is still very much an open one and further evidence for and against the role of $K^+$ ions is discussed by Briskin (1986), who appears to be generally in favour of a direct cotransport mechanism, and Serrano (1988), who is not.

The number of protons transported for each ATP used is also unknown. In *Neurospora crassa* the stoichiometry has been shown by current voltage analysis to be 1:1 (Slayman 1987) and the similarity with results from root hair cells of *Sinapsis alba* (Felle 1982) suggests that the stoichiometry in higher plants may be the same (Sussman and Surowy 1987).

### 1.2.1.5. Evolution.

The plant and fungal $H^+$–ATPases belong to a class of cation–transporting ATPases, the $E_1/E_2$ type ATPases. These enzymes have similar reaction mechanisms and inhibitor sensitivities and also a similar 100 kDa polypeptide with stretches of sequence homology (see 1.2.1.3.). It is thought that the $E_1/E_2$ type ATPases evolved as a result of the inability of the $F_0F_1$ ATPases (*e.g.* the mitochondrial ATP synthase) or the respiratory and photosynthetic proton pumps (*e.g.* cytochrome c oxidase) to develop as active transporters of cations other than protons (Serrano 1988).

There are four other known members of this class of enzymes, the $Na^+,K^+$–ATPase on the plasma membrane of animal cells, the $Ca^{2+}$–ATPase from sarcoplasmic reticulum of muscle cells, the $H^+,K^+$–ATPase from stomach gastric mucosa and the bacterial plasma membrane $K^+$–ATPase. The plant $H^+$–ATPase probably represents a late development in this class of ATPases and the ancestral pump may be a $H^+,K^+$–ATPase (Nelson 1988, Serrano 1989). An interesting question that this raises is whether the small activation by $K^+$ ions represents binding to an ancestral site that is no longer required for activity.

## 1.2.2. The tonoplast $H^+$–ATPase.

The tonoplast $H^+$–ATPase represents a third distinct class of proton transporters (Rea and Sanders 1987, Schneider 1987), which include $H^+$–ATPases present in lysosomes, plant and fungal vacuoles, Golgi membranes, clatharin coated vesicles, synaptic vesicles and some secretory granules (Nelson 1988). The subunit structure of this class of ATPase varies greatly, but in plants, the tonoplast

$H^+$–ATPase is comprised of three polypeptides of about 70, 60 and 16 kDa. The functional molecule probably has one or more of these subunits in more than one copy (Rea and Sanders 1987). The 16 kDa subunit is thought to function as the proton channel because of its reactivity with DCCD (Sussman and Surowy 1987) and the 70 kDa subunit is probably catalytic (Bowman *et al* 1986, Griffith *et al* 1986). The role of 60 kDa subunit is not so clear. There is some evidence for a regulatory nucleotide binding site (Mandala and Taiz 1986) but it may also participate in catalysis (Rea and Sanders 1987).

The tonoplast $H^+$–ATPase is characterized by sensitivity to $NO_3^-$ ions and activation by $Cl^-$ ions. It is not inhibited by vanadate and has a higher pH optimum than the plasma membrane $H^+$–ATPase making it comparatively easy to distinguish it from this enzyme in mixed vesicle populations (Sze 1985). The physiological role of the tonoplast $H^+$–ATPase is essentially to drive the active transport of solutes across the tonoplast. However, there is also a $H^+$–PPase located at this membrane (Rea and Poole 1985) and the relative importance of each proton pump in the generation of the electrochemical gradient is unknown (Rea and Sanders 1987).

### 1.2.3. Other ion-transporting ATPases.

The most abundant $H^+$–transporting ATPase in the plant cell is the mitochondrial $H^+$–ATPase. *In situ* this enzyme functions as an ATP synthase. It is characterized in membrane systems by sensitivity to azide or oligomycin, anion stimulation and by a high pH optimum of pH 8.0–9.0 (Sze 1985). For a more detailed discussion of the structure and function of this enzyme the reader is referred to Bowman and Bowman (1986) and Pedersen and Carofoli (1987a,b).

In addition to the three considered above, plant cells contain many more ATPases some of which are ion transporters. Chloroplasts, for example, possess a similar enzyme to the mitochondrial ATP synthase. There is also a second ion transporting ATPase located at the plasma membrane, the calmodulin–stimulated $Ca^{2+}$–ATPase. $Ca^{2+}$ transport was demonstrated in membrane vesicles before proton transport (Gross and Marme 1978) but progress in the characterization of this enzyme at the molecular level has only been made recently (Briars *et al* 1988, Briars and Evans 1989). The activity of this enzyme is two or three times lower than the $H^+$–ATPase and also has a higher pH optima (>7.0), but it may still be a major contaminating component of pure plasma membrane preparations with respect to the investigation of the $H^+$–ATPase. One final consideration is that the plasma membrane $H^+$–ATPase may be transiently active in other membranes, such as the Golgi membrane system, during

transportation to the plasma membrane from the endoplasmic reticulum.

## 1.3. PHYTOCHROME.

For photosynthetic plants light is the most important environmental factor and it is therefore crucial that the plant is able to perceive changes in both light quantity and quality. This is achieved by using photoreceptors which transduce changes in light energy into biochemical signals. Phytochrome is the best characterized of the plant photoreceptors and was first demonstrated to be present in higher plants by the measurement of action spectra for the inhibition of flowering in short-day plants (Parker *et al* 1946). Since this time there has been a considerable amount of research into both the molecular characterization of phytochrome and the characterization of phytochrome-mediated responses. These approaches have undoubtedly advanced our knowledge of this field but as yet the mechanism of phytochrome action has still to be elucidated.

There is an enormus body of literature on phytochrome and the reader is referred to Lagarias (1985), Jordan *et al* (1986) and Colbert (1988) for reviews which cover the topic quite broadly and to Shropshire and Mohr (1983), Kendrick and Kronenberg (1986) and Furuya (1987) for a more complete discussion of all aspects of phytochrome and plant photomorphogenesis.

### 1.3.1. Physiological role.

Photomorphogenesis is the term given to all plant development that is influenced by light (Smith 1984) and includes many different types of responses. Phytochrome is the photoreceptor through which many of these responses are mediated and is involved in the regulation of all stages of plant development from seed germination to the induction of flowering and senescence. The action of phytochrome is, perhaps, most evident in the de-etiolation of dark-grown plants after they are placed into light. This response can be very rapid and includes the synthesis of chlorophyll, stimulation of chloroplast and leaf development and inhibition of stem elongation (Hart 1988).

The role of phytochrome in light grown plants is not so clear. Certainly a primary function of phytochrome is in the determination of day length (photoperiodism) and this is important in flowering (Hart 1988). In addition, phytochrome has been shown to be important in shade avoidance responses (Smith 1982) and also in the perception of neighbour proximity and the orientation of photoreceptive surfaces (Smith *et al* 1990).

### 1.3.2. Properties of phytochrome.

#### 1.3.2.1. Photochemical properties.

Phytochrome can exist in two forms, $P_r$ and $P_{fr}$. It is synthesized as $P_r$ and can be photoconverted by red light (R, 660nm) to $P_{fr}$ *via* several intermediates (Rudiger 1987). The $P_{fr}$ form can be photoconverted back to $P_r$ by far-red light (FR, 730nm) or, in certain species including all dicotyledons except the *Centrospermae*, it can thermally revert back to the $P_r$ form in the dark (Butler and Lane 1965). In etiolated tissue $P_{fr}$ is rapidly degraded ($t_{0.5}$ =1-2h, Jabben and Holmes 1983). $P_r$ can also be degraded but only after existing in the $P_{fr}$ form (Dooskin and Mancinelli 1968). Photoconversion between these two forms is mediated by a linear tetrapyrrole chromophore that is attatched to the phytochrome apoprotein by a thioether linkage (Lagarias and Rapoport 1980, Song 1988). Light-induced changes in chromophore structure lead to conformational changes in the structure of the whole phytochrome molecule as a result of interactions between the chromophore and the apoprotein.

Figure 1.2 shows the absorbance and difference spectra of highly purified oat phytochrome. The difference spectrum is obtained by subtracting the absorbance spectrum of $P_r$ from that of $P_{fr}$. Because $P_{fr}$ also absorbs at 660nm it is impossible to photoconvert all $P_r$ to the $P_{fr}$ form and at photoequilibrium approximately 86% of the total phytochrome exists as $P_{fr}$ (Vierstra and Quail 1983, Lagarias *et al* 1987). The extinction coefficients for phytochrome vary between phytochrome preparations but by using the photoequilibrium value of Kelly and Lagarias (1985) the extinction coefficient of undegraded oat phytochrome after a saturating R irradiation can be calculated to be $7.9 \times 10^7$ mol $m^{-3}$ $cm^{-1}$ (Jordan *et al* 1986).

#### 1.3.2.2. Molecular properties.

The predominant phytochrome species in etiolated tissue was first purified to homogeneity in 1983 (Viersta and Quail 1983) and has an apparent monomeric molecular mass which ranges from 120 kDa in *Zucchini* to 127 kDa in corn (Vierstra *et al* 1984, Vierstra and Quail 1986) with approximately 1100 amino acid residues. The $NH_2$-terminus is susceptible to rapid degradation by a PMSF-sensitive protease to give 118 and 114 kDa species. These species are termed 'large' phytochrome and for many years were considered to be the native phytochrome species. Further proteolytic degradation gives a 60 kDa 'small' phytochrome. This was the species first purified (Butler *et al* 1959).

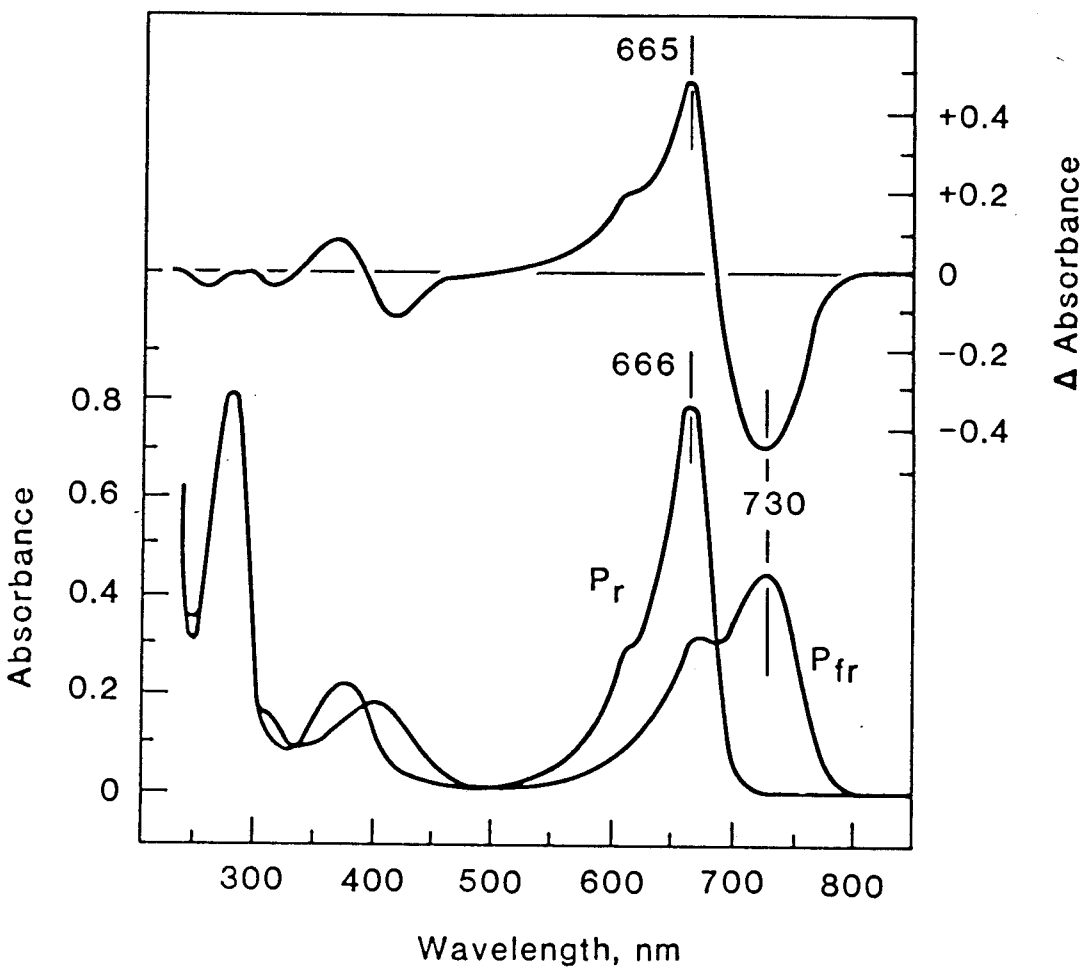


Figure 1.2: The absorbance (lower) and difference (upper) spectra of highly purified, undegraded phytochrome from etiolated shoots of *Avena sativa*. From Vierstra and Quail (1983).

Phytochrome behaves as a soluble globular protein. Although there is considerable evidence for the presence of hydrophobic regions on the molecule (Jordan *et al* 1986) and for a membrane association (see Chapter 6), it is unlikely that it is an intrinsic membrane protein. The observation that phytochrome is a polar, soluble molecule is borne out by the hydropathic properties of the protein as predicted from the primary sequence (Vierstra and Quail 1986). The surface is generally hydrophillic with the chromophore housed in a hydrophobic pocket within the protein between residues 80 and 315 (Vierstra and Quail 1986). A simple model of the phytochrome molecule is shown in Figure 1.3.

It appears that during photoconversion between $P_r$ and $P_{fr}$ there are conformational changes in protein structure. A number of techniques have been used to investigate this process. Differential proteolysis of the two spectral forms has been very successful in mapping conformational changes and it seems that two regions in particular are involved, the $NH_2$-terminus and a more centrally located region on the chromophore-bearing domain (Vierstra and Quail 1986). These results have been confirmed by studies on the differential binding of monoclonal antibodies (Cordonnier *et al* 1985, Partis and Thomas 1985, Thomas *et al* 1986). A variety of other techniques have also been used to demonstrate structural differences between $P_r$ and $P_{fr}$; these include size exclusion chromotography, circular dichroism and differential reactivity with residue specific reagents (Vierstra and Quail 1986 for review). Phytochrome behaves as a dimer in solution (Lagarias and Mercurio 1985, Jones and Quail 1986) and is therefore probably a dimer *in vivo*.

### 1.3.3. Detection of phytochrome.

#### 1.3.3.1. Spectrophotometric assay.

In etiolated tissues phytochrome can be measured spectrophotometrically by measuring the photoreversible changes in absorbance between $P_r$ and $P_{fr}$ ($\Delta(\Delta A)$). As chlorophyll also absorbs in this region the assay cannot be used for samples from light-grown plants.

The spectrophotometric assay can be accomplished using any of three pairs of wavelengths, R (660nm) and FR (730nm), R and near infra-red (880nm) or FR and near infra-red. The use of R and FR is the most sensitive assay but the FR, near infra-red combination is more accurate as interference by chlorophyll or protocholorophyll is reduced (Jose *et al* 1977). The $\Delta(\Delta A)$ value is determined by measuring the difference in absorbance at 730 and 800nm after R and FR irradiations of the sample and is equal to the sum of these values (Pratt 1983).

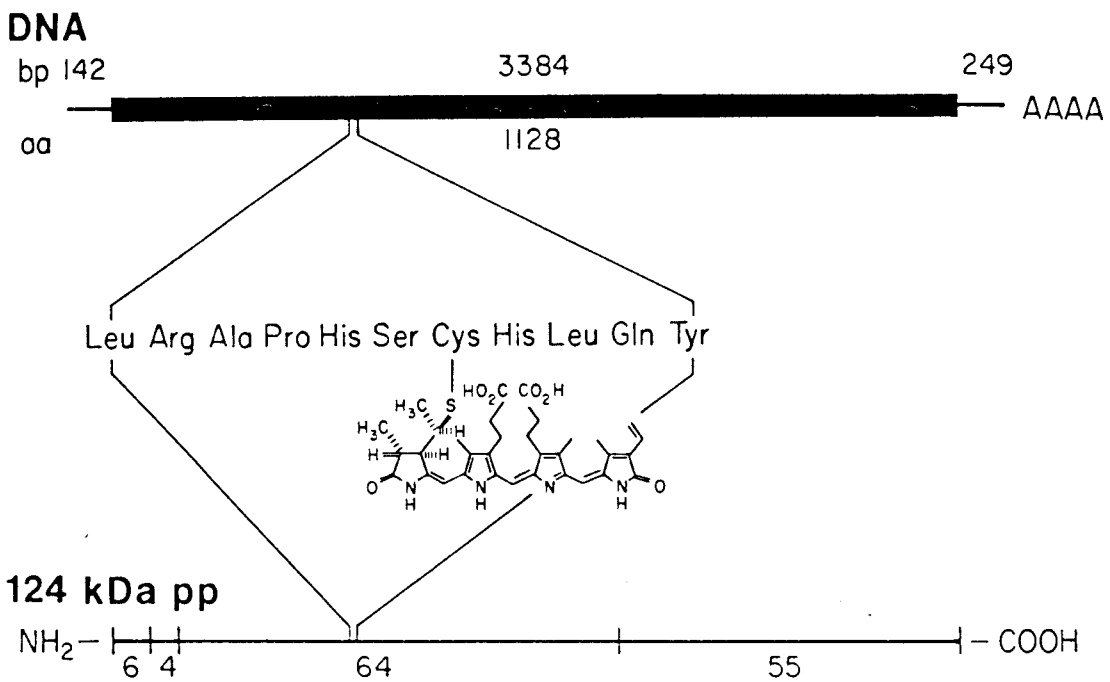


Figure 1.3: Schematic representation of the 124–kDa phytochrome polypeptide from *Avena sativa* (labelled 124 kDa pp) and of the nucleotide sequence derived from analysis of cDNA and genomic clones (labelled DNA). The DNA sequence has the 5′ end to the left and includes the coding region which is 3384 base pairs (1128 amino acids) long (solid bar) and untranslated sequences at either end (narrow bar). The schematic of the polypeptide includes the positions of the major proteolytic cleavage sites (vertical lines) and the molecular mass of the derived peptides. The position of the 11–amino–acid, chromophore attatchment sequence as derived from the nucleotide sequence is indicated, together with the structure of the chromophore itself. From Vierstra and Quail (1986).

The quantity of phytochrome in the sample is linearly proportional to the $\Delta(\Delta A)$ value and the concentration can be calculated using the extinction coefficient of phytochrome.

There are a number of limitations inherent in using a spectrophotometric assay. The absorbance of light by other pigments has already been mentioned. In addition, there are problems of fluorescence, which can be induced by irradiation from the measuring beam(s), and of changes in phytochrome extinction, which can be altered reversibly in the FR region as a function of a variety of perturbants such as mulivalent cations. The sensitivity of the assay is low but this can be overcome in part by the use of a scattering agent which increases the light path of the sample and therefore the absorbance. Calcium carbonate is commonly used, but there are artifactual problems associated with this technique (Watson and Smith 1982a). A further limitation of the assay is that only spectrally active phytochrome can be detected.

The advantages of the assay is that it is quick and easy to do and also that it allows the measurement of phytochrome *in vivo*, although there are also problems associated with this application (Gross *et al* 1984). The practicalities and limitations of this assay technique are discussed fully by Pratt in a series of reviews (Pratt 1978, 1979, 1983).

### 1.3.3.2. Immunological assays.

The first use of antibodies to assay for phytochrome was by Hunt and Pratt (1979) who developed a radioimmunoassay (RIA). This assay depends upon the displacement of $^{3}$H–phytochrome from antiphytochrome IgG by non–radioactive phytochrome. The amount of radioactivity released is proportional to the amount of phytochrome in the sample. The assay is more sensitive than spectrophotometric methods and also unaffected by other pigments. However, it is technically difficult and slow to perform, and has only been used for phytochrome quantitation in a few studies (Hunt and Pratt 1980, Gottman and Schafer 1983).

More recently, a double–antibody sandwich enzyme–linked immunosorbent assay (ELISA) for phytochrome was developed independently by Thomas *et al* (1984a) and Shimazaki *et al* (1983). This technique is based on the immunoabsorption of phytochrome from a plant extract followed by the detection of the antigen by a subsequent incubation with excess antiphytochrome IgG. In the formative experiments polyclonal antibodies were used for both stages but it is now more common to use a monoclonal antibody as the detecting antibody. This detecting antibody is linked either directly or indirectly, *via* a second antibody, to an enzyme which uses an added substrate to give a coloured product. Exact

protocols are given in Chapter 2 (see 2.12.2.) and Thomas *et al* (1986). The ELISA technique is as equally sensitive as RIA, is unaffected by other pigments and gives rapid, reproducible results, making it the most widely used quantitative immunological assay (Jordan *et al* 1986, Thomas *et al* 1986, Cordonnier 1989).

Antibodies have also been used for the qualitative assay of phytochrome. Early experiments utilized both immunoelectrophoresis and Ouchterlony double immunodiffusion (Pratt 1983 for review) but these methods have been superceded by western blotting techniques. A western blot is the transfer of proteins from a polyacrylamide gel to an immobilizing membrane, usually nitrocellulose. Proteins are then identified using enzyme-linked antibodies by methods analagous to those used for the detecting stage of an ELISA. This approach is superior as it affords much greater resolution and the ability to examine a greater number of samples at a time.

Further qualitative detection of phytochrome can be achieved using an immunocytochemical assay. This technique probes thin tissue sections with antibodies linked to enzymic or fluorescent markers (for light microscopy) or a ferritin label (for electronmicroscopy). Immunocytochemistry has been useful in providing important information about phytochrome distribution at both the tissue and subcellular level (Thomas *et al* 1986).

### 1.3.3.3. Applications of Immunological assays.

Perhaps the most important advance in phytochrome research, dependent on the use of immunological assays, has been the demonstration that there are two immunochemically distinct phytochrome species (Shimazaki *et al* 1983, Hilton and Thomas 1985, 1987, Konomi *et al* 1987). These two species have been designated type I phytochrome which is present at high levels in etiolated plants and is well characterized (see 1.3.2.) and type II or green phytochrome which is predominant in light-grown plants and is less well understood. In addition to providing evidence for the existence of these two phytochrome pools, immunological techniques have also made possible comparative studies of the two phytochrome types (*e.g.* Cordonnier *et al* 1986). One notable difference is in the apparent molecular mass which appears to be lower (118 kDa) for type II phytochrome (Tokuhisa *et al* 1985, Tokuhisa and Quail 1989).

A major advantage of the immunological assay is that it is able to detect phytochrome apoprotein. This feature has been particularly useful in evaluating the action of inhibitors of chromophore biosynthesis and this approach has yielded important information on both the intermediates of the phytochrome biosynthetic pathway (Elich and Lagarias 1987) and the conditions required for

phytochrome holoprotein assembly (Lagarias and Lagarias 1989).

Qualitative immunoassays have allowed structure–function studies to be performed on the phytochrome molecule (Cordonnier 1989). This approach initially requires the mapping of the epitopes on the protein to which the monoclonal antibodies bind. This is usually achieved by using proteolytically derived peptide fragments that are separated by PAGE before western blotting (*e.g.* Pratt *et al* 1988). Subsequently the antibodies can be used to evaluate the role of these domains in the function of the protein *i.e.* do these domains undergo light–induced conformational changes (see 1.3.2.)? Are they required for dimerization or ubiquitination? Are the domains evolutionarily conserved? This technique has been used effectively to provide information in all these areas (Lagarias *et al* 1987, Yamamoto 1987, Cordonnier 1989) and immunological assays have proved to be a very powerful investigative tool for the elucidation of the molecular biology of phytochrome.

### 1.3.4. Mechanism of phytochrome action.

Although the view that $P_{fr}$ is the active form of phytochrome is widely held (Shropshire and Mohr 1983), it has also been proposed that phytochrome responses are determined by the proportion of $P_{fr}$ in the total phytochrome pool, $P_{fr}/P_{total}$ (Smith 1981, 1983). Evidence for both of these hypotheses comes from correlations between spectrophotometrically determined phytochrome levels and physiological responses and there are obvious problems associated with this approach. There are at least two spectrally similar forms of phytochrome (types I and II) and these may have different functions. In addition, certain responses may be dependent on localized phytochrome populations (*e.g.* membrane bound phytochrome?). In both cases, correlations with total cell phytochrome would be uninformative and the question will only be resolved unequivocally when the reaction partner(s) of phytochrome is identified.

Phytochrome responses can be classified into three groups. The high irradiance response (HIR) which is irradiance dependent but does not show reciprocity between fluence intensity and irradiation time or FR reversibility, the inductive or low fluence response which is repeatedly photoreversible and does show reciprocity and the very low fluence response (VLFR) which is a special case of the inductive response where the amount of $P_{fr}$ required to saturate the response can be produced by a FR irradiation alone. As mentioned above there has been considerable effort directed at the characterization of these phytochrome responses and at the elucidation of the molecular mechanism(s) of phytochrome action. However, as yet this mechanism is essentially unknown.

It is possible that this great variation in phytochrome-mediated responses could be generated by a small number of primary phytochrome actions. Undoubtedly the regulation of gene expression is a central feature of many of the responses that have so far been described (Tobin and Silverthorne 1985). However, there are numerous phytochrome-mediated responses that are too rapid to be attributable to changes in gene expression (Quail 1983). Many of these involve rapid changes in membrane-related processess and it was proposed as early as 1967 that the primary action of phytochrome is through an interaction with a cell membrane (Hendricks and Borthwick 1967). It is important to realise that these two mechanisms need not be exclusive and may involve the same molecular interaction at different cellular locations.

### 1.3.4.1. Phytochrome control of gene expression.

Phytochrome has been shown to play a role in the light regulation of both nuclear (Tobin and Silverthorne 1985, Jenkins 1988, Thompson 1988) and plastid (Tobin and Silverthorne 1985, Link 1988) gene expression. Although there are numerous recorded light-regulated changes in the amounts or activities of gene products (Schopfer 1977, DeVries *et al* 1982, Lamb and Lawton 1983, Tobin and Silverthorne 1985) only a few of these have been extensively characterized. Much research has centred on the regulation of two nuclear-encoded chloroplast proteins, the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbcS* or SSU) and the major light-harvesting, chlorophyll *a*/*b*-binding protein (*cab* or LHCP). The expression of both of these genes is light stimulated. Phytochrome can also mediate the inhibition of gene expression by light, for example in the gene coding for NADPH-protochlorophyllide oxidoreductase (PCR) and, most interestingly, in the expression of the phytochrome gene(s) itself (Quail 1984, Quail *et al* 1986).

Phytochrome regulates the rate of gene transcription and this regulation can be very rapid. In oats, transcription of phytochrome mRNA is reduced to one third of the dark level within 15 min of a red light pulse (Colbert *et al* 1985). However, this reduction in transcription rate is both too small and too slow to account for the observed decrease in the levels of phytochrome mRNA and this has led to the proposal that phytochrome also regulates gene expression by post-transcriptional modification, *i.e.* by altering mRNA stability (Quail *et al* 1986). More recent examples have suggested that phytochrome-mediated regulation of mRNA abundance by this mechanism may be quite widespread (Thompson 1988).

The components of the signal transduction chain between $P_{fr}$ and changes in gene expression are largely unknown. It has been shown that there are sequences of DNA that are required for phytochrome regulation of the expression

of some genes (Jenkins 1988, Nagy *et al* 1988). These *cis*-acting factors have been identified by reintroducing defined DNA sequences into an *in vivo* expression system and by examining the interaction of nuclear protein factors by gel retardation assays and DNase I footprinting experiments. The picture beginning to emerge is a very complicated one with many interacting DNA regulatory elements upstream of a single light-regulated gene. Evidence for the identity of the corresponding DNA-binding proteins (*trans*-acting factors) is more limited. Green *et al* (1987) have shown that one such protein, GT-1, is involved in the regulation of the *rbcS* gene and there are reports that *de novo* protein synthesis is required for the light regulation of certain genes (Lam *et al* 1989) although not for the oat phytochrome gene, *phy* (Lissemore and Quail 1988).

### 1.3.4.2. Phytochrome control of membrane processes.

Given the properties of the phytochrome molecule it would seem unlikely that phytochrome interacts directly with a cellular membrane. However, as stated earlier, there is considerable evidence that a phytochrome-membrane association may exist and this is reviewed more fully in Chapter 6. What is clear is that phytochrome regulates a number of membrane functions. Rapid, phytochrome-regulated changes in membrane potential, as measured by external electrodes, were first demonstrated by Newman and Briggs in 1972. In this and later reports (Racusen 1976, Wiesenseel and Ruppert 1977) a R-induced depolarization was detected, but hyperpolarizations have also been recorded (Racusen and Satter 1975, Newman and Sullivan 1976, Newman 1981). The identity of the ions involved in these changes in membrane potential are unknown, but $K^+$, $Cl^-$, $Ca^{2+}$ and protons (see 5.1.) have all been implicated.

Most attention has been focussed on the possible role of $Ca^{2+}$ ions in phytochrome-mediated responses and there are numerous reports of $Ca^{2+}$ influx into R-irradiated cells (*e.g.* Wiesenseel and Ruppert 1977, Das and Sopory 1985, Bossen *et al* 1988). In addition, there are a number of phytochrome-mediated responses which have been shown to be $Ca^{2+}$ dependent (Hepler and Wayne 1985, Roux *et al* 1986). For example the R-induced germination of *Onoclea* spores requires external $Ca^{2+}$ with half-maximal response supported by $3 \times 10^{-3}$ mol $m^{-3}$, a concentration in the same order of magnitude as that required for $Ca^{2+}$ modulation of hormone-stimulated responses in animal cells (Wayne and Hepler 1984). In the same system it has been shown, by atomic absorption spectroscopy, that there is a FR reversible increase in intracellular $Ca^{2+}$ after R irradiation (Wayne and Hepler 1985) and that the response can be mimicked by the addition of the $Ca^{2+}$ ionophore, A23187 or blocked by $La^{3+}$. Whether this influx is

dependent on the direct interaction of $P_{fr}$ or whether intermediate steps, such as the breakdown of inositol phospholipids, are required is not known.

Another example of the role of $Ca^{2+}$ ions is in the R-induced chloroplast rotation response in *Mougeotia*. Serlin and Roux (1984) were able to show, by the selective use of A23187 (*via* coated microtips of glassrods), that the chloroplast rotated away from localized $Ca^{2+}$ influx. This response requires external $Ca^{2+}$ but can be blocked by depleting internal $Ca^{2+}$ stores (Wagner and Rossbacher 1980) and by calmodulin antagonists. In addition, $Ca^{2+}$ ions have also been shown to stimulate the phosphorylation of several proteins in *Mougeotia*, indicating a possible regulatory mechanism (Roux *et al* 1986).

### 1.3.4.3. The protoplast swelling response.

One well characterized phytochrome-mediated response that can be localized to the plasma membrane is the R-induced swelling of leaf protoplasts. The response is thought to be associated with phytochrome regulation of leaf unrolling and was originally demonstrated in etiolated wheat by Blakeley *et al* (1983) who proposed that the swelling was due to a change in plasma membrane permeability. The stimulation of the response by $K^+$ ions together with an inhibition by vanadate suggested that the plasma membrane $H^+$-ATPase could be involved. This response has also been detected in oat leaf protoplasts where cAMP was also found to be able to induce the swelling response (Kim *et al* 1986, Chung *et al* 1988). A subsequent paper, again using wheat leaves, failed to detect any $K^+$ stimulation but did show that the response was dependent on the presence of external $Ca^{2+}$ ions (Bossen *et al* 1988). The involvement of $Ca^{2+}$ ions was further demonstrated by the ability of A23187 to mimick the response and $La^{3+}$ or the $Ca^{2+}$-channel blocker, verapamil, to block it. More recent work suggests that both GTP-binding proteins (G-proteins) and phosphatidyl inositol turnover may be involved in the regulation of this response (Bossen 1990).

## 1.4. Aims.

The phytochrome-regulated protoplast swelling response has been described briefly above. Because the response itself and components of a possible signal transduction chain have been extensively characterized in wheat leaves, they provide a model system in which to study the action of phytochrome at the plasma membrane and, for this reason wheat leaves were chosen for this project.

There were two principle aims of this research project. The first of these was to provide a better understanding of the nature of the ATPase activity

associated with wheat leaf membrane preparations. In particular, to characterize the $K^+$-stimulated, $Mg^{2+}$-dependent ATPase of the plasma membrane. The second, and perhaps more important, aim was to investigate the mechanism of action of phytochrome at the plasma membrane. The purification of wheat leaf plasma membranes and the characterization of the associated ATPase activity would then provide an ideal system with which to do this. The approach taken to investigate the action of phytochrome in this response was two-fold. It was proposed firstly to examine the photoregulation of the plasma membrane $H^+$-ATPase *in vitro* and subsequently to characterize, both qualitatively and quantitatively, the association of phytochrome with plasma membrane preparations.

# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1. Plant material

Southampton: seed of winter wheat (*Triticum aestivum* L. cv Maris Huntsman, from NSDO Ltd., Newton, Cambridge) was imbibed in running water overnight, sown in moist vermiculite and grown in the dark at 25°C. Wheat leaves were harvested when the shoots had reached a height of ~8–10 cm. Seed of cucumber (*Cucumis sativus* L. cv Long Green Ridge, from Clause (UK) Ltd., Charvil, Reading) was grown in moist perlite in the dark at 27°C. Cucumber hypocotyls were harvested after 6 days.

Littlehampton: seed of winter wheat (*Triticum aestivum* L. cv Avalon, from Bartholomews, Chichester, West Sussex) was grown in moist vermiculite at 25°C either in the dark or with specified light treatments.

## 2.2. Preparation of a microsomal fraction.

Wheat leaves were homogenized in a precooled pestle and mortar with a ratio of 1g fresh weight tissue to 4 $cm^3$ of ice–cold homogenization medium, containing 250 mol $m^{-3}$ sucrose, 50 mol $m^{-3}$ Tris–Mes buffer (pH 7.0 or pH 8.2 for phytochrome binding experiments, Chapter 6), 3 mol $m^{-3}$ EGTA, 2 mol $m^{-3}$ DTT and 0.5% (w/v) PVP. For membrane preparations used on some western blots (Figures 6.11 and 6.13) the homogenisation medium included 4 mol $m^{-3}$ PMSF and 1.9 $g/dm^3$ sodium metabisulphite. The homogenate was squeezed through a layer of gauze and centrifuged at 12,000g for 10 min at 4°C in a fixed–angle rotor (Sorvall SS34). The resulting supernatant was centrifuged at 100,000g ($r_{av}$) for 30 min at 4°C using either a Sorvall A–841 rotor (Southampton) or a Beckman 55.2Ti rotor (Littlehampton) to give the microsomal pellet. A microsomal fraction from cucumber hypocotyls was prepared similarly except that the ratio of tissue to homogenization medium was 3g to 1 $cm^3$.

## 2.3. Preparation of plasma membranes.

### 2.3.1. Separation by sucrose gradient.

A discontinuous sucrose density gradient was prepared by successive layering of 5 $cm^3$ each of 38, 34, 25 and 20% (w/w) sucrose in 2 mol $m^{-3}$

Tris–Mes buffer (pH 7.0) and 1 mol $m^{-3}$ DTT. The microsomal membrane fraction was layered onto the gradient in 2 $cm^3$ of resuspension medium containing 250 mol $m^{-3}$ sucrose, 5 mol $m^{-3}$ Tris–Mes buffer (pH 7.0) and 1 mol $m^{-3}$ DTT and the gradient was centrifuged at 80,000g for 2h at 4°C using a Sorvall AH–629 rotor. After centrifugation, membrane fractions were removed from the gradient interfaces with a Pasteur pipette and diluted with resuspension medium containing 250 mol $m^{-3}$ sucrose, 5 mol $m^{-3}$ Tris–Mes buffer (pH 7.0) and 1 mol $m^{-3}$ DTT.

A number of variations to this basic separation technique were used. When an extra gradient step was included the volume of each sucrose solution was reduced to 4 $cm^3$. For KI treatments given to isolated membranes, the membranes were incubated for 20 min on ice in the usual resuspension medium with 250 mol $m^{-3}$ KI included. They were then repelleted at 100,000g before being layered onto the sucrose gradient in the normal way. For the experiment where KI was included in the homogenization medium, the medium used was based on the KI–washing medium described by DeMichelis and Spanswick (1986) and contained 250 mol $m^{-3}$ sucrose, 25 mol $m^{-3}$ Tris–Mes buffer (pH 7.0), 2 mol $m^{-3}$ EGTA, 2 mol $m^{-3}$ DTT, 2 mol $m^{-3}$ $MgSO_4$, 2 mol $m^{-3}$ ATP, 10% (w/v) glycerol, 0.5% (w/v) BSA and 250 mol $m^{-3}$ KI.

The method used in an attempt to separate $Ca^{2+}$–phosphatase activity from $Mg^{2+}$–ATPase activity was as described by DuPont and Hurkman (1985). Wheat leaves were homogenized in 250 mol $m^{-3}$ sucrose, 25 mol $m^{-3}$ Tris–Mes buffer (pH 8.0), 4 mol $m^{-3}$ EDTA and 2 mol $m^{-3}$ DTT in a ratio of 8 $cm^3$ homogenization medium to 1g fresh weight tissue. The resulting microsomal pellet was layered onto a sucrose gradient containing 5 mol $m^{-3}$ Tris–Mes buffer (pH 7.2), 1 mol $m^{-3}$ EDTA and 1 mol $m^{-3}$ DTT and including an extra step of 15% (w/w) sucrose.

### 2.3.2. Separation by phase partition.

The phase partition technique followed that described by Lundborg *et al* (1981) and, more generally, by Larsson (1985). The microsomal membrane fraction was resuspended in 250 mol $m^{-3}$ sucrose and 5 mol $m^{-3}$ potassium phosphate buffer (pH 7.8). These resuspended microsomal membranes (1g) were then added to 7g of phase mixture to give final concentrations of 6.5% (w/w) Dextran T500 (Pharmacia Ltd., Milton Keynes), 6.5% (w/w) PEG 3350, 3 mol $m^{-3}$ KCl, 250 mol $m^{-3}$ sucrose and 5 mol $m^{-3}$ potassium phosphate buffer (pH 7.8) although in some early experiments the phase composition varied as detailed in Chapter 3. This phase system was mixed by inversion (20–30 times) and centrifuged for 5 min at ~1500g to facilitate phase separation. The top 90% of the upper phase (U)

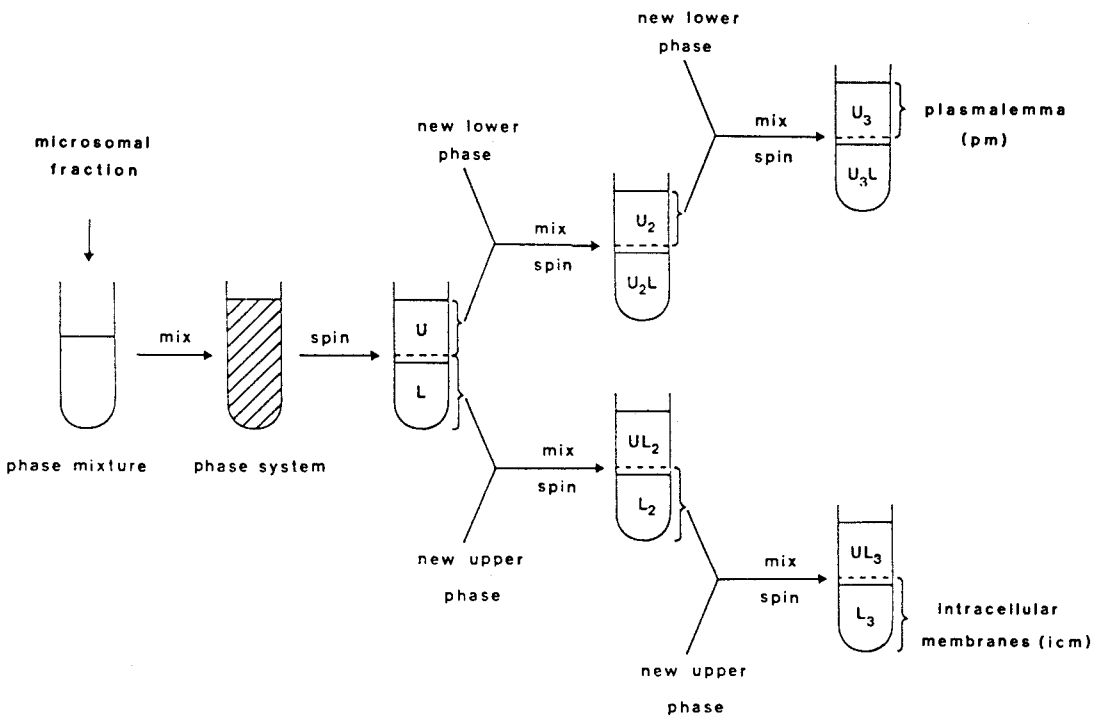


Figure 2.1: Scheme showing a batch procedure for the preparation of plasma membrane vesicles by partition in a Dextran–PEG two–phase system. From Widell (1987).

was then added to fresh lower phase and made up to 8g with fresh upper phase. The mixing and separation process was repeated to give $U_2$ and subsequently $U_3$. A scheme for such a batch procedure is outlined diagrammatically in Figure 2.1. The lower phase (L) can also be re-extracted twice with fresh upper phase to give a membrane fraction ($L_3$) which is largely free of plasma membrane. The fresh upper and lower phases were prepared previously as a 200g phase system using the same constituents at the same concentrations as used for the 8g phase system. All procedures were carried out as close to 4°C as possible.

The membrane fractions $U_3$ and $L_3$ were diluted in a medium containing 250 mol $m^{-3}$ sucrose, 10 mol $m^{-3}$ Tris-Mes buffer (pH 7.4) and 1 mol $m^{-3}$ EGTA and centrifuged at 100,000g for 40 min. The resulting pellet was resuspended in 250 mol $m^{-3}$ sucrose, 5 mol $m^{-3}$ Tris-Mes buffer (pH 7.0) and 2 mol $m^{-3}$ DTT and used immediately. For the phytochrome binding experiments (Chapter 6), a resuspension medium of 250 mol $m^{-3}$ sucrose, 5 mol $m^{-3}$ Tris-Mes buffer (pH 7.8) and 10% (w/v) glycerol was used and the membranes were frozen in liquid $N_2$ before storage at -70°C. These membrane preparations were used within two weeks.

An important consideration is that Dextran T500 contains a small quantity of water which, if not allowed for, will alter the phase composition, and therefore the membrane separation. The water content can be determined by using a polarimeter to measure the optical rotation at 589nm of a Dextran solution, as described by Larsson (1985). The Dextran T500 used in this study was found to contain 3.4% (w/w) water and the concentration of Dextran was adjusted accordingly.

## 2.4. Preparation of membranes for proton transport experiments.

A microsomal membrane fraction was prepared after gentle homogenization in 250 mol $m^{-3}$ mannitol, 25 mol $m^{-3}$ Hepes-BTP buffer (pH 7.4), 3 mol $m^{-3}$ EGTA, 1 mol $m^{-3}$ DTT and 0.1% (w/v) BSA. These microsomal membranes were then resuspended in 250 mol $m^{-3}$ mannitol, 2 mol $m^{-3}$ BTP-Mes buffer (pH 7.0), 1 mol $m^{-3}$ DTT, 0.2% (w/v) BSA and 10% (w/w) glycerol using a process called re-annealing in which the membranes are resuspended by repeatedly drawing them gently through a pipette tip at 37°C. This process is designed to enhance vesicle integrity and thereby increase the amount of measurable proton transport.

## 2.5. ATPase assay.

ATPase activity was determined by measuring the release of inorganic phosphate ($P_i$) from ATP using a method based on that of Sumner (1944). The assay was carried out in either a 1 $cm^3$ (Southampton) or a 0.5 $cm^3$ (Littlehampton) reaction volume at 37°C for 30 or 40 min. Over this time period $P_i$ release was linear (data not shown). The standard assay medium contained 40 mol $m^{-3}$ Tris-Mes buffer, 2 mol $m^{-3}$ $MgCl_2$, 2 mol $m^{-3}$ ATP, 0.1 mol $m^{-3}$ ammonium molybdate, 50 mol $m^{-3}$ KCl and 50 $mm^3$ of membranes (5-50 $\mu$g protein/$cm^3$). When assaying ATPase activity associated with plasma membranes prepared by phase partition 0.01% (w/w) Triton X-100 was included in the assay medium. The reaction was started by the addition of the membranes and stopped by adding 1.6 $cm^3$ (or 0.8 $cm^3$) of 625 mol $m^{-3}$ $H_2SO_4$ and 1.25% (w/v) ammonium molybdate. The colour was developed by the addition of 8% (w/v) ferrous sulphate and precipitates, formed by the acid denaturation of proteins, were removed by centrifugation before the absorbance was read at 620nm after 10 min. If Triton was present in the assay medium, SDS at a final concentration of 0.33% (w/v) was added to prevent interference of the Triton in the colour reaction (Peterson 1978). Vanadate was added in the form $Na_3VO_4$ and SW26 was used in 10% (v/v) DMSO.

## 2.6. Latent IDPase assay.

IDPase activity was measured at pH 6.0 as for ATPase activity in the presence and absence of 0.01% (w/w) Triton X-100 after the method of Ray *et al* (1969).

## 2.7. Glucan synthetase II assay.

Glucan synthetase activity was measured by the incorporation of $^{14}C$-labelled UDP-glucose by the method of Ray (1979). 100 $mm^3$ of membranes were added to 40 $mm^3$ of 50 mol $m^{-3}$ Tris-Mes buffer (pH 8.0) containing 648 Bq of UDP-[$^{14}C$]glucose (specific activity =20.42 GBq/mol $m^{-3}$, Amersham International plc, Amersham, Bucks.) and 0.5 mol $m^{-3}$ of unlabelled UDP-glucose. Cellobiose (300 mol $m^{-3}$) and Triton X-100 (0.01% (w/w)) were also included in the assay. Samples were incubated for 15 or 30 min at 25°C over which period incorporation of UDP-[$^{14}C$]glucose was linear (data not shown). The reaction was stopped with 1 $cm^3$ of 70% (v/v) ethanol and 50 $mm^3$ of 50 mol $m^{-3}$ $MgCl_2$. 150 $mm^3$ of boiled microsomal membranes (4g fresh wt tissue/$cm^3$) were added to

improve the recovery of the labelled products. The mixture was boiled for 1 min and left to stand overnight at 4°C. It was then centrifuged at 1000g for 5 min and the precipitated material washed four times in 70% (v/v) ethanol before being suspended in 10 $cm^3$ of scintillation fluid. Incorporated radioactivity was measured by liquid scintillation spectrometry with a Beckman counter.

### 2.8. Cytochrome c oxidase assay.

Cytochrome c oxidase activity was measured by following the oxidation of reduced cytochrome c according to the method of Hodges and Leonard (1974). The assay medium contained 45 mol $m^{-3}$ phosphate buffer (pH 7.5), 0.01% (w/v) digitonin, $15x10^{-3}$ mol $m^{-3}$ reduced cytochrome c and 100 $mm^3$ of membranes in a 3 $cm^3$ reaction volume. Cytochrome c was reduced chemically using sodium dithionite, the excess dithionite being removed by passing air through the solution. The assay was started by the addition of the cytochrome c and the initial rate of oxidation was followed spectrophotometrically at 550nm at room temperature (RT) using either a Varian DMS 90 (Southampton) or a Perkin-Elmer 554 (Littlehampton) spectrophotometer.

### 2.9. NADH-cytochrome c reductase assay.

NADH-cytochrome c reductase activity was measured by following the reduction of cytochrome c according to the method of Hodges and Leonard (1974). The assay medium contained 41.67 mol $m^{-3}$ phosphate buffer (pH 7.5), 1.67 mol $m^{-3}$ NaCN, $30x10^{-3}$ mol $m^{-3}$ cytochrome c, $1x10^{-3}$ mol $m^{-3}$ antimycin A (to eliminate the mitochondrial enzyme) and 100 $mm^3$ of membranes in a 3 $cm^3$ reaction volume. The assay was started by the addition of 0.1 mol $m^{-3}$ NADH and the initial rate of cytochrome c reduction was followed spectrophotometrically as for the cytochrome c oxidase assay.

### 2.10. Measurement of proton transport.

Proton transport was measured by the quenching of quinacrine fluorescence using a method similar to that described by Sze (1983). Fluorescence quenching was followed at RT in a Perkin-Elmer LS3 spectrofluorimeter using excitation and emission wavelengths of 420nm and 495nm respectively. The standard assay medium contained 200 mol $m^{-3}$ mannitol, 15 mol $m^{-3}$ Hepes-BTP buffer (pH 6.5), 0.35 mol $m^{-3}$ EGTA, 50 mol $m^{-3}$ KCl, 50 mol $m^{-3}$ Cl-BTP, 5 mol $m^{-3}$ $MgCl_2$, 5 mol $m^{-3}$ ATP-BTP, $5x10^{-3}$ mol $m^{-3}$ quinacrine and 100

$mm^3$ of membranes in a 0.5 $cm^3$ reaction volume. The reaction was started with the addition of ATP-BTP at which point the fluorescence intensity was set to 100%. The degree of quenching measured initially was subsequently amplified to allow a more accurate determination of the effects of inhibitors on proton transport activity.

### 2.11. Light sources.

For both *in vivo* and *in vitro* red and far-red light treatments a light box was used. This contained, for red light treatments, Grolux fluorescent lamps filtered through one layer of Plexiglas R 501 and, for far-red light treatments, 60-W tungsten-filament strip lights filtered through three layers of Plexiglas blue 627 and one layer of Strand Cinemoid deep orange (no. 58). Details of the spectral properties of these light sources are shown in Chapter 5 (5.3.1.) and were determined using a cosine corrected receiver attatched by a quartz fibre probe to an Optronic 740A spectroradiometer (Glen Creston Instruments, London). This was interfaced with a Hewlett Packard 9815 A/S Desktop computer.

For light treatments at other wavelengths a 250-W quartz halogen projector lamp was used with appropriate interference filters (Type B-40, half band width 10nm, Balzers High Vacuum, Berkhampsted, Herts.). The green safelight comprised two 15-W fluorescent lamps filtered through three layers of Strand Cinemoid primary green (no. 39).

### 2.12. Phytochrome assays.

#### 2.12.1. Spectrophotometric assay.

The concentration of phytochrome present in a 1 $cm^3$ sample was determined spectrophotometrically by measuring at 4 °C the $\Delta(\Delta A)$ between $P_r$ and $P_{fr}$ at 730 and 800nm in a modified Perkin-Elmer 557 double wavelength, double beam spectrophotometer interfaced to a Commodore 3032 computer.

The spectral properties of this phytochrome sample were measured by scanning the sample with phytochrome in both the $P_r$ and $P_{fr}$ forms to give an absorbtion spectrum for each between 600 and 800nm. The absorbance spectrum of $P_r$ was then subtracted from that of $P_{fr}$ to give a difference spectrum for the phytochrome sample.

### 2.12.2. Enzyme-linked immunosorbent assay (ELISA).

Phytochrome was quantitated immunochemically with a double-antibody sandwich ELISA using 96-well microtitre plates (Nunc Immunoplate 1F, Gibco-Europe). The assay procedure required the sequential addition of,

1. 50 $mm^3$ per well of 5 $\mu g/cm^3$ rabbit anti-phytochrome immunoglobulin G (IgG) in 50 mol $m^{-3}$ sodium carbonate buffer (pH 9.6) for 2h at 25°C. The polyclonal antibody, 39/6, had been raised to oat phytochrome previously as described in Thomas *et al* (1984a).
2. 200 $mm^3$ per well of 2% (w/v) BSA in phosphate buffered saline (PBS) (pH 7.4) consisting of 8 $g/dm^3$ NaCl, 0.2 $g/dm^3$ $KH_2PO_4$, 1.44 $g/dm^3$ $Na_2HPO_4.2H_2O$ and 0.2 $g/dm^3$ KCl. The plates were blocked overnight at 4°C.
3. 50 $mm^3$ per well of either membrane samples or partially purified wheat phytochrome standards in PBS containing 0.05% (v/v) Tween 20 (PBS-Tween) and 1% (w/v) BSA for 2h at 4°C.
4. 50 $mm^3$ per well of 0.3 $\mu g/cm^3$ rat anti-phytochrome monoclonal antibody ARC MAC 56 (raised previously to oat phytochrome as described in Thomas *et al* 1984b) in PBS-Tween containing 1% (w/v) BSA for 2h at 25°C.
5. 50 $mm^3$ per well of a 1 in 4000 dilution of anti-rat IgG horse radish peroxidase (HRP) conjugate (ICN Biomedicals Ltd., High Wycombe, Bucks.) in PBS-Tween containing 1% (w/v) BSA for 1h at 25°C.
6. 100 $mm^3$ per well of peroxidase substrate consisting of 0.01% (w/v) 3,3',5,5'-tetramethylbenzidine (TMB) and 0.3% (w/v) hydrogen peroxide in 100 mol $m^{-3}$ sodium acetate adjusted to pH 6.0 with citric acid.

Between each stage the ELISA plates were washed three times with PBS-Tween, once briefly followed by two 5 min washes. The reaction was stopped after ~30 min with 25 $mm^3$ per well of $2x10^3$ mol $m^{-3}$ $H_2SO_4$ and the absorbance was measured at 450nm with a Bio-Rad model 2550 EIA reader.

### 2.13. Polyacrylamide gel electrophoresis (PAGE).

Samples were separated by SDS-PAGE according to the method of Laemmli (1970) and using a Bio-Rad mini protean II dual slab cell. 10% (w/v) acrylamide gels were used with 3% (w/v) stacking gels. The composition of the gel mixtures are given below.

| | running gel ($15cm^3$) | stacking gel ($10cm^3$) |
|---|---|---|
| acrylamide–bis (30% w/v) | 5.00 $cm^3$ | 1.00 $cm^3$ |
| $1.5x10^3$ mol $m^{-3}$ Tris–HCl pH 8.8 | 3.75 $cm^3$ | |
| $0.5x10^3$ mol $m^{-3}$ Tris–HCl pH 6.8 | | 2.25 $cm^3$ |
| 10% (w/v) SDS | 0.15 $cm^3$ | 0.05 $cm^3$ |
| 10% (w/v) ammonium persulphate | 0.15 $cm^3$ | 0.15 $cm^3$ |
| TEMED | 7.5 $mm^3$ | 2.5 $mm^3$ |
| water | 5.95 $cm^3$ | 6.50 $cm^3$ |

The acrylamide comprised of 28.8% (w/v) acrylamide and 1.2% (w/v) N,N' methylene bisacrylamide. The ammonium persulphate was added last and the gels poured immediately. After setting, the gels were placed in precooled running buffer containing 14.4 $g/dm^3$ glycine, 3.025 $g/dm^3$ Tris and 0.1% (w/v) SDS, (pH 8.3). The cell was surrounded with ice and run at 150V for ~1h using a Bio–Rad model 1000/500 power supply.

### 2.13.1. Preparation of samples.

Protein samples were diluted in $8x10^3$ mol $m^{-3}$ urea, 5% (v/v) 2–mercaptoethanol and 1% (w/v) SDS. Sample buffer at 5x the final concentration was then added. This contained 125 mol $m^{-3}$ Tris–HCl buffer (pH 6.8), 5% (w/v) SDS, 25% (v/v) glycerol, 2.5% (v/v) 2–mercaptoethanol and 1 drop of bromophenol blue. The samples were then boiled for at least 1 min, cooled and loaded using a volume of 5–15 $mm^3$ (1–3 $mg/cm^3$) per lane.

Prestained molecular weight markers (SDS 7B, Sigma Chemical Company Ltd, Poole, Dorset) were used with a loading of 3 $mm^3$ per lane. The standard molecular weights used were,

| protein | molecular weight (Da) |
|---|---|
| $\alpha_2$–macroglobulin | 180,000 |
| $\beta$–galactosidase | 116,000 |
| fructose–6–phosphate kinase | 84,000 |
| pyruvate kinase | 58,000 |
| fumarase | 48,500 |
| lactate dehydrogenase | 36,500 |
| triosephosphate isomerase | 26,600 |

## 2.14. Visualization of phytochrome on western blots.

### 2.14.1. Transfer to nitrocellulose.

Proteins separated on SDS-gels were transferred to nitrocellulose using a Bio-Rad mini trans-blot cell. Transfer was at 100V for 75 min using either a Bio-Rad model 1000/500 or a model 250/2.5 power supply. Cold blotting buffer containing 14.4 g/dm$^3$ glycine, 3.025 g/dm$^3$ Tris and 20% (v/v) methanol (pH 8.3) was used and the cell was surrounded with ice to prevent overheating.

### 2.14.2. Immunostaining of nitrocellulose.

After transfer the nitrocellulose was incubated sequentially with the following treatments.

1. 3% (w/v) dried, skimmed milk with 3 mol m$^{-3}$ sodium azide in Tris buffered saline (TBS) containing 50 mol m$^{-3}$ Tris-HCl buffer (pH 7.4) and 200 mol m$^{-3}$ NaCl overnight at 4°C.
2. Goat serum for 1h at RT.
3. Anti-oat phytochrome monoclonal antibodies in TBS containing 0.05% (v/v) Tween 20 (TBS-Tween) and 1% (w/v) BSA for 2-3h at RT. The antibodies and concentrations used are given in the appropriate figure legends.
4. A 1 in 2000 dilution of either anti-rat IgG HRP conjugate or anti-rat IgG alkaline phosphatase conjugate (Sigma Chemical Company Ltd., Poole, Dorset) in TBS-Tween containing 1% (w/v) BSA for 2h at RT.
5. The appropriate substrate. For the peroxidase reaction; 3 mg/cm$^3$ chloronapthol in methanol diluted 1 in 6 with TBS with 0.006% (w/v) hydrogen peroxide. For the phosphatase reaction; 150 mol m$^{-3}$ sodium barbitone (pH 9.6 with acetic acid) containing 4 mol m$^{-3}$ $MgCl_2$, 0.01% (w/v) nitroblue tetrazolium (NBT) and 0.05% (w/v) 5-bromo-4-chloro-3-indolylphosphate (BCIP) in dimethylformamide.

Between each stage the nitrocellulose was washed for three 5 min periods in TBS-Tween. The final wash, before the addition of the substrate, was in TBS or sodium barbitone (pH 9.6) as appropriate. Once the colour had developed, the reaction was stopped by placing the nitrocellulose into distilled water.

For some later experiments (see figure legends) a biotin-streptavidin amplification system was used. Instead of incubating directly with an anti-rat IgG enzyme conjugate, biotinylated goat anti-rat IgG (ICN Biomedicals Ltd., High

Wycombe, Bucks.) was added, diluted 1 in 2000 with TBS-Tween containing 1% (w/v) BSA. After incubation for 2h at RT, preformed streptavidin-biotinylated peroxidase complex was added, also for 2h at RT. The peroxidase substrate was then added as normal.

Quantitative data from western blots was obtained using a Bio-Rad model 620 video densitometer. This was interfaced to an Elonex PC-286 computer running a Bio-Rad 1-D Analyst program for IBM PC compatible computers.

### 2.15. Filtration of partially purified phytochrome.

Partially purified phytochrome (a gift from H. Carr-Smith) was equilibrated in a spin column consisting of Sephadex G-25 in a 2 $cm^3$ syringe. The Sephadex was pre-equilibrated with resuspension medium containing 250 mol $m^{-3}$ sucrose and 5 mol $m^{-3}$ Tris-Mes buffer (pH 7.4) and centrifuged at ~1500g for 3 min. A 0.2 $cm^3$ sample of partially purified phytochrome was added to the spin column which was again centrifuged at ~1500g for 3 min to give sample 1. The phytochrome was eluted with successive 0.2 $cm^3$ volumes of resuspension medium and samples 1-5 were assayed for phytochrome content, free phosphate ($P_i$) and phosphatase activity.

### 2.16. Chlorophyll determination.

The concentration of chlorophyll was determined by the method of Arnon (1949) after extraction in 80% (v/v) acetone overnight at 4°C.

### 2.17. Protein determination.

Protein was determined by the method described by Bradford (1976) using either Coomassie Brilliant Blue G-250 (Pierce Chemical Company, Rockford, Illinois, USA) for experiments conducted at Southampton or Bio-Rad Protein Assay (diluted 1:1 with water) for those at Littlehampton. BSA was used as the standard.

### 2.18. Chemicals.

Unless stated chemicals were analar grade and obtained form the Sigma Chemical Company Ltd (Poole, Dorset) or BDH Ltd. (Poole, Dorset). Purified Triton X-100 (see 5.3.4.) was obtained from the Pierce Chemical Company, Rockford, Illinois, USA. The inhibitor, SW26 was a gift from Dr. J-P. Blein, Institut National de la Recherche Agronomique, France.

# CHAPTER THREE

# CHARACTERIZATION OF MICROSOMAL ATPASE ACTIVITY AND PRELIMINARY ATTEMPTS TO PURIFY PLASMA MEMBRANES

## 3.1 INTRODUCTION

There are only a few reports concerning the nature of ATPase activity in wheat. Of these, most have been conducted with roots and very little is known about ATPase activity in wheat coleoptiles or leaves. The first study of ATPase activity in wheat was made by Kylin and Kahr (1973) who compared the properties of ATPases in roots from wheat and oat. They showed that microsomal activity from wheat was preferentially stimulated by $Ca^{2+}$ ions over $Mg^{2+}$ ions, a result that was in direct contrast to that for oat roots. Furthermore, the amount of ATPase activity in wheat was considerably greater. They also demonstrated a striking difference in the pH profiles of the ATPase activities between the two species. In wheat roots, both $Mg^{2+}$– and $Ca^{2+}$–dependent activities were highest at pH 5.2 (the lowest pH examined) declining steadily as the pH increased. The $Mg^{2+}$–dependent activity in oat roots was lowest at pH 5.2, rising sharply to a maximum at pH 6.5, where it remained up to pH 7.7, the highest pH value examined. Only very low levels of $Ca^{2+}$–dependent activity were found in the oat roots. In a more detailed study of the effects of $Mg^{2+}$ and $Ca^{2+}$ ions, Kahr and Moller (1976) supported these earlier findings and also demonstrated that the wheat enzyme was highly active in the absence of either cation.

Confirmation that ATPase activity from wheat shoots also exhibits unusual properties came from Hall *et al* (1982). They looked at ATPase activity from wheat coleoptiles and also measured more $Ca^{2+}$–dependent activity than $Mg^{2+}$–dependent activity. There was little stimulation by $K^+$ ions and the pH optimum was found to be 5.5.

There have been a number of attempts to purify plasma membranes from wheat. Pomeroy and McMurchie (1982) used a discontinuous sucrose gradient to compare plasma membrane preparations from wheat shoots and cauliflower florets. In wheat shoots presumptive plasma membranes from a 34/45% (w/w) fraction exhibited properties that were similar to those described for microsomal preparations. There was more activity with $Ca^{2+}$ than $Mg^{2+}$ ions and $K^+$ stimulation was very low (≤25%). Substrate specificity was low and activity was almost as high with ADP as it was with ATP. After using similar isolation methods with cauliflower the plasma membrane ATPase activity had the properties normally associated with the plasma membrane $H^+$–ATPase. These included high

$K^+$ stimulation, inhibition by $Ca^{2+}$ ions and little activity with substrates other than ATP. This study also included a comparison of plasma membranes from wheat shoots and roots after homogenization in a variety of conditions. In general, ATPase activity from roots was similar to that of shoots although there was slightly more $K^+$ stimulation of the root plasma membrane ATPase.

Plasma membranes have also been prepared from wheat roots by aqueous polymer two-phase partition (Lundborg *et al* 1981, Sommarin *et al* 1985). In the first of these papers it was reported that plasma membrane ATPase activity was stimulated by $Ca^{2+}$ ions more than $Mg^{2+}$ or $Mn^{2+}$ ions. There was no separation of these activities by differential centrifugation or by phase partition using a range of phase systems and counter-current distribution. In the second paper, Sommarin *et al* (1985) compared ATPase activity from plasma membranes of wheat and oat roots. The wheat root ATPase had an unusual pH profile but did exhibit $K^+$ stimulation of the $Mg^{2+}$-dependent activity. This stimulation was maximal (80%) at pH 6.2. The substrate specificity was also examined. Again the wheat root enzyme had considerable activity with all nucleoside di- and triphosphates with the surprising exception of ADP.

The importance of working with a purified plasma membrane preparation, free from contaminating ATPase activity, to investigate the effect of light on the plasma membrane $H^+$-ATPase has already been outlined (see Chapter 1). The work in this chapter describes the characterization of the microsomal ATPase activity from wheat leaves and also experiments undertaken to isolate a plasma membrane-enriched membrane fraction. All experiments were performed at Southampton.

## 3.2. EXPERIMENTAL

All ATPase assays were performed at pH 6.0 unless stated in the appropriate figure legend. Where the results are presented as mean % of control, the control values were as follows: For divalent cations (or no addition) the upper value was the control value. For the addition of monovalent cations or inhibitors the control value was the activity with the respective divalent cation alone. When the inhibitor $NO_3^-$ was used it was added as $KNO_3$ and the control value was the activity measured with KCl. Molybdate (0.1 mol $m^{-3}$) was routinely included in all ATPase assays. Inhibition by molybdate is expressed as a % of activity when molybdate was omitted from the assay. The control value for alternative substrates was the activity in the presence of ATP under the same assay conditions.

## 3.3. RESULTS

### 3.3.1. Characterization of microsomal ATPase activity.

In order to understand more clearly the nature of the ATPase activity present in wheat leaves a preliminary characterization of the microsomal ATPase activity was undertaken. Table 3.1 shows the specific activities of the microsomal ATPase measured in the presence and absence of divalent cations. A number of important points are immediately obvious. The specific activities measured were very high and there was considerable variation between preparations. There were also very high levels of activity in the absence of a divalent cation which is unusual in microsomal preparations from higher plants.

#### 3.3.1.1. Ion specificity.

The activity in the presence of both monovalent and divalent cations is shown in more detail in Table 3.2. So that the variation in specific activities between preparations does not obscure the error within treatments, these data are presented as a percentage of the control activity. $Ca^{2+}$ ions stimulated ATPase activity to a greater degree than $Mg^{2+}$ ions (see also Table 3.1). Both were equally effective in the presence of $SO_4^-$ ions and $Cl^-$ ions and did not appear to be additive, suggesting that they were both stimulating the same enzyme. There was no stimulation with the addition of any monovalent cation and in some membrane preparations they were inhibitory. Only a small proportion of the total ATPase activity was inhibited, and inhibition by KCl never exceeded 25%, even with the addition of 500 mol $m^{-3}$ KCl (data not shown).

Figure 3.1 shows the effect on microsomal ATPase activity of changing $Mg^{2+}$ and $Ca^{2+}$ ion concentrations. Activation by both $Ca^{2+}$ and $Mg^{2+}$ was saturated at low concentrations ($\leq$0.5 mol $m^{-3}$) and at higher concentrations ($>$5 mol $m^{-3}$) $Mg^{2+}$ was inhibitory.

#### 3.3.1.2. The effect of pH.

Figure 3.2 shows the pH profile for microsomal ATPase activity. Activity was highest at pH 6.0 and the $Mg^{2+}$–stimulated component had a pH optimum of 5.5. At higher pH values the activity was more dependent on $Ca^{2+}$ ions than $Mg^{2+}$ ions.

| Ions | Specific activity ($\mu$mol $P_i$/mg protein/h) |
|---|---|
| none | 146.84 ± 30.24 |
| $MgCl_2$ | 184.12 ± 49.23 |
| $CaCl_2$ | 244.79 ± 50.57 |

Table 3.1: Microsomal ATPase activity from wheat leaves measured in the presence and absence of divalent cations (2 mol $m^{-3}$) and in the absence of KCl. Results are from several experiments and values are expressed as mean specific activity ± standard error ($n \geq 3$).

| Ions | % of control |
|---|---|
| none | 100.00 ± 1.28 |
| $MgCl_2$ | 120.20 ± 4.20 |
| $MgSO_4$ | 131.22 ± 10.82 |
| $CaCl_2$ | 156.74 ± 4.18 |
| $CaSO_4$ | 145.16 ± 7.47 |
| $MgCl_2$ + $CaCl_2$ | 159.44 ± 4.41 |
| $MgCl_2$ | 100.00 ± 0.73 |
| $MgCl_2$ + KCl | 89.62 ± 4.41 |
| $MgCl_2$ + RbCl | 98.85 ± 2.56 |
| $MgCl_2$ + NaCl | 91.53 ± 3.32 |
| $MgCl_2$ + LiCl | 98.12 ± 1.22 |
| $CaCl_2$ | 100.00 ± 0.81 |
| $CaCl_2$ + KCl | 93.61 ± 1.59 |
| $CaCl_2$ + RbCl | 92.93 ± 2.19 |
| $CaCl_2$ + NaCl | 94.63 ± 1.90 |
| $CaCl_2$ + LiCl | 90.10 ± 3.54 |

Table 3.2: The effects of divalent (2 mol $m^{-3}$) and monovalent (50 mol $m^{-3}$) cations on microsomal ATPase activity from wheat leaves. Results are from several experiments and values are expressed as mean % of control ± standard error ($n \geq 3$).

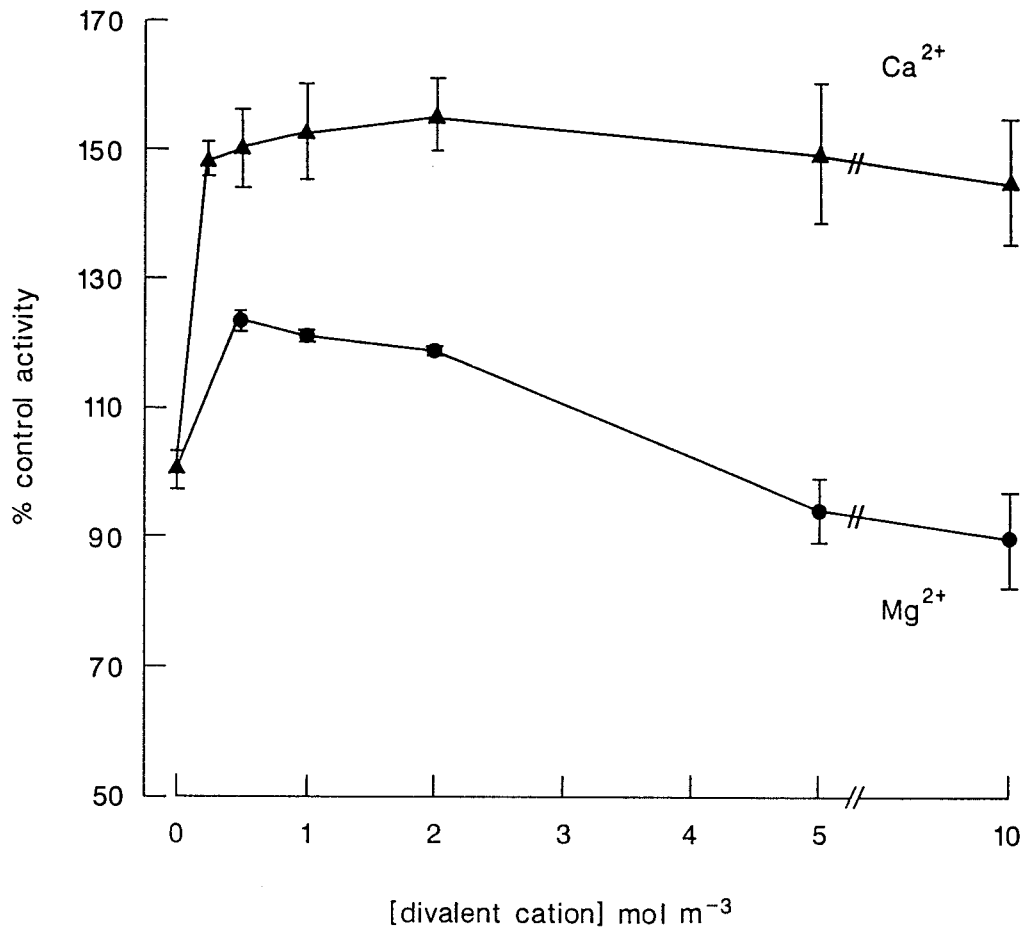


Figure 3.1: The effect of $Mg^{2+}$ and $Ca^{2+}$ ion concentration on microsomal ATPase activity from wheat leaves measured in the absence of KCl. Results are from two ($Mg^{2+}$) or three ($Ca^{2+}$) experiments and points are expressed as mean % of control ± standard error ($n \geq 2$).

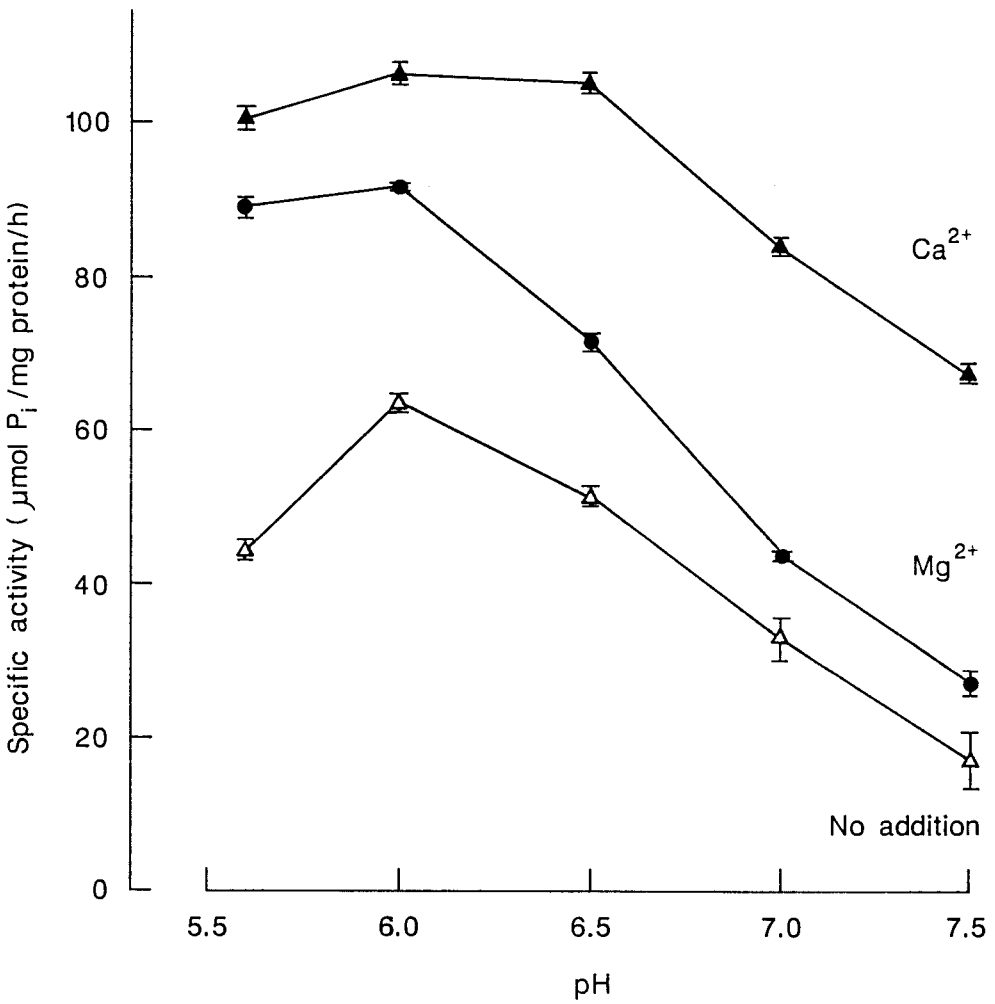


Figure 3.2: The effect of pH on microsomal ATPase activity from wheat leaves measured in the presence of different divalent cations (2 mol $m^{-3}$) and in the absence of KCl. Results are from a single experiment and points are expressed as mean specific activity ± standard error (n=3).

### 3.3.1.3. The effect of inhibitors.

A number of common ATPase inhibitors were tested in an attempt to identify the origin of the ATPase activity measured (Table 3.3). Ammonium molybdate is an inhibitor of acid phosphatases (Gallagher and Leonard 1982) and was routinely included in all ATPase assays. It was found to have a limited inhibitory effect under all conditions examined. The mitochondrial $H^+$–ATPase inhibitor, sodium azide, did not inhibit activity under any conditions nor did the tonoplast inhibitor, nitrate. Vanadate was the most effective inhibitor tested, with activity in the presence of $Mg^{2+}$ ions the most sensitive to it. The action of vanadate was examined more closely in Figure 3.3 which shows the inhibition of both $Mg^{2+}$– and $Ca^{2+}$–dependent ATPase activity at different vanadate concentrations.

### 3.3.1.4. Substrate specificity.

Table 3.4 demonstrates that the microsomal ATPase activity had very low substrate specificity, and was active with all nucleoside phosphates tested. There was, however, no activity with pyrophosphate and little with $\rho$–nitrophenyl phosphate or $\beta$–glycerophosphate which indicates that, in the presence of 0.1 mol $m^{-3}$ ammonium molybdate, acid phosphatase activity is a small component of the total ATPase activity measured in this preparation. One interesting point is that activity in the absence of a divalent cation can utilize ADP and AMP more effectively than $Mg^{2+}$– or $Ca^{2+}$–dependent activity. This trend is reversed with GTP as the substrate.

## 3.3.2. Characterization of proton transport activity.

To help clarify the nature of the microsomal ATPase activity, the proton transport activity of the microsomal membrane preparation was investigated. Under the conditions used only a small amount of ATP–driven proton transport was detected, but by amplifying the fluorescence quench measured some useful qualitative information was obtained.

Figure 3.4 shows the fluorescence quench in the presence of $MgSO_4$ and ATP. It was demonstrated that this was due to the transport of protons by the addition of the protonophore, gramicidin, which dissipates the proton gradient. $CaCl_2$ could not replace $MgSO_4$ and also prevented proton transport in the presence $MgSO_4$. The complexity of the situation was increased by the finding that $CaCl_2$ could dissipate all of the $Mg^{2+}$–dependent gradient but that, in the absence

| Ions | Inhibitor | % of control |
|---|---|---|
| none | none | 100.00 ± 0.86 |
| none | vanadate | 87.62 ± 1.00 |
| none | azide | 99.68 ± 2.38 |
| none | molybdate | 85.62 ± 4.21 |
| $MgCl_2$ | none | 100.00 ± 0.77 |
| $MgCl_2$ | vanadate | 65.28 ± 1.15 |
| $MgCl_2$ | azide | 102.97 ± 1.22 |
| $MgCl_2$ | molybdate | 86.39 ± 1.56 |
| $MgCl_2$ + KCl | none | 100.00 ± 2.02 |
| $MgCl_2$ + KCl | vanadate | 73.91 ± 2.14 |
| $MgCl_2$ + KCl | azide | 98.83 ± 1.02 |
| $MgCl_2$ + KCl | molybdate | 91.96 ± 0.69 |
| $MgCl_2$ + $K^+$ | $NO_3^-$ | 100.27 ± 3.12 |
| $CaCl_2$ | none | 100.00 ± 0.65 |
| $CaCl_2$ | vanadate | 83.61 ± 1.72 |
| $CaCl_2$ | azide | 101.56 ± 1.45 |
| $CaCl_2$ | molybdate | 92.52 ± 0.84 |
| $CaCl_2$ + $K^+$ | $NO_3^-$ | 98.70 ± 1.62 |

Table 3.3: The effects of inhibitors on microsomal ATPase activity from wheat leaves. Inhibitors used were sodium vanadate (0.1 mol $m^{-3}$), sodium azide (1 mol $m^{-3}$), ammonium molybdate (0.1 mol $m^{-3}$) and potassium nitrate (50 mol $m^{-3}$). Results are from several experiments and values are expressed as mean % of control ± standard error ($n \geq 3$).

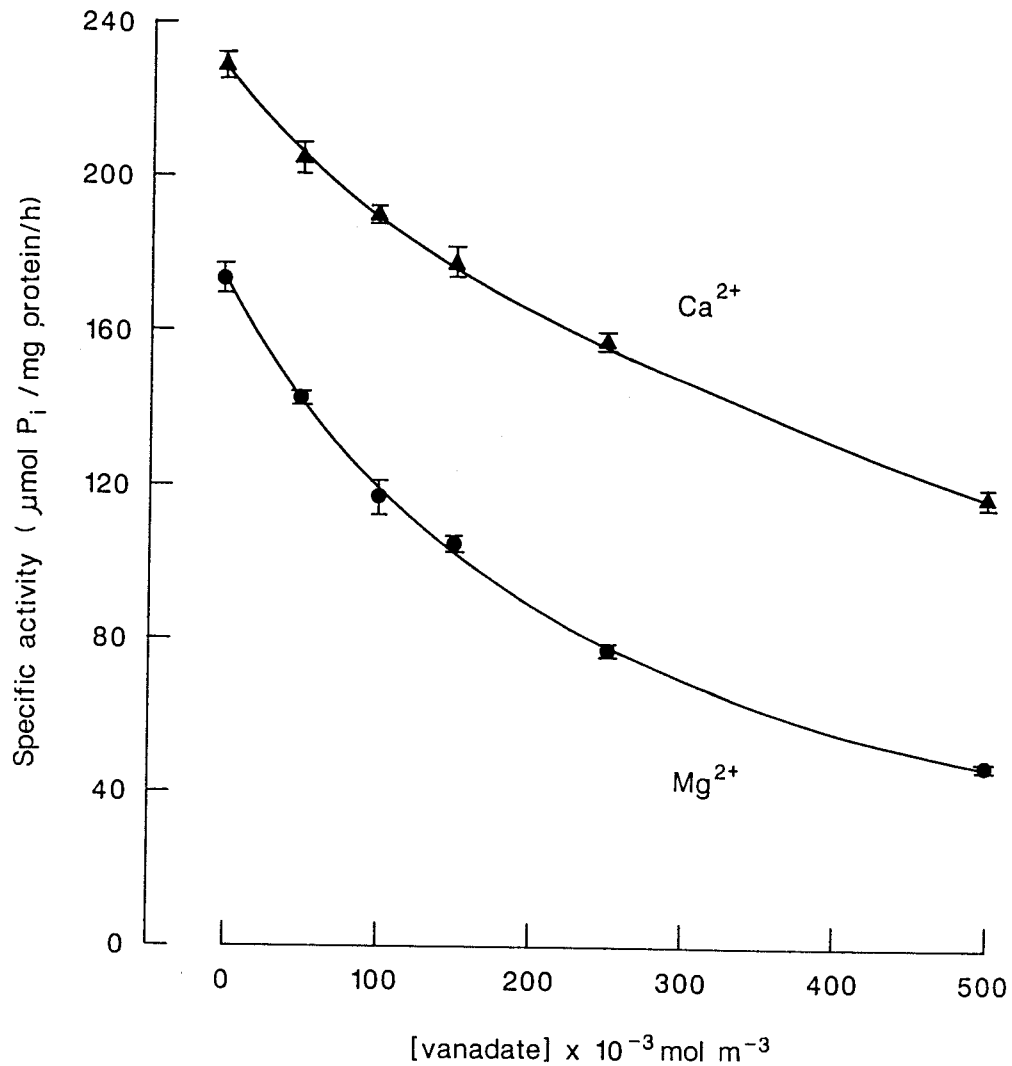


Figure 3.3: The effect of sodium vanadate concentration on microsomal ATPase activity from wheat leaves measured in the presence of $Mg^{2+}$ and $Ca^{2+}$ ions (2 mol $m^{-3}$) and in the absence of KCl. Results are from a single experiment and points are expressed as mean specific activity ± standard error ($n \geq 2$).

| Substrate | % of control | | |
|---|---|---|---|
| | None | $MgCl_2$ | $CaCl_2$ |
| ATP | 100.00 ± 1.56 | 100.00 ± 1.00 | 100.00 ± 1.62 |
| GTP | 44.17 ± 4.41 | 87.94 ± 4.57 | 75.99 ± 3.77 |
| ITP | 79.82 ± 5.08 | 79.22 ± 5.26 | 87.57 ± 4.65 |
| CTP | - | 79.80 ± 1.71 | - |
| ADP | 140.59 ± 7.02 | 103.79 ± 1.01 | 98.86 ± 2.07 |
| AMP | 89.00 ± 1.43 | 55.17 ± 0.18 | 41.21 ± 0.41 |
| $\rho$-NPP | 26.43 ± 0.66 | 24.91 ± 0.70 | 11.54 ± 0.34 |
| $\beta$-GP | - | 2.27 ± 0.18 | - |
| $PP_i$ | - | 0.00 ± 0.00 | - |

Table 3.4: The substrate specificity of microsomal ATPase activity from wheat leaves measured in the presence of different divalent cations and in the absence of KCl. Results are from several experiments and values are expressed as mean % of control ± standard error ($n \geq 2$).

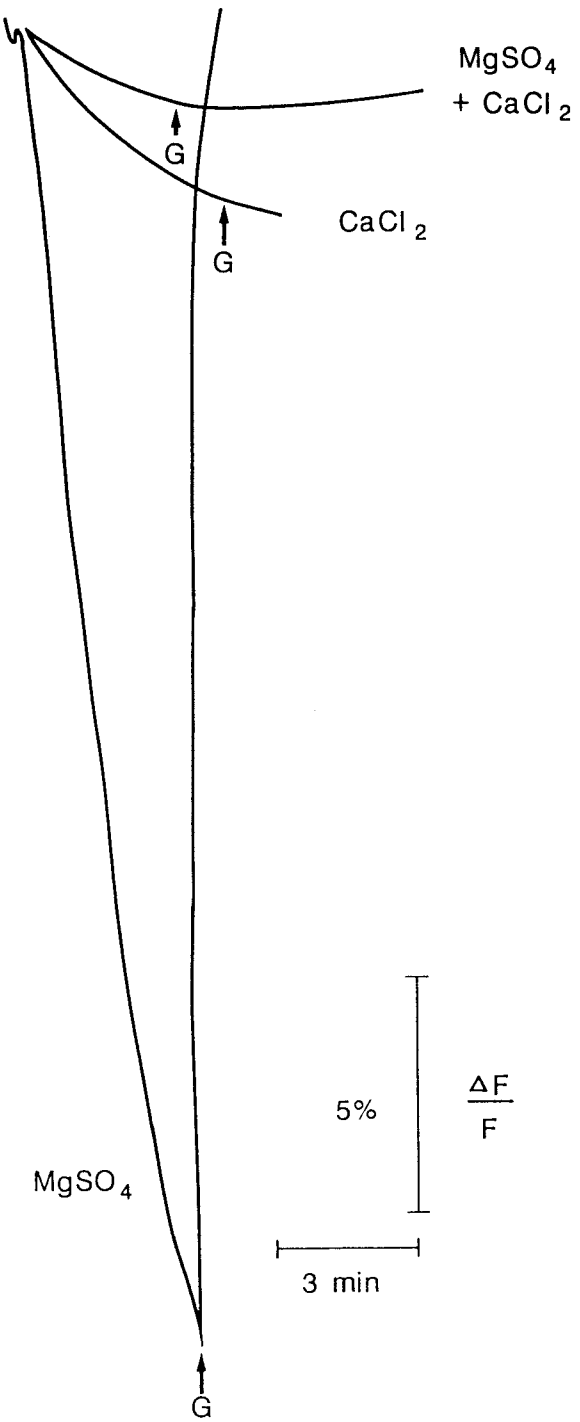


Figure 3.4: The effect of divalent cations (2 mol $m^{-3}$) on ATP-dependent quinacrine fluorescence quenching in a microsomal fraction from wheat leaves. G marks the point of addition of gramicidin at a final concentration of $2x10^{-2}$ mol $m^{-3}$. Results are from a single representative experiment.

of KCl, a small $Ca^{2+}$–dependent gradient could be generated (Figure 3.5).

ATP–dependent proton pumping in plant cells is principally attributable to the plasma membrane and tonoplast $H^+$–ATPases. The use of the inhibitors vanadate and nitrate demonstrated that proton transport in wheat leaf microsomal preparations was also mediated through these two proton pumps (Figure 3.6). Over 3 experiments vanadate and nitrate inhibition was 70±8% and 71±4% of the total proton transport activity respectively. It is not clear why the combined inhibition exceeds 100%. One of the inhibitors could be acting at both pumps, although there are no previous reports of this. Indeed, $NO_3^-$ ions have been shown to stimulate the plasma membrane $H^+$–ATPase in some systems (*e.g.* Giannini *et al* 1987). An alternative explanation is that, because the tonoplast $H^+$–ATPase is stimulated by $Cl^-$ ions (O'Neill *et al* 1983), the addition of nitrate also results in the loss of $Cl^-$ stimulation, causing an overestimation of nitrate inhibition. A third possibility is that it is due to error. Because the signal has been amplified, any error associated with the measurement of inhibitor action will also have been amplified.

Despite this inconsistency, it was still clear that the greater proportion of the ATPase activity measured by the release of inorganic phosphate did not originate from the two proton pumps. In order that the ATPase activity associated with these proton pumps could be characterized, it was necessary to remove this additional non–specific ATPase activity.

### 3.3.3. KI wash of the microsomal preparation.

Washing membrane preparations with KI results in the removal of proteins loosely associated with the membranes. KI can break both hydrophobic and hydrophilic bonds (Maddy and Dunn 1976) and the treatment is designed to remove any non–specific phosphatase activity that might be associated with the membrane preparations (Briskin and Poole 1983). The treatment has also been shown to inhibit the tonoplast $H^+$–ATPase (De Michelis and Spanswick 1986).

Microsomal membranes were treated with 250 mol $m^{-3}$ KI. This resulted in a loss of 74% of the total microsomal ATPase activity. Table 3.5 shows the effect of a KI wash on the properties of the remaining activity. There was a decrease in both $Mg^{2+}$ and $Ca^{2+}$ stimulation but no significant increase in vanadate inhibition in the presence of $Mg^{2+}$. There was, however, an increase in vanadate sensitivity with the $Ca^{2+}$–dependent ATPase activity.

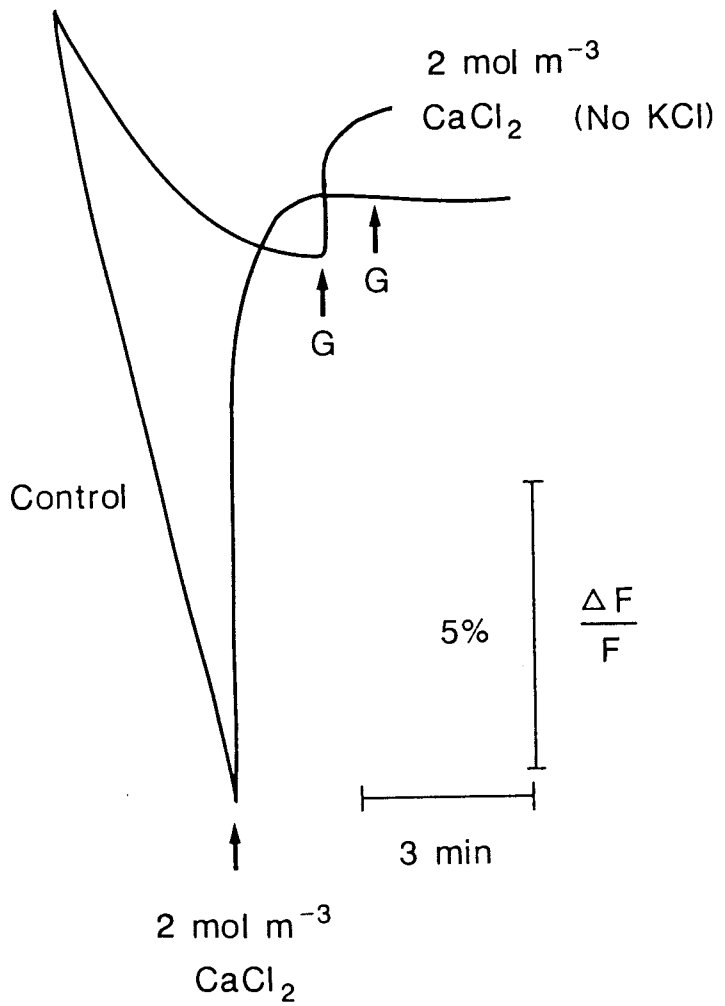


Figure 3.5: The effect of either replacing $MgSO_4$ with $CaCl_2$ (in the absence of KCl) or adding $CaCl_2$ on $Mg^{2+}$– and ATP–dependent quinacrine fluorescence quenching in a microsomal fraction from wheat leaves. G marks the point of addition of gramicidin at a final concentration of $2x10^{-2}$ mol $m^{-3}$. Results are from a single experiment.

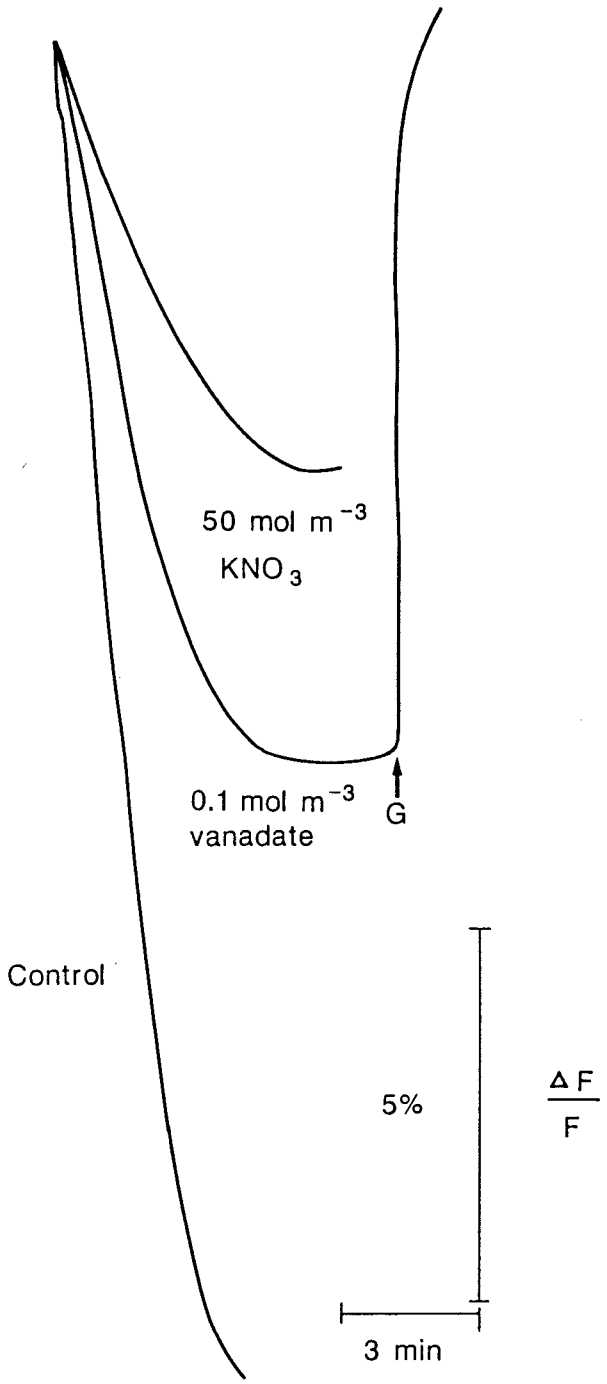


Figure 3.6: The effect of sodium vanadate (0.1 mol $m^{-3}$) and $KNO_3$ (50 mol $m^{-3}$) on $Mg^{2+}$– and ATP–dependent quinacrine fluorescence quenching in a microsomal fraction from wheat leaves. G marks the point of addition of gramicidin at a final concentration of $2x10^{-2}$ mol $m^{-3}$. Results are from a single representative experiment.

| Assay conditions | % of control | |
|---|---|---|
| | -KI | +KI |
| no addition | 100.00 ± 0.38 | 100.00 ± 1.40 |
| $MgCl_2$ | 112.88 ± 9.08 | 72.23 ± 8.43 |
| $CaCl_2$ | 149.69 ± 7.95 | 114.87 ± 6.83 |
| $MgCl_2$ + vanadate | 65.62 ± 1.88 | 60.19 ± 7.28 |
| $CaCl_2$ + vanadate | 82.92 ± 2.51 | 52.65 ± 4.81 |

Table 3.5: The effect of washing microsomal membranes from wheat leaves with 250 mol $m^{-3}$ KI on ATPase activity assayed in the absence of KCl. Results are from two experiments and values are expressed as mean % of control ± standard error ($n \geq 5$).

### 3.3.4. Separation of microsomal membranes by discontinuous sucrose density gradients.

Because a substantial proportion of the non-specific ATPase activity appeared to be membrane associated, membrane purification techniques were used in an attempt to remove this activity. A microsomal fraction was loaded onto a discontinuous sucrose gradient comprised of 4 steps. In the standard gradient these were 20, 25, 34 and 38% (w/w) sucrose (see 2.3.1.). The properties of the ATPase activity at the 3 interfaces were investigated and are shown in Table 3.6. Plasma membranes have a density of 1.13-1.17 g/cm$^3$ and would be expected to band at the 34/38% interface (Hall 1983). There was some indication of plasma membrane enrichment in this fraction. Inhibition by vanadate in the presence of $Mg^{2+}$ was greater than in microsomal preparations (with or without a KI treatment) and KCl no longer inhibited ATPase activity. The $Ca^{2+}$-stimulated activity was also more vanadate sensitive.

The distribution of $Mg^{2+}$- and $Ca^{2+}$-dependent activity was surprising. At the top of the gradient (the 20/25% interface) both $Mg^{2+}$ and $Ca^{2+}$ stimulation was greater than in microsomal preparations. However, further down the gradient stimulation, particularly by $Mg^{2+}$, was considerably reduced. The absence of any $Mg^{2+}$-dependent activity is not consistent with an enrichment of plasma membranes in the 34/38% fraction. There was no nitrate or azide inhibition in any fraction with the single exception of the azide inhibition of the $Ca^{2+}$-dependent activity from the 34/38% interface. It is unlikely that this is due to inhibition of the mitochondrial $H^+$-ATPase as this enzyme is not stimulated by $Ca^{2+}$ ions and has a higher pH optimum (Sze 1985).

To try to enhance the possible plasma membrane-enrichment an extra step (30% w/w) was included in the sucrose gradient. This did not result in any apparent improvement in membrane separation (data not shown).

### 3.3.5. Separation of KI-treated microsomal membranes by sucrose density gradients.

Table 3.7 shows ATPase activity from the standard sucrose gradient after the microsomal membranes had been washed in 250 mol m$^{-3}$ KI prior to loading onto the gradient. There was a small increase in vanadate sensitivity in the 34/38% fraction in the presence of $Ca^{2+}$ ions but no change with $Mg^{2+}$. There was also a general increase in vanadate inhibition throughout the whole gradient and in $K^+$ stimulation of the $Mg^{2+}$-dependent activity. In particular, the level of $K^+$ stimulation in the 34/38% fraction was considerably higher than for previous separations.

| Assay conditions | % of control | | |
|---|---|---|---|
| | 20/25% | 25/34% | 34/38% |
| no addition | 100.00 ± 2.03 | 100.00 ± 1.32 | 100.00 ± 1.19 |
| $MgCl_2$ | 127.22 ± 4.18 | 95.02 ± 2.69 | 91.39 ± 2.00 |
| $CaCl_2$ | 161.96 ± 6.92 | 118.48 ± 2.82 | 111.28 ± 2.92 |
| $MgCl_2$ + KCl | 95.72 ± 2.82 | 106.45 ± 1.84 | 104.47 ± 2.12 |
| $MgCl_2$ + vanadate | 63.46 ± 2.67 | 70.17 ± 4.36 | 58.67 ± 3.70 |
| $MgCl_2$ + azide | 98.89 ± 2.33 | 103.12 ± 8.27 | 103.06 ± 9.44 |
| $MgCl_2$ + $KNO_3$ | 105.77 ± 3.38 | 99.47 ± 1.37 | 99.31 ± 2.13 |
| $CaCl_2$ + KCl | 103.06 ± 3.31 | 103.08 ± 1.86 | 102.78 ± 1.21 |
| $CaCl_2$ + vanadate | 85.44 ± 2.36 | 85.94 ± 5.78 | 70.03 ± 2.93 |
| $CaCl_2$ + azide | 108.18 ± 3.29 | 100.04 ± 9.15 | 87.00 ± 5.10 |
| $CaCl_2$ + $KNO_3$ | 97.51 ± 4.45 | 100.26 ± 1.63 | 99.45 ± 0.98 |

Table 3.6: The separation of ATPase activity from wheat leaves by a discontinuous sucrose density gradient. ATPase activity was assayed using samples taken from 3 interfaces on the gradient. Results are from seven experiments and values are expressed as mean % of control ± standard error ($n \geq 6$).

| Assay conditions | % of control | | |
|---|---|---|---|
| | 20/25% | 25/34% | 34/38% |
| no addition | 100.00 ±12.70 | 100.00 ± 1.37 | 100.00 ± 6.37 |
| $MgCl_2$ | 134.35 ± 5.98 | 94.44 ± 5.25 | 78.11 ± 7.89 |
| $CaCl_2$ | 203.04 ± 3.13 | 148.81 ± 3.15 | 113.70 ± 8.83 |
| $MgCl_2$ + KCl | 102.91 ± 2.24 | 112.61 ± 5.11 | 119.59 ± 6.23 |
| $MgCl_2$ + vanadate | 58.57 ± 0.65 | 60.50 ± 4.43 | 58.97 ± 3.44 |
| $MgCl_2$ + $KNO_3$ | 101.88 ± 3.40 | 96.27 ± 2.33 | 85.91 ± 9.78 |
| $CaCl_2$ + KCl | 100.43 ± 1.19 | 103.47 ± 0.27 | 97.65 ± 5.82 |
| $CaCl_2$ + vanadate | 77.94 ± 2.78 | 80.80 ± 4.00 | 63.57 ± 6.35 |
| $CaCl_2$ + $KNO_3$ | 100.64 ± 2.16 | 109.54 ± 3.23 | 111.71 ±10.43 |

Table 3.7: The separation of microsomal ATPase activity from wheat leaves by a sucrose density gradient after treating the membrane preparation with 250 mol $m^{-3}$ KI. ATPase activity was assayed using samples taken from 3 interfaces on the gradient. Results are from a single experiment and values are expressed as mean % of control ± standard error (n>2).

The possibility that homogenization in the presence of 250 mol $m^{-3}$ KI would be more effective than washing the isolated membranes was also investigated. The results from these experiments are shown in Table 3.8. There did not appear to be any plasma membrane enrichment in the 34/38% fraction with the 20/25% fraction exhibiting both the highest $K^+$ stimulation and the most vanadate sensitivity.

### 3.3.6. A method to separate $Ca^{2+}$-phosphatase activity.

$Ca^{2+}$-phosphatase activity was successfully separated from $Mg^{2+}$-ATPase activity in barley roots by DuPont and Hurkman (1985). Many of the characteristics of microsomal ATPase activity from barley are similar to those found in wheat (see 3.4.) and it was thought that this technique might be successfully applied to the wheat leaf system.

The technique required the tissue to be homogenized at a higher pH (8.0), with a buffer to tissue ratio of 8:1, before separation on a sucrose gradient. EDTA was included in both the homogenization medium and the gradient (see 2.3.1. for details). The $Ca^{2+}$-phosphatase activity is found at the top of the gradient and to trap this activity an extra sucrose step (15% w/w) was included.

The results of this treatment are shown in Table 3.9. At the 3 interfaces normally examined, the properties and distribution of the ATPase activity measured remained essentially unchanged from those of other sucrose gradients.

### 3.3.7. Plasma membrane purification by aqueous polymer two-phase partition.

#### 3.3.7.1. Selection of a suitable phase system.

Table 3.10 shows the results from a preliminary experiment performed to assess the correct phase system to use. From these data the membrane fraction separated by 6.3% phase system appeared to be the most plasma membrane enriched. ATPase activity from this fraction had most vanadate sensitivity, showed the highest stimulation by $KNO_3$ and a low specific activity with $Mg^{2+}$ ions, a characteristic that appeared to be associated with high vanadate inhibition, and therefore with plasma membranes, on sucrose gradients. The 6.3% phase system was chosen for further examination.

#### 3.3.7.2. Plasma membrane isolation using a 6.3% phase system.

Table 3.11 shows the properties of the ATPase activity present in the

| Assay conditions | % of control | | |
|---|---|---|---|
| | 20/25% | 25/34% | 34/38% |
| no addition | 100.00 ± 4.88 | 100.00 ± 1.02 | 100.00 ± 4.11 |
| $MgCl_2$ | 95.93 ± 0.81 | 91.65 ± 0.61 | 84.54 ± 1.00 |
| $CaCl_2$ | 160.97 ± 3.72 | 124.03 ± 1.76 | 109.82 ± 2.09 |
| $MgCl_2$ + KCl | 111.86 ± 1.47 | 110.67 ± 2.78 | 107.81 ± 2.67 |
| $MgCl_2$ + vanadate | 60.17 ± 5.56 | 76.67 ± 0.38 | 71.81 ± 0.69 |
| $MgCl_2$ + $KNO_3$ | 117.42 ± 0.76 | 105.02 ± 1.12 | 100.53 ± 0.71 |
| $CaCl_2$ + KCl | 102.35 ± 2.62 | 99.84 ± 3.23 | 100.29 ± 2.03 |
| $CaCl_2$ + vanadate | 66.67 ± 2.31 | 78.62 ± 0.34 | 80.06 ± 1.55 |
| $CaCl_2$ + $KNO_3$ | 107.43 ± 2.16 | 103.78 ± 0.72 | 105.70 ± 1.54 |

Table 3.8: The separation of microsomal ATPase activity from wheat leaves by a sucrose density gradient after homogenization in the presence of 250 mol $m^{-3}$ KI. ATPase activity was assayed using samples taken from 3 interfaces on the gradient. Results are from a single experiment and values are expressed as mean % of control ± standard error (n=3).

| Assay conditions | % of control | | | |
|---|---|---|---|---|
| | 15/20% | 20/25% | 25/34% | 34/38% |
| no addition | 100.00 ± 5.54 | 100.00 ± 2.58 | 100.00 ± 1.76 | 100.00 ± 2.39 |
| $MgCl_2$ | 154.16 ±16.40 | 108.19 ± 4.78 | 92.83 ± 1.46 | 90.57 ± 2.24 |
| $CaCl_2$ | 216.98 ±24.45 | 157.20 ±11.54 | 121.90 ± 1.58 | 112.97 ± 2.86 |
| $MgCl_2$ + vanadate | 67.64 ± 2.58 | 74.89 ± 1.35 | 72.26 ± 2.87 | 75.07 ± 3.64 |
| $MgCl_2$ + $KNO_3$ | 104.95 ± 4.12 | 112.49 ± 3.83 | 102.26 ± 1.56 | 103.61 ± 1.17 |

Table 3.9: The separation of microsomal ATPase activity from wheat leaves by a sucrose density gradient containing EDTA, after homogenization at pH 8.0 with EDTA and a high buffer to tissue ratio. The control value for the addition of $KNO_3$ is $MgCl_2$ alone. Results are from two experiments and values are expressed as mean % of control ± standard error (n=6).

| Assay conditions | % of control | | | | |
|---|---|---|---|---|---|
| | 5.7% | 5.9% | 6.1% | 6.3% | 6.5% |
| no addition | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| $MgCl_2$ | 86.3 | 89.9 | 84.6 | 76.8 | 90.4 |
| $CaCl_2$ | 111.2 | 110.7 | 126.7 | 134.6 | 112.9 |
| $MgCl_2$ + vanadate | 76.1 | 78.9 | 64.7 | 53.6 | 76.7 |
| $MgCl_2$ + $KNO_3$ | 100.3 | 107.5 | 112.1 | 117.4 | 98.6 |
| Specific activity ($\mu$mol $P_i$/mg protein/h) | 73.4 | 66.2 | 43.8 | 44.2 | 76.2 |

Table 3.10: The properties of ATPase activity from wheat leaves in $U_1$ after partitioning in a range of phase systems. The control value for the addition of $KNO_3$ is $MgCl_2$ alone. Results are from a single experiment and values are expressed as mean % of control and specific activity (n=2).

| Assay conditions | % of control |
|---|---|
| $MgCl_2$ | 100.00 ± 1.03 |
| no addition | 81.05 ± 5.56 |
| $CaCl_2$ | 106.00 ± 4.08 |
| KCl | 74.67 ± 9.50 |
| $MgCl_2$ + KCl | 108.87 ± 2.31 |
| $MgCl_2$ + vanadate | 62.69 ± 4.79 |
| $MgCl_2$ + molybdate | 89.63 ± 1.58 |
| $MgCl_2$ + $KNO_3$ | 97.93 ± 0.59 |
| $MgCl_2$ + KCl + vanadate | 60.09 ± 3.89 |
| $MgCl_2$ + KCl + molybdate | 87.24 ± 3.77 |
| ADP + $MgCl_2$ | 105.25 ± 3.88 |
| ADP + $MgCl_2$ + KCl | 104.67 ± 2.09 |

Table 3.11: The properties of ATPase activity from wheat leaves in $U_3$ after partitioning in a 6.3% phase system. Results are from several experiments and values are expressed as mean % of control ± standard error ($n \geq 4$).

$U_3$ fraction after partitioning in a 6.3% two-phase system which included 3 mol $m^{-3}$ KCl. The addition of KCl was previously found to improve separation (data not shown). This $U_3$ fraction did not appear to be more plasma membrane enriched than the 34/38% fraction from the standard sucrose gradient. Both inhibition by vanadate and $K^+$ stimulation were similar, but some $Mg^{2+}$-dependent activity was now present. As with microsomal membranes, ADP was able to substitute for ATP.

In view of the widespread success in isolating plasma membrane vesicles using this technique (see 1.1.2.), confirmation was sought that the conditions used in this study were suitable for plasma membrane isolation. Cucumber hypocotyls were chosen because of their ready availability in the laboratory at that time. Table 3.12 demonstrates that plasma membranes from cucumber hypocotyls can be purified using a 6.3% phase system. Vanadate inhibition (88%) was remarkably high for a membrane preparation. There was also little nitrate inhibition (2%) and no azide inhibition indicating the absence of tonoplast and mitochondrial contamination respectively. There was no $K^+$ stimulation but this is quite usual for plasma membrane preparations from cucumber hypocotyls (Ball 1988).

Because the plasma membrane preparation isolated from cucumber hypocotyls appeared to be highly purified, it was considered probable that the wheat leaf preparation was also plasma membrane enriched. This assumption was tested by examining some other membrane markers (see 1.1.3.). Cytochrome c oxidase and NADH cytochrome c reductase both had lower activities in $U_3$ than $L_3$ (Table 3.13). This indicated that membranes from mitochondria and the endoplasmic reticulum were being selectively excluded from the upper fraction. The plasma membrane marker glucan synthetase II was enriched in $U_3$ but by less than two-fold.

### 3.3.7.3. Plasma membrane isolation using a 6.5% phase system.

In some previous studies on plasma membrane isolation from wheat roots by phase partitioning a 6.5% phase system has been used (Sommarin *et al* 1985, Berczi and Moller 1986). Because plasma membrane isolation using a 6.3% phase system had only resulted in limited success, it was decided to continue the investigation with a 6.5% phase system.

Table 3.14 shows the distribution of marker enzymes after membrane separation in a 6.5% phase system. There was less cytochrome c oxidase and NADH cytochrome c reductase in $U_3$ even though the total activity recovered was considerably greater and there was also more glucan synthetase II activity. In addition, there was less of the Golgi marker, latent IDPase activity, in $U_3$ than

| Assay conditions | Specific activity ($\mu$mol $P_i$/mg protein/h) |
|---|---|
| no addition | 0.55 |
| $MgCl_2$ | 49.37 |
| $MgCl_2$ + KCl | 47.08 |
| $MgCl_2$ + azide | 49.87 |
| $MgCl_2$ + vanadate | 16.84 |
| $MgCl_2$ + $KNO_3$ | 46.20 |
| $MgCl_2$ + KCl + vanadate | 5.58 |
| no addition - molybdate | 9.13 |
| $MgCl_2$ - molybdate | 53.91 |

Table 3.12: The properties of ATPase activity from cucumber hypocotyls in $U_3$ after partitioning in a 6.3% phase system. ATPase activity was assayed at pH 6.75. Results are from a single experiment and values are expressed as mean specific activity (n=2).

| Enzyme | Specific activity | |
|---|---|---|
| | $U_3$ | $L_3$ |
| Cytochrome c Oxidase ($\mu$mol/mg protein/min) | 0.086 ± 0.013 | 0.314 ± 0.046 |
| NADH Cytochrome c Reductase ($\mu$mol/mg protein/min) | 0.065 ± 0.014 | 0.152 ± 0.009 |
| Glucan Synthetase II (nmol/mg protein/min) | 0.030 ± 0.008 | 0.018 ± 0.004 |

Table 3.13: The distribution of marker enzymes from wheat leaves between $U_3$ and $L_3$ after partitioning in a 6.3% phase system. Results are from several experiments and values are expressed as mean specific activity ± standard error ($n \geq 2$).

| Enzyme | Specific activity | |
|---|---|---|
| | $U_3$ | $L_3$ |
| Cytochrome c Oxidase ($\mu$mol/mg protein/min) | 0.078 ± 0.007 | 0.671 ± 0.034 |
| NADH Cytochrome c Reductase ($\mu$mol/mg protein/min) | 0.058 ± 0.007 | 0.352 ± 0.021 |
| Glucan Synthetase II (nmol/mg protein/min) | 0.096 ± 0.003 | 0.027 ± 0.001 |
| Latent IDPase ($\mu$mol $P_i$/mg protein/h) | 14.20 ± 0.54 | 17.73 ± 4.15 |

Table 3.14: The distribution of marker enzymes from wheat leaves between $U_3$ and $L_3$ after partitioning in a 6.5% phase system. Results are from several experiments and values are expressed as mean specific activity ± standard error ($n \geq 3$).

$L_3$. However, the reliability of this result may be affected by any non-specific ATPase activity present in the preparation as this will hydrolyse nucleoside diphosphates.

Although there appears to be a higher plasma membrane-enrichment in $U_3$ after separation in a 6.5% phase system there was no major change apparent in the properties of ATPase activity associated with this fraction (Table 3.15). Vanadate inhibition and $K^+$ stimulation were similar but the small nitrate inhibition was no longer present. The only significant improvement could be seen in the reduced activity in the presence of ADP. As this activity is not $K^+$ stimulated and shows little inhibition by vanadate (26%) it is a good indicator of the presence of non-specific ATPase activity.

### 3.3.7.4. Further characterization of the ATPase activity associated with membranes after separation using a 6.5% phase system.

Figures 3.7 and 3.8 show pH profiles of ATPase activity in $U_3$ and $L_3$ after separation in a 6.5% phase system. In general there was considerable similarity between $U_3$ and $L_3$ and also with the pH profile for microsomal ATPase activity (Figure 3.3). The principle difference between the profiles for $U_3$ and $L_3$ was the level of $K^+$ stimulation. In $U_3$ there was stimulation at all pH values below pH 7.5 with a sharp peak at pH 6.0. In contrast, the $L_3$ fraction showed little or no $K^+$ stimulation at any pH value. In comparison to the ATPase activity of microsomal membranes there was a reduction in $Mg^{2+}$- and $Ca^{2+}$-dependent activity in both partitioned fractions.

The substrate specificity for the ATPase activity present in $U_3$ is shown in Table 3.16. There was activity with all nucleotides examined but in the presence of $K^+$ ions this activity was uniformly lower. This finding suggests that the lack of substrate specificity is due to non-specific ATPase activity still present in this membrane fraction and not to the plasma membrane $H^+$-ATPase.

### 3.3.8. Detergent treatment of the $U_3$ fraction from a 6.5% phase system.

The purified membranes were washed with Triton X-100 (0.01% w/w) to (a) release any non-specific ATPase activity trapped within vesicles during homogenization and (b) to dissociate any proteins which may be loosely associated with the membranes. Once treated the membranes were centrifuged and the ATPase activity from the pellet and supernatant was examined (Table 3.17).

The properties of the pelleted fraction showed little change from those of $U_3$ after a normal phase separation. There was a slight increase in vanadate

| Assay conditions | % of control |
|---|---|
| $MgCl_2$ | 100.00 ± 0.34 |
| no addition | 89.76 ± 4.39 |
| $CaCl_2$ | 115.86 ± 4.99 |
| $MgCl_2$ + KCl | 111.18 ± 7.38 |
| $MgCl_2$ + azide | 112.04 ± 6.33 |
| $MgCl_2$ + $KNO_3$ | 105.31 ± 4.48 |
| $MgCl_2$ + KCl + vanadate | 61.86 ± 2.92 |
| ADP + $MgCl_2$ | 89.56 ± 5.19 |
| ADP + $MgCl_2$ + KCl | 79.33 ± 3.05 |

Table 3.15: The properties of ATPase activity from wheat leaves in $U_3$ after partitioning in a 6.5% phase system. Results are from several experiments and values are expressed as mean % of control ± standard error ($n \geq 2$).

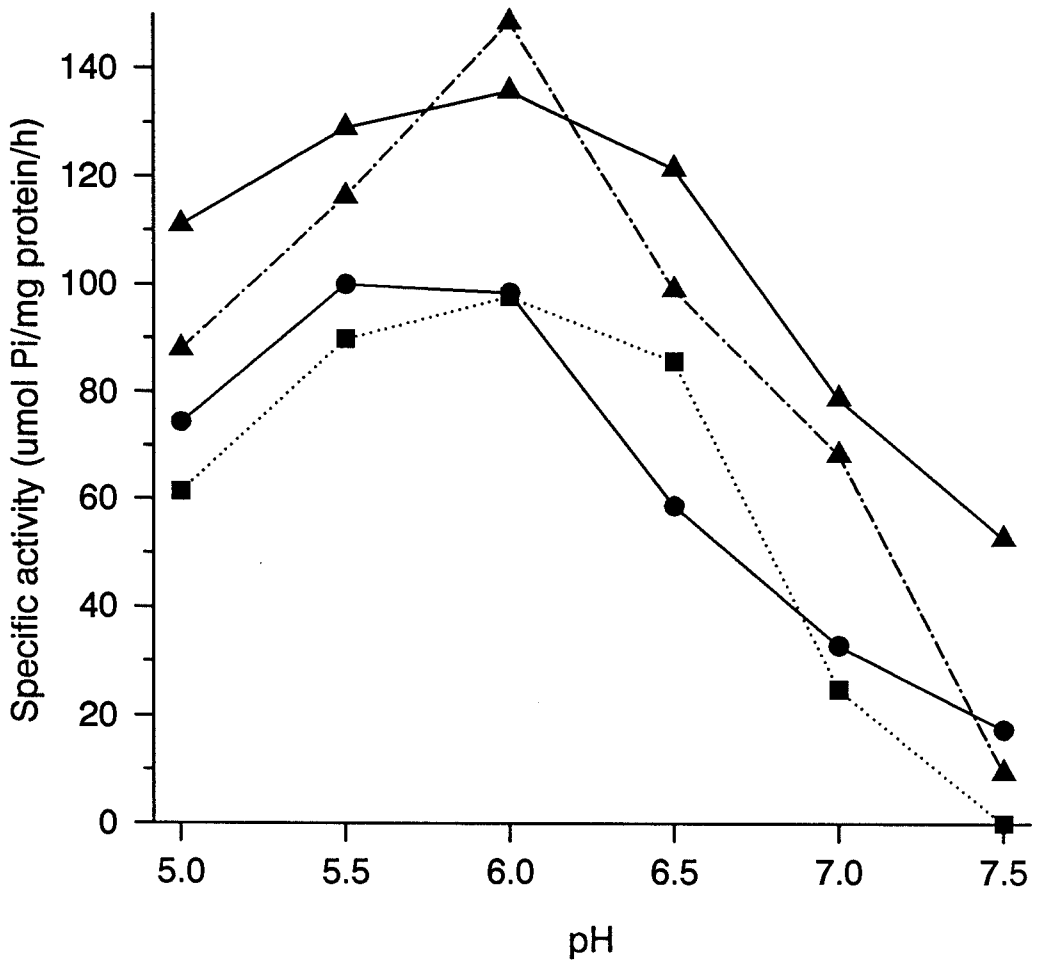


Figure 3.7: The effect of pH on ATPase activity from wheat leaves in $U_3$ after partitioning in a 6.5% phase system. ATPase activity was measured in the absence of a divalent cation (··· ■ ···) or in the presence of 2 mol $m^{-3}$ $MgCl_2$ (—●—), 2 mol $m^{-3}$ $MgCl_2$ and 50 mol $m^{-3}$ KCl (–·– ▲ –·–) and 2 mol $m^{-3}$ $CaCl_2$ (—▲—). Results are from a single experiment and points are expressed as mean specific activity (n=3).

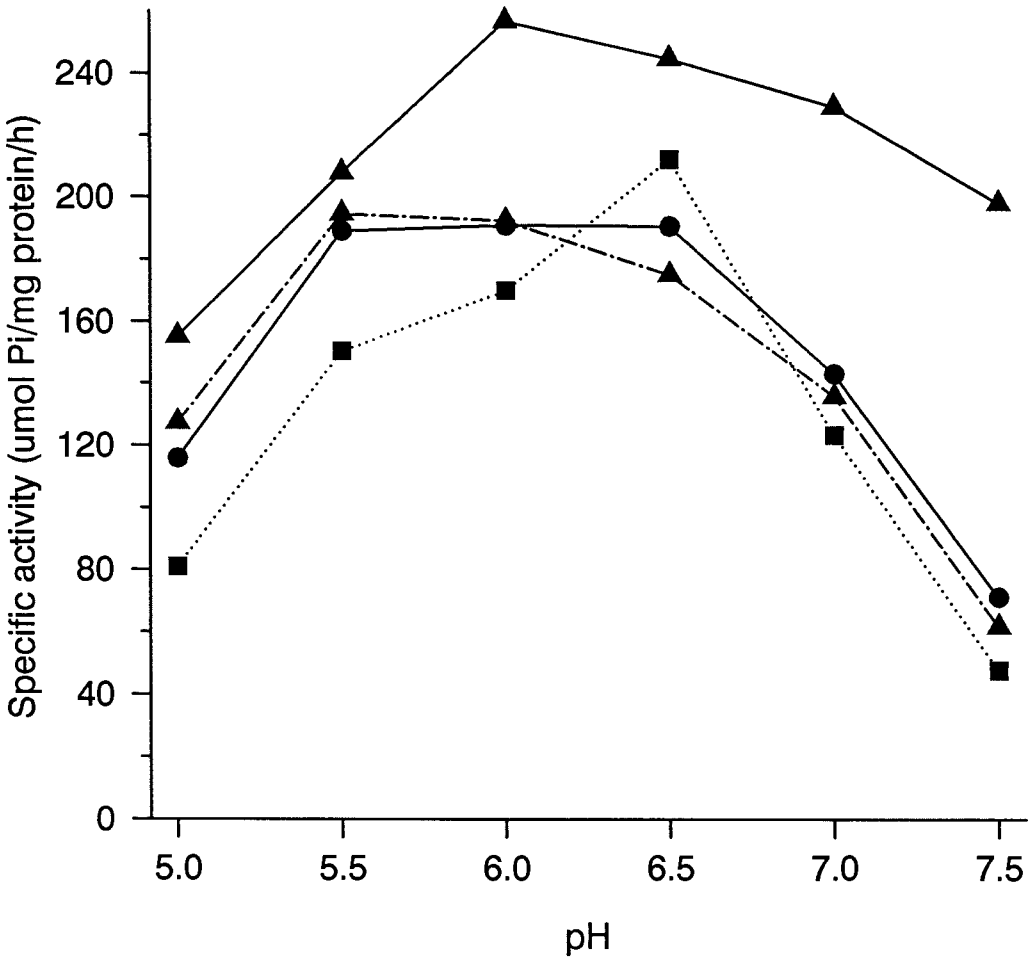


Figure 3.8: The effect of pH on ATPase activity from wheat leaves in $L_3$ after partitioning in a 6.5% phase system. ATPase activity was measured in the absence of a divalent cation (··· ■ ···) or in the presence of 2 mol $m^{-3}$ $MgCl_2$ (—●—), 2 mol $m^{-3}$ $MgCl_2$ and 50 mol $m^{-3}$ KCl (–·– ▲ ·–·) and 2 mol $m^{-3}$ $CaCl_2$ (—▲—). Results are from a single experiment and points are expressed as mean specific activity (n=3).

| Substrate | % of control | |
|---|---|---|
| | $MgCl_2$ | $MgCl_2$ + KCl |
| ATP | 100.00 ± 1.42 | 100.00 ± 0.00 |
| GTP | 65.77 ± 1.28 | 60.37 ± 0.53 |
| ITP | 72.59 ± 0.71 | 58.65 ± 0.67 |
| ADP | 93.61 ± 5.54 | 83.12 ± 5.19 |
| IDP | 35.09 ± 0.71 | - |
| AMP | 25.71 ± 0.14 | 16.49 ± 0.00 |
| $PP_i$ | 0.00 ± 0.00 | 0.00 ± 0.00 |

Table 3.16: The substrate specificity of ATPase activity from wheat leaves in $U_3$ after partitioning in a 6.5% phase system. Results are from a single experiment and values are expressed as mean % of control ± standard error (n=2).

| Assay conditions | % of control | |
|---|---|---|
| | pellet | supernatant |
| $MgCl_2$ | 100.00 ± 5.10 | 100.00 ± 1.24 |
| no addition | 108.87 ± 2.00 | 101.25 ± 1.87 |
| $CaCl_2$ | 120.18 ± 1.78 | 138.82 ± 0.93 |
| $MgCl_2$ + KCl | 116.19 ± 2.22 | 80.44 ± 1.56 |
| $MgCl_2$ + molybdate | 74.06 ± 3.77 | 63.89 ± 0.79 |
| $MgCl_2$ + KCl + vanadate | 59.16 ± 1.53 | 52.51 ± 6.95 |
| ADP + $MgCl_2$ | 95.35 ± 0.89 | 134.16 ± 1.86 |

Table 3.17: The effect on ATPase activity from wheat leaves of washing $U_3$, after partitioning in a 6.5% phase system, with 0.01% (w/w) Triton X-100. Results are from a single experiment and values are expressed as mean % of control ± standard error (n=2).

inhibition and $K^+$ stimulation. In contrast, the properties of the ATPase activity in the supernatant fraction were quite different. It was more sensitive to molybdate, had greater activity with ADP than ATP and was inhibited by $K^+$ ions. It was also the most vanadate–sensitive ATPase fraction yet characterized in this study which suggests that the non–specific ATPase is itself inhibited by vanadate.

$U_3$ was also treated with KI in an attempt to remove this non–specific activity. As with previous KI treatments (Tables 3.5, 3.7 and 3.8) there was no significant enrichment of the properties usually associated with the plasma membrane $H^+$–ATPase (data not shown).

## 3.4. DISCUSSION

The results presented in this chapter confirm that the ATPase activity associated with microsomal membranes from wheat leaves has different properties to those reported for a variety of other species. The specific activities measured are very high, even in the absence of a divalent cation. This is in agreement with previous reports. Kahr and Moller (1976) showed that, in the presence of $Mg^{2+}$ ions, the specific ATPase activity in wheat was up to seven times higher than that for oat and Pomeroy and McMurchie (1982) found a twenty–fold increase over ATPase activity from cauliflower. There is also more activity in the presence of $Ca^{2+}$ ions than $Mg^{2+}$ ions and this result confirms the findings of all the previous studies where activity with both divalent cations was measured. Microsomal ATPase activity has a very broad pH profile with maximum activity in the range pH 5.5 to 6.5. This pH profile is similar to that described by Kylin and Kahr (1973). A further characteristic of this activity is the low specificity for ATP as the substrate.

Despite problems with low levels of measurable proton transport, these experiments are very revealing. The degree of inhibition by vanadate and nitrate indicates that the proton gradient measured is generated primarily by the plasma membrane and tonoplast proton pumps (Sze 1985). Interestingly, the gradient can be collapsed by the addition of $CaCl_2$. There are two possible explanations for this. The first is that the $Ca^{2+}$ ions are exchanging with the protons in the vesicles *via* an antiport. There is evidence for a $Ca^{2+}/H^+$ antiport in the tonoplast of oat roots (Schumaker and Sze 1985) but not in the plasma membrane. Under these conditions the gradient would only be partially abolished and the subsequent addition of gramicidin demonstrates that this is not the case. An alternative explanation is that $Ca^{2+}$ is inhibiting the proton pumps so allowing the gradient to dissipate by the leakage of protons from the vesicles. $Ca^{2+}$ ions have been shown to inhibit the plasma membrane $H^+$–ATPase directly (see 4.3.11. and Leonard and Hotchkiss 1976) but may also inhibit both pumps by competing with $Mg^{2+}$ for

ATP and thereby reducing the concentration of the true substrate, $MgATP^{2-}$ (Bennett *et al* 1985). It is likely that the collapse of the Mg:ATP-generated proton gradient is due to a combination of these factors.

In contrast to the effect of nitrate on proton transport there is no apparent nitrate inhibition of microsomal ATPase activity. This finding, together with the absence of azide inhibition and the reduction in vanadate sensitivity, suggests that the predominant ATPase activity measured by the release of inorganic phosphate is not that of the $H^+$-ATPases. The activity of these enzymes, if indeed they are present in wheat, is being masked by the high levels of ATPase activity that has been shown to be present in microsomal membrane preparations. For the purposes of this, and future discussions, the ATPase activity described here has been termed non-specific ATPase activity.

A variety of membrane purification techniques have been used in an attempt to isolate plasma membranes that are not contaminated by non-specific ATPase activity. Initially discontinuous sucrose density gradients were used but these were unable to separate a membrane fraction which exhibited ATPase activity consistent with the properties normally associated with a pure plasma membrane preparation. Namely, almost complete dependence on the presence of $Mg^{2+}$ ions; stimulation by $K^+$ ions and inhibition by $Ca^{2+}$ ions and vanadate. By these criteria the most successful procedure was to wash the isolated microsomal membranes with 250 mol $m^{-3}$ KI before loading them onto a normal sucrose gradient (Table 3.7).

Early experiments using the technique of aqueous polymer two-phase partition have proved no more successful. However, the use of other membrane markers has demonstrated that the membranes in $U_3$ are predominantly of plasma membrane origin. Because of this, the success with preliminary experiments using cucumber hypocotyls and the potential for isolating very pure plasma membranes (Larsson 1985), it was decided to continue with this technique (see Chapter 4).

The origin of the non-specific activity is unknown. Obvious candidates are the non-specific acid phosphatases which are common in higher plant cells (*e.g.* Leigh and Walker 1980, Gallagher and Leonard 1982) and have suitably low pH optima. However, the addition of ammonium molybdate (0.1 mol $m^{-3}$), an inhibitor of acid phosphatases (Gallagher and Leonard 1982, Van Etten *et al* 1974), resulted in less than 15% inhibition of ATPase activity and this, together with the absence of any activity with $\beta$-glycerophosphate or pyrophosphate, indicates that acid phosphatases do not contribute significantly to the total ATPase activity measured. This conclusion is supported by the inability of KI treatments to remove all the non-specific activity from membrane preparations (Tables 3.5, 3.7 and 3.8) and by the demonstration that it is not due to soluble proteins trapped

within vesicles (Table 3.17).

Although detergent treatment of plasma membrane-enriched vesicles was unsuccessful in removing the majority of the non-specific ATPase activity, an examination of the ATPase activity of the supernatant does provide some information on the properties of the non-specific ATPase activity without the presence of contaminating $H^+$-ATPases (Table 3.17). It is stimulated by $Ca^{2+}$ ions but not by $Mg^{2+}$ ions. There is still substantial activity in the absence of divalent cations but $Ca^{2+}$ ions associated with membranes *in vivo* may be sufficient for partial or complete activation of the enzyme. $K^+$ ions are inhibitory, as is vanadate and molybdate and ADP is the preferred substrate over ATP.

The evidence presented in this chapter indicates that the non-specific ATPase activity is associated with a membrane or membranes. If the activity is due to a single enzyme, is this an integral or peripheral membrane protein? Unfortunately this question is beyond the scope of this investigation but the stringency of the KI treatments suggests that the membrane association is, at least, a very stable one. A second important question is whether the activity is associated with one or more (or indeed all) cellular membranes and which one(s)? Results presented in Chapter 4 demonstrate that this activity can be separated from plasma membranes by phase partition and is therefore not associated with all cellular membranes. This finding also suggests that the non-specific ATPase is not a soluble cytosolic protein which is sticking to the nearest available membrane during homogenization.

One suitable candidate for the cellular location of this enzyme is the tonoplast. Diaz de Leon and Wyn Jones (1985) reported a dominant $Ca^{2+}$-ATPase activity in vacuolar preparations from wheat mesophyll cells. This activity was not an acid phosphatase and had a broad pH optima between pH 5.0 and 7.0, with the $Ca^{2+}$-stimulated component having two peaks of activity at these two values respectively. $Mg^{2+}$ ions were able to substitute for $Ca^{2+}$ ions, particularly at lower pH values and substrate specificity for ATP was low. These properties are similar in many respects to those of the microsomal activity described here and it is likely that this vacuolar $Ca^{2+}$-ATPase contributes to the non-specific activity measured. It is not, however, the only contributor. Tonoplast membranes are less dense than than plasma membranes (Sze 1985) and would be expected to band near the top of a sucrose density gradient. The relatively high $Ca^{2+}$ stimulation at the 20/25% interface of the sucrose gradients used in this study (Tables 3.6, 3.7 and 3.8) is probably attributable to this vacuolar $Ca^{2+}$-ATPase activity. Non-specific ATPase activity is still present throughout the remainder of the gradient. Furthermore, plasma membranes purified by phase partition have previously been shown to be free from tonoplast contamination (Sommarin *et al* 1985) and the results obtained

in this study using cucumber hypocotyls (Table 3.12) support this evidence. It is therefore unlikely that the non–specific ATPase activity detected in the plasma membrane–enriched preparations derives from the presence of tonoplast.

Evidence of a second possible location for non–specific ATPase activity was provided by Hall and Dymott (1980). In this investigation using wheat coleoptiles they showed that at pH 5.5 staining for ATPase activity was heavy and specific in both the endoplasmic reticulum and the dictyosomes of epidermal and outer cortical cells. At pH 7.0 there was little staining at these sites but ATPase activity was detectable at the plasma membrane.

In contrast to the majority of higher plants, barley possesses a non–specific ATPase activity that is almost identical to that of wheat (Caldwell and Haugh 1980, 1982, Nagahashi *et al* 1978). Nagahashi *et al* (1978) were unable to separate this activity from a plasma membrane–enriched sucrose gradient fraction that was between 60 and 70% pure as determined by phosphotungstic chromic acid staining. Caldwell and Haugh (1980, 1982) were also unable to separate this activity from plasma membranes isolated using a combination of sucrose density gradients followed by a primitive phase partition method. However, DuPont and Hurkman (1985) were able to remove this activity from cellular membranes by homogenizing at a higher pH, and in the presence of EDTA, before separation on a modified sucrose density gradient (see 2.3.1.). They concluded that the predominant non–specific ATPase activity was a soluble enzyme with a strong tendency to adhere to microsomal membranes. Unfortunately, this technique was unsuccessful when applied to wheat (Table 3.9). It is still probable, however, that the non–specific activity in both barley and wheat is due to a very similar enzyme(s) that does not appear to be present in such quantity in other species. Kahr and Moller (1976) attributed this to a nutritional requirement for $Ca^{2+}$ in both barley and wheat.

Hall (1971) was able to show that a cell wall fraction from barley contained an ATPase activity that was preferentially stimulated by $Ca^{2+}$ ions. Support for a localization of non–specific ATPase activity in the cell wall comes from Blakeley (1983). He reported that a microsomal preparation from wheat leaf protoplasts contained ATPase activity that was largely dependent on the presence of a divalent cation (<20% of control activity without $Mg^{2+}$) and could be stimulated by $K^+$ ions (102%). Vanadate inhibition was also very high (66% with $50x10^{-3}$ mol $m^{-3}$ vanadate). These properties are consistent with those of the plasma membrane $H^+$–ATPase, although some properties associated with non–specific ATPase activity remained. For example, $Ca^{2+}$ ions were able to replace $Mg^{2+}$ ions and substrate specificity for ATP was poor, particularly in the absence of $K^+$ ions. Even so, it is quite clear that some of the non–specific

ATPase activity associated with conventionally prepared microsomes is not associated with microsomal membranes prepared under these conditions. This may be due to the prior removal of the cell wall but it is possible that the gentle conditions required for protoplast lysis, which would result in minimal subcellular damage, prevented the release of non-specific activity.

An interesting candidate for the identity of the non-specific ATPase activity was reported by Tognoli and Marre (1981). They purified and characterized a divalent cation-activated ATP-ADPase from pea stem microsomes. This enzyme was more active with ADP than ATP, more active with $Ca^{2+}$ ions than $Mg^{2+}$ ions and had a low pH optimum. Unfortunately there was no indication of the cellular location of the ATP-ADPase except that the microsomal membranes used contained no mitochondrial membranes.

Even though, for reasons stated earlier, it is unlikely that the non-specific activity is a soluble acid phosphatase there are a number of similarities in their properties. Gallagher and Leonard (1982) described an acid phosphatase activity from corn roots that had a very similar pH profile and substrate specificity to that of wheat leaf microsomal ATPase activity. The enzyme was also inhibited equally well by vanadate as by molybdate. The difference appears to be in the sensitivity to molybdate. In corn roots inhibition by molybdate was approximately 50% at a concentration of 0.1 mol $m^{-3}$. In other species it is even higher. In oat roots and coleoptiles acid phosphatase activity was almost completely inhibited at this concentration (Gallagher and Leonard 1982) as was acid phosphatase in roots of red beet (Leigh and Walker 1980). The possibility that the non-specific ATPase could be a form of membrane-associated acid phosphatase should not be ruled out.

A final comment on the origin of the non-specific ATPase activity. $Mg^{2+}$-dependent, $Ca^{2+}$-transporting ATPases have been reported at both the endoplasmic reticulum and the plasma membrane (Lew *et al* 1986, Robinson *et al* 1988). It is highly unlikely that these $Ca^{2+}$-transporting ATPases are responsible for the activity seen in microsomal preparations because they have (a) very low specific activities and (b) higher pH optima. The same arguments also apply to the ATPase activity located on the chloroplast envelope membrane (McCarty *et al* 1984). In summary, the non-specific ATPase activity is probably located at more than one site in the cell and is possibly the summation of two or more different enzymes with differing properties.

In this study both conventional and modified sucrose density gradients were unable to separate a plasma membrane-enriched fraction as shown by the absence of the plasma membrane marker, vanadate-sensitive, $K^+$-stimulated $Mg^{2+}$-ATPase activity. Subsequent experiments using phase partitioning

demonstrated that plasma membrane preparations that were considerably enriched, as shown by a variety of membrane markers, still did not exhibit ATPase properties consistent with those of the plasma membrane $H^+$–ATPase. Future experimenters investigating membrane–associated ATPase activities in wheat should be careful to use very pure membrane systems, determined, where possible, by markers other than those requiring the measurement of ATP hydrolysis.

# CHAPTER FOUR

# CHARACTERIZATION OF PLASMA MEMBRANE ATPASE ACTIVITY

## 4.1. INTRODUCTION

It has been demonstrated in Chapter 3 that a plasma membrane–enriched fraction from wheat leaves, isolated by phase partitioning, does not exhibit the properties normally associated with plasma membrane preparations from other species. As discussed these results were in general agreement with those obtained using wheat root tissue (Lundborg *et al* 1981, Sommarin *et al* 1985) and subsequent papers using the same preparatory procedure failed to clarify the situation with regard to the origin of the ATPase activity measured (Berczi and Moller 1986, Berczi and Moller 1987, Berczi 1988). The results presented in this chapter show that wheat leaf plasma membrane preparations contain an ATPase activity with properties that are consistent with those of the plasma membrane $H^+$–ATPase of other higher plants (Sze 1985). This activity has been characterized and the effects of four inhibitors investigated. The inhibitors chosen were: vanadate, commonly used to identify the presence of this enzyme (Hall 1983); $Ca^{2+}$ ions, an inhibitor that is present *in vivo*; and erythrosin B and SW26, two new, but as yet not widely used inhibitors of the plasma membrane $H^+$–ATPase (see Ball *et al* 1987). All experiments were performed at Littlehampton.

## 4.2. EXPERIMENTAL

All ATPase assays were performed at pH 6.0 unless stated in the appropriate figure legend. Where the results are presented as mean % of control, the control values were as follows: For divalent cations (or no addition) the upper value was the control value. For the addition of monovalent cations or inhibitors the control value was the activity with the respective divalent cation alone. When the inhibitor $NO_3^-$ was used it was added as $KNO_3$ and the control value was the activity measured with KCl. Molybdate (0.1 mol $m^{-3}$) was routinely included in all ATPase assays. Inhibition by molybdate is expressed as a % of activity when molybdate was omitted from the assay. The control value for alternative substrates was the activity in the presence of ATP under the same assay conditions.

## 4.3. RESULTS

### 4.3.1. Plasma membrane purity.

With no apparent change in the isolation procedure there was a considerable improvement in the purity in the plasma membrane fraction ($U_3$) compared with the plasma membrane fraction described in Chapter 3. This is illustrated in Tables 4.1 and 4.2. Mitochondrial contamination as determined by cytochrome c oxidase activity was almost completely abolished. This finding was supported by the absence of any azide inhibition of ATPase activity. The Golgi-associated latent IDPase activity was also greatly reduced in this preparation although this may reflect the absence of non-specific ATPase contamination rather than an increase in the exclusion of Golgi membranes from $U_3$ (Figure 4.1 and Table 3.16). Antimycin A-resistant, NADH-cytochrome c reductase activity was still present which may indicate some contamination by the endoplasmic reticulum.

The plasma membrane marker, glucan synthetase II, was more active in this preparation than in the preparations described in Chapter 3 and vanadate sensitivity was also markedly increased. The nitrate sensitivity of the plasma membrane ATPase activity suggests the presence of tonoplast. In earlier plasma membrane preparations there was little or no detectable nitrate-inhibited ATPase activity (Tables 3.11 and 3.15). It is likely that the removal of the non-specific ATPase activity has now permitted the detection of tonoplast contamination that was also present previously.

### 4.3.2. The effect of pH.

The pH profiles for ATPase activity from $U_3$ and $L_3$ are shown in Figures 4.1 and 4.2. There was a marked contrast between the two profiles. The profile for $L_3$ closely resembled that previously shown (Figure 3.9) and was essentially the same as that for the microsomal preparation without the $Mg^{2+}$-stimulated component. Perhaps the most striking difference between the profiles for $U_3$ and $L_3$ was the absence, in $U_3$, of almost any ATPase activity in the absence of a divalent cation. A further important difference was the presence of $K^+$ stimulation in the $U_3$ fraction over a broad pH range.

### 4.3.3. Cation specificity.

Table 4.3 shows the effect of substituting both monovalent and divalent cations for $K^+$ and $Mg^{2+}$ ions. $Mn^{2+}$ ions substituted well for $Mg^{2+}$ ions in the

| Enzyme | Specific activity | |
|---|---|---|
| | $U_3$ | $L_3$ |
| Cytochrome c Oxidase ($\mu$mol/mg protein/min) | 0.013 ± 0.002 | 1.011 ± 0.057 |
| NADH Cytochrome c Reductase ($\mu$mol/mg protein/min) | 0.054 ± 0.006 | 0.108 ± 0.028 |
| Glucan Synthetase II (nmol/mg protein/min) | 0.154 ± 0.035 | 0.005 ± 0.002 |
| Latent IDPase ($\mu$mol $P_i$/mg protein/h) | 0.46 ± 0.25 | 8.41 ± 1.52 |

Table 4.1: The distribution of marker enzyme activities between $U_3$ and $L_3$ after partitioning in a 6.5% phase system. Results are from several experiments and values are expressed as mean specific activity ± standard error ($n \geq 5$).

| Inhibitor | % of control |
|---|---|
| none | 100.00 ± 0.69 |
| vanadate | 29.52 ± 2.58 |
| azide | 99.54 ± 3.05 |
| molybdate | 71.87 ± 1.32 |
| nitrate | 86.29 ± 3.79 |

Table 4.2: The effects of inhibitors on ATPase activity from $U_3$ after partitioning in a 6.5% phase system. The inhibitors used were sodium vanadate (0.1 mol $m^{-3}$), sodium azide (1 mol $m^{-3}$), ammonium molybdate (0.1 mol $m^{-3}$) and potassium nitrate (50 mol $m^{-3}$). Results are from several experiments and values are expressed as mean % of control ± standard error ($n \geq 6$).

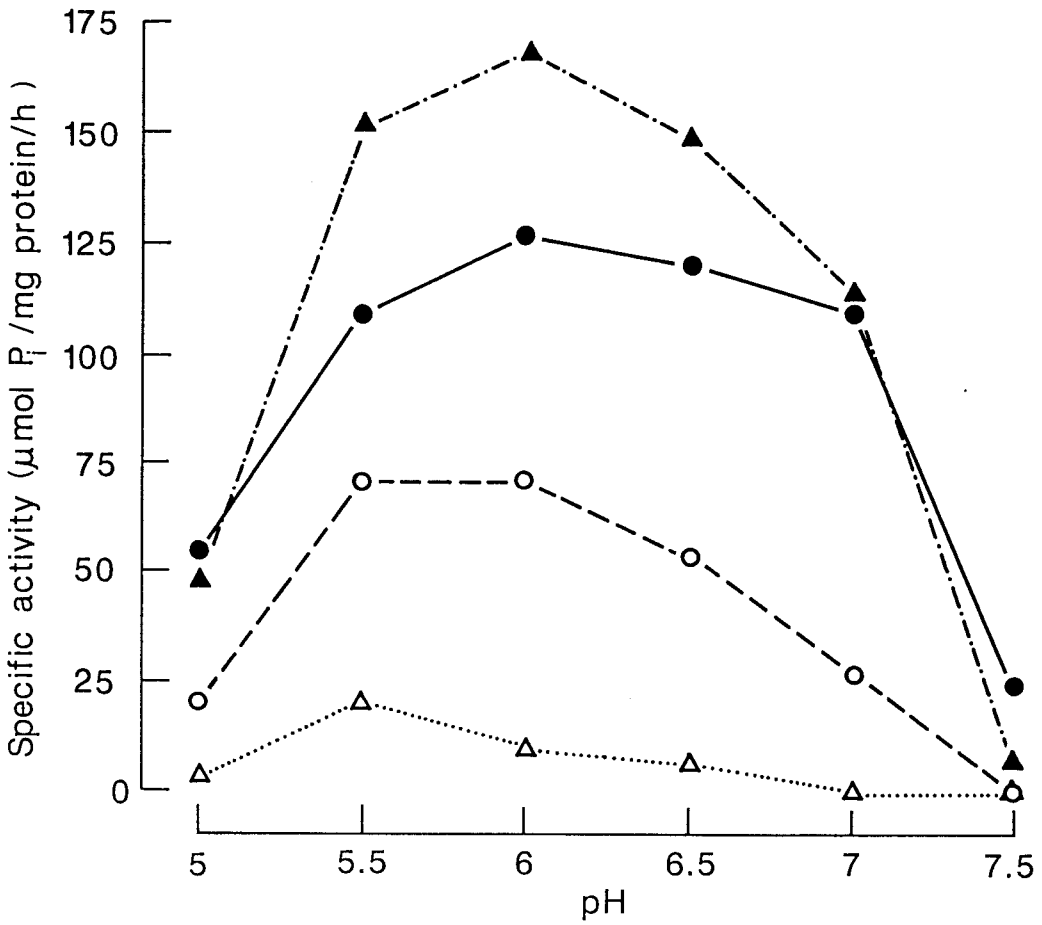


Figure 4.1: The effect of pH on ATPase activity from $U_3$ after partitioning in a 6.5% phase system. ATPase activity was measured in the absence of a divalent cation (···△···) or in the presence of 2 mol $m^{-3}$ $MgCl_2$ (—●—), 2 mol $m^{-3}$ $MgCl_2$ and 50 mol $m^{-3}$ KCl (-·-▲-·-) and 2 mol $m^{-3}$ $MgCl_2$, 50 mol $m^{-3}$ KCl and 0.1 mol $m^{-3}$ vanadate (--O--). Results are from a single representative experiment and points are expressed as mean specific activity (n=3).

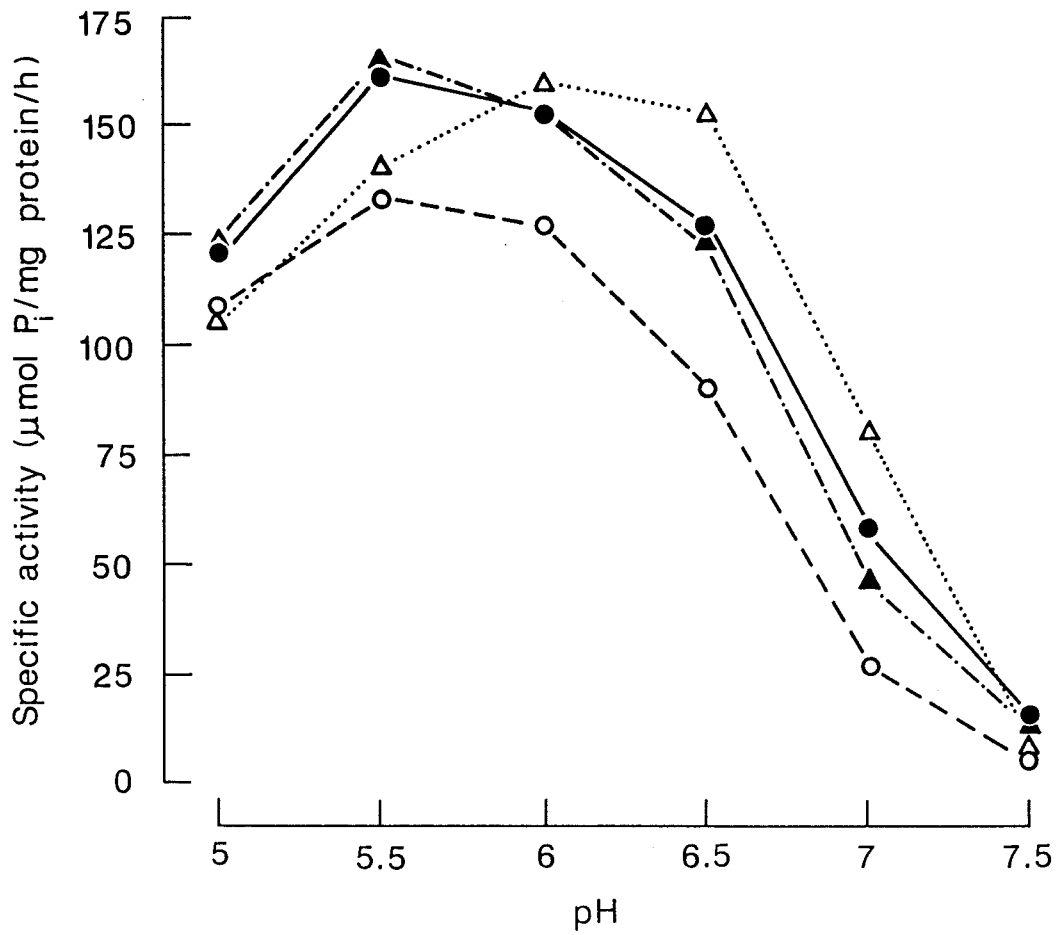


Figure 4.2: The effect of pH on ATPase activity from $L_3$ after partitioning in a 6.5% phase system. ATPase activity was measured in the absence of a divalent cation (··· Δ ···) or in the presence of 2 mol $m^{-3}$ $MgCl_2$ (— ● —), 2 mol $m^{-3}$ $MgCl_2$ and 50 mol $m^{-3}$ KCl (-·- ▲ -·-) and 2 mol $m^{-3}$ $MgCl_2$, 50 mol $m^{-3}$ KCl and 0.1 mol $m^{-3}$ vanadate (--O--). Results are from a single representative experiment and points are expressed as mean specific activity (n=3).

| Ions | % of control |
|---|---|
| $MgCl_2$ | 100.00 ± 1.00 |
| $MgSO_4$ | 115.33 ± 0.99 |
| $MnCl_2$ | 101.73 ± 2.66 |
| $CaCl_2$ | 49.58 ± 6.00 |
| $MgCl_2$ + KCl | 100.00 ± 1.08 |
| $MgSO_4$ + KCl | 115.93 ± 1.76 |
| $MgCl_2$ + RbCl | 117.99 ± 1.65 |
| $MgCl_2$ + $NH_4Cl$ | 101.48 ± 3.33 |
| $MgCl_2$ + NaCl | 92.57 ± 3.51 |
| $MgCl_2$ + LiCl | 76.89 ± 2.13 |
| $MnCl_2$ + KCl | 79.34 ± 2.73 |
| $CaCl_2$ + KCl | 23.75 ± 0.18 |

Table 4.3: Specificity for divalent (2 mol $m^{-3}$) and monovalent (50 mol $m^{-3}$) cations of ATPase activity from $U_3$ after partitioning in a 6.5% phase system. Results are from several experiments and values are expressed as mean % of control ± standard error ($n \geq 6$).

absence of $K^+$ but the enzyme did not appear to be stimulated by $K^+$ ions as effectively when using Mn:ATP as a substrate. Activity in the presence of $MgSO_4$ was slightly higher than that with $MgCl_2$, with or without $K^+$ ions. This is not an inhibition by $Cl^-$ ions as $K^+$ ions are added as KCl in much greater concentration than $MgCl_2$. Substituting $CaCl_2$ for $MgCl_2$ gave approximately 50% and 24% of control activity in the absence and presence of $K^+$ ions respectively. Most of the monovalent cations tested were able to replace $K^+$ ions with the rank order for ATPase stimulation being $Rb^+ > K^+ = NH_4^+ > Na^+ > Li^+$.

### 4.3.4. The effect of $K^+$ ions.

The effect of increasing $K^+$ ion concentrations on plasma membrane ATPase activity is demonstrated in Figure 4.3. The mean stimulation by 50 mol $m^{-3}$ KCl was 35.4 ± 2.9% at pH 6.0 with the mean specific activity in the presence of $K^+$ ions for these experiments being 97.69 ± 7.84 $\mu$mol $P_i$/mg protein/h (n=14). The pH profile of the $K^+$ stimulation is shown in Figure 4.4 with stimulation over the range pH 5.0 to 7.0 and maximum stimulation at pH 5.5.

### 4.3.5. Latency.

Plasma membrane vesicles purified by phase partition are considered to be orientated as they would be in the cell, *i.e.* right-side out. Because the location of the substrate binding site of the ATPase is on the cytoplasmic side of the plasma membrane, ATPase assays using right-side out plasma membrane vesicles are performed in the presence of a detergent to make the substrate binding site accessible. In this study 0.01% (w/w) Triton X-100 was routinely included in the assay.

Figure 4.5 shows the latency of the ATPase activity from $U_3$, with an approximate 3-fold stimulation in the presence of 0.01% Triton and $K^+$ ions. This would correspond to 68% of the membrane population being sealed, right-side out vesicles, which is in general agreement with other preparations (Larsson *et al* 1984). Sandstrom *et al* (1987) have recently compared the effect of Triton X-100 and lysolecithin on the latency of plasma membrane ATPase activity from oat roots. They concluded that, as the Triton concentration required to disrupt vesicle integrity was 10-fold less than that resulting in the maximum stimulation of ATPase activity, the Triton itself was stimulating the ATPase. The Triton may act either by altering the lipid environment of the enzyme or possibly by removing an inhibitory component of the regulatory system. Direct stimulation of ATPase activity by Triton would lead to an overestimate of the proportion of sealed,

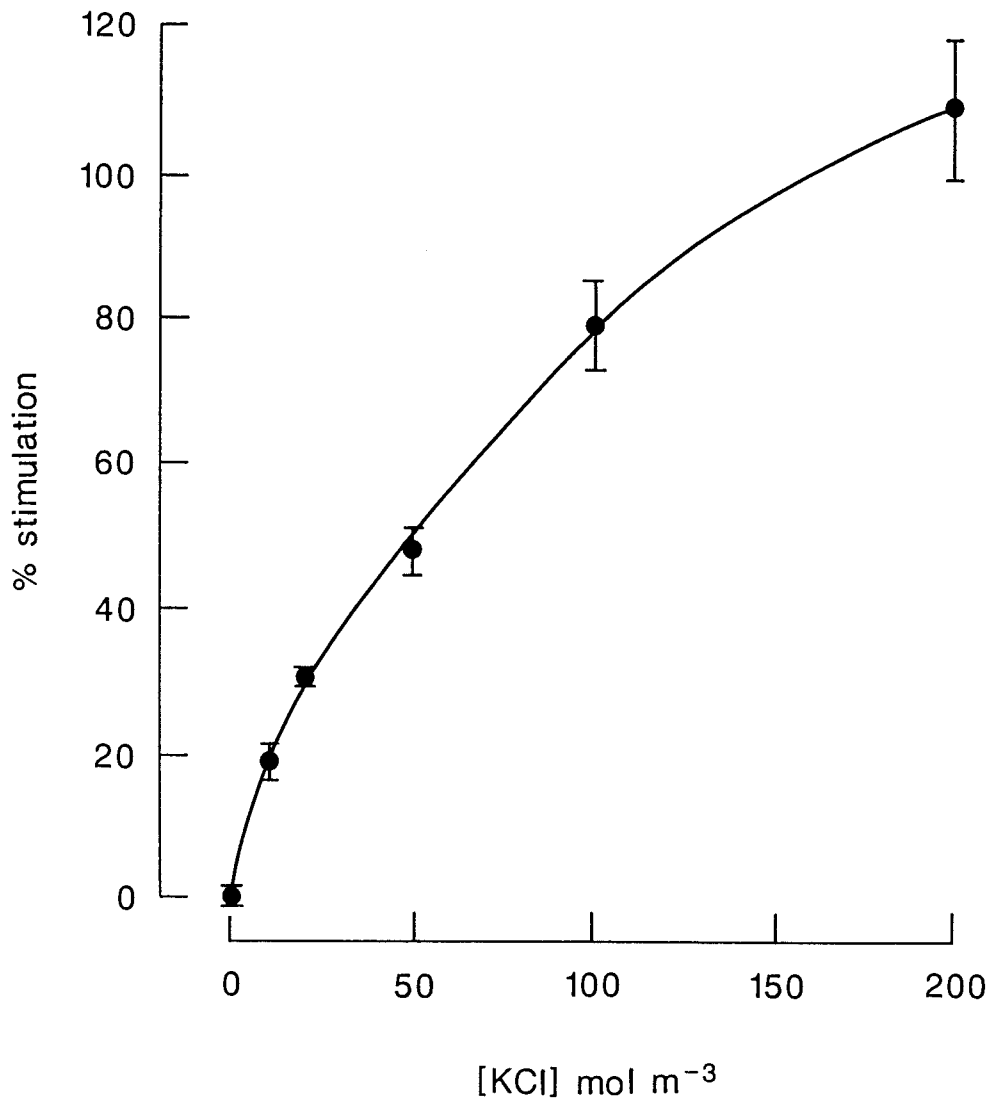


Figure 4.3: The effect of $K^+$ ion concentration on ATPase activity from $U_3$ after partitioning in a 6.5% phase system. Results are from two experiments and points are expressed as mean % stimulation ± standard error (n>5).

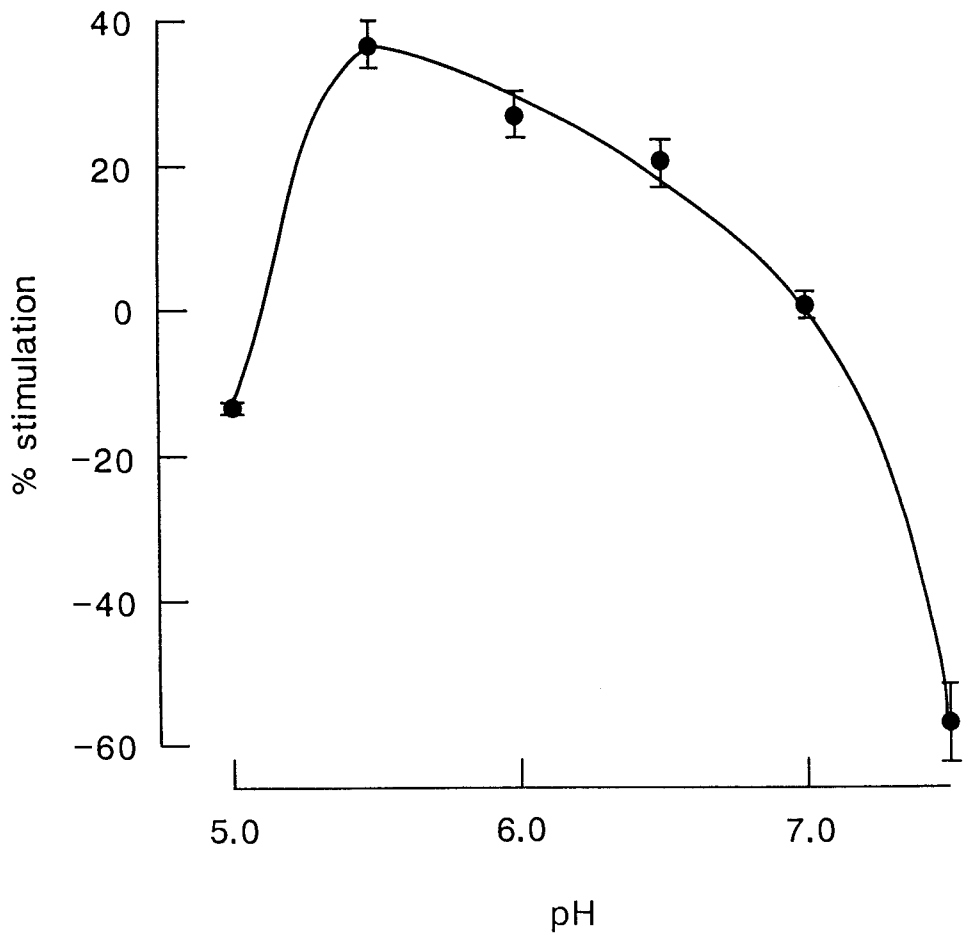


Figure 4.4: The effect of pH on stimulation by 50 mol $m^{-3}$ KCl of ATPase activity from $U_3$ after partitioning in a 6.5% phase system. Results are from three experiments and points are expressed as mean % stimulation ± standard error (n≥3).

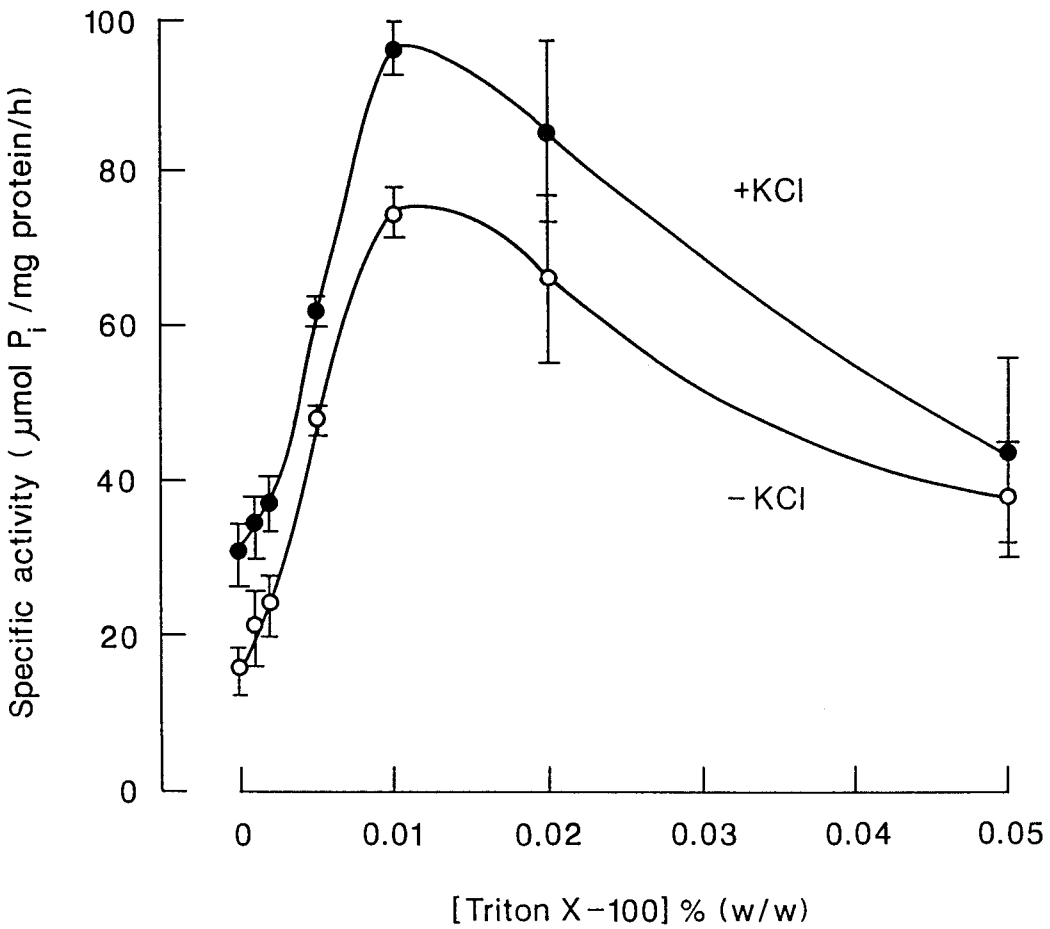


Figure 4.5: The effect of Triton X-100 concentration on ATPase activity from $U_3$, after partitioning in a 6.5% phase system, measured in the absence and presence of 50 mol $m^{-3}$ KCl. Results are from two experiments and points are expressed as mean specific activity ± standard error (n≥3).

right–side out plasma membrane vesicles.

### 4.3.6. Substrate specificity.

In contrast to all previous wheat leaf plasma membrane–enriched preparations substrate specificity for ATP was very high (Table 4.4). There was little activity with any of the alternative substrates tested with the exception of GTP. The low level of activity with ADP is another good indicator that the non–specific ATPase activity had been removed from this preparation. The absence of any hydrolysis of ρ–nitrophenyl phosphate confirmed that, in the presence of ammonium molybdate, acid phosphatase activity had been completely inhibited.

### 4.3.7. Determination of apparent $K_m$ and $V_{max}$.

Figure 4.6a shows the effect of increasing substrate (Mg:ATP) on ATPase activity. The enzyme appeared to show simple Michaelis–Menten kinetics. By representing these data using a double reciprocal, Lineweaver–Burk plot (Figure 4.6b) the kinetic parameters of the enzyme can be determined. Over 3 experiments the $K_m$ and $V_{max}$ values measured for Mg:ATP at 37°C were 0.54 ± 0.06 mol $m^{-3}$ and 106.5 ± 20.1 μmol $P_i$/mg protein/h respectively with $K^+$ ions and 0.73 ± 0.26 mol $m^{-3}$ and 80.2 ± 15.7 μmol $P_i$/mg protein/h without $K^+$ ions.

### 4.3.8. The effect of sodium vanadate.

The pH profile for vanadate inhibition is shown in Figure 4.7. Inhibition appeared to be greatest at higher pH values with 100% inhibition in the presence of 0.1 mol $m^{-3}$ at pH 7.5. This result may be an artifact of the lower activities measured or could represent the absence of any remaining non–specific ATPase activity at these higher pH values.

Figure 4.8 shows the effect of increasing vanadate concentration on plasma membrane ATPase activity. From this graph the $I_{50}$ for inhibition was estimated to be approximately $38x10^{-3}$ mol $m^{-3}$ and, at $250x10^{-3}$ mol $m^{-3}$ vanadate, ATPase activity was almost completely abolished. The kinetics of inhibition were subsequently studied in more detail (Figure 4.9). Vanadate inhibition was of the non–competitive type with a $K_i$ value of $37.3x10^{-3}$ mol $m^{-3}$.

### 4.3.9. The effect of SW26.

Figure 4.8 shows the effect of SW26 on plasma membrane ATPase

| Substrate | % of control |
|---|---|
| ATP | 100.00 ± 0.72 |
| GTP | 35.15 ±11.07 |
| CTP | 8.90 ± 3.63 |
| ITP | 3.95 ± 0.42 |
| ADP | 12.12 ± 0.98 |
| AMP | 0.00 ± 0.00 |
| ρ-NPP | 0.00 ± 0.00 |

Table 4.4: Substrate specificity of ATPase activity from $U_3$ after partitioning in a 6.5% phase system. Results are from several experiments and values are expressed as mean % of control ± standard error ($n \geq 6$).

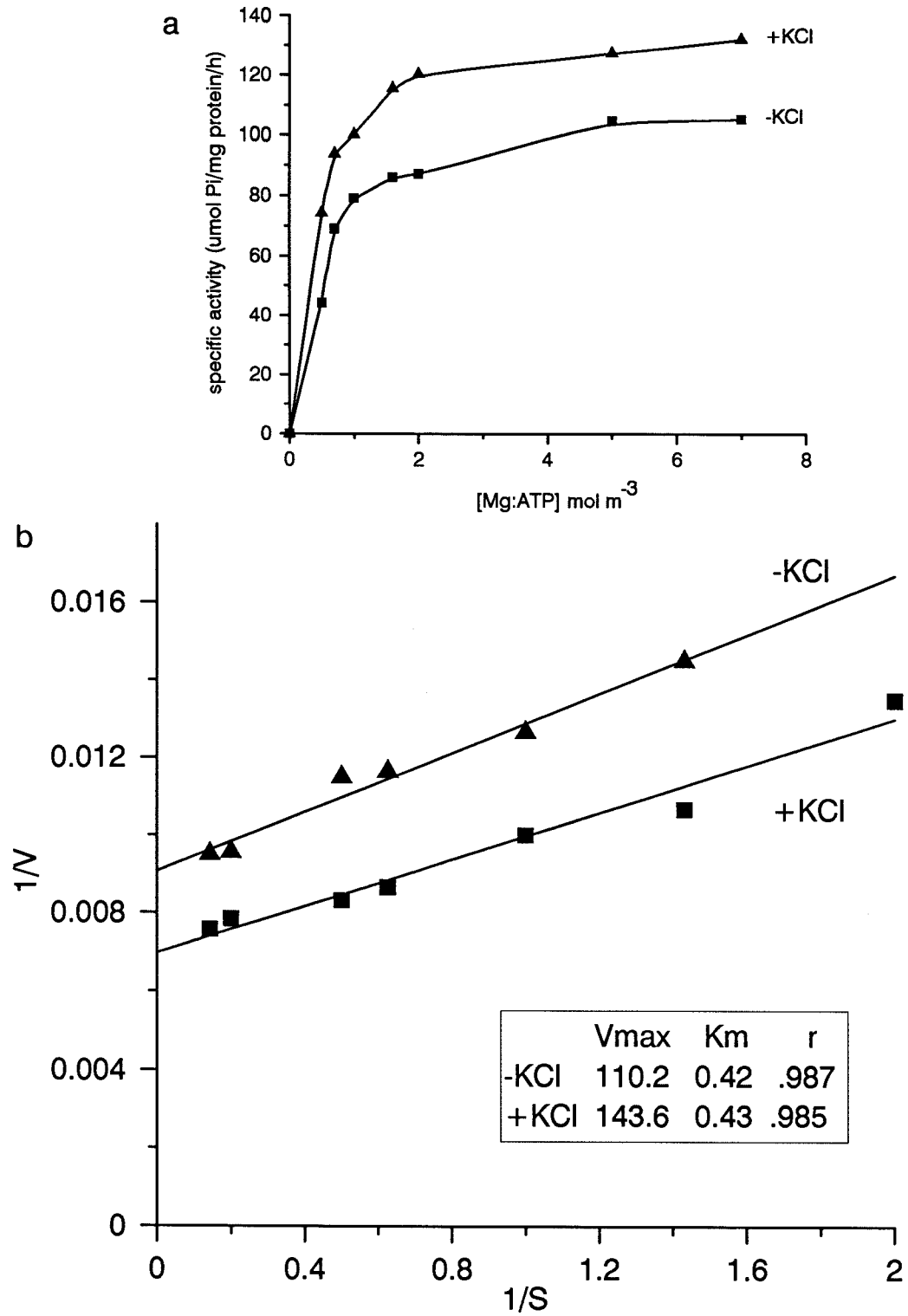


Figure 4.6a: The effect of Mg:ATP concentration on ATPase activity from $U_3$, after partitioning in a 6.5% phase system, measured in the absence and presence of 50 mol $m^{-3}$ KCl. Results are from one of three experiments and points are expressed as mean specific activity (n=3).

Figure 4.6b: Lineweaver–Burk plot of data from Figure 4.6a. Kinetic parameters were calculated by linear regression.

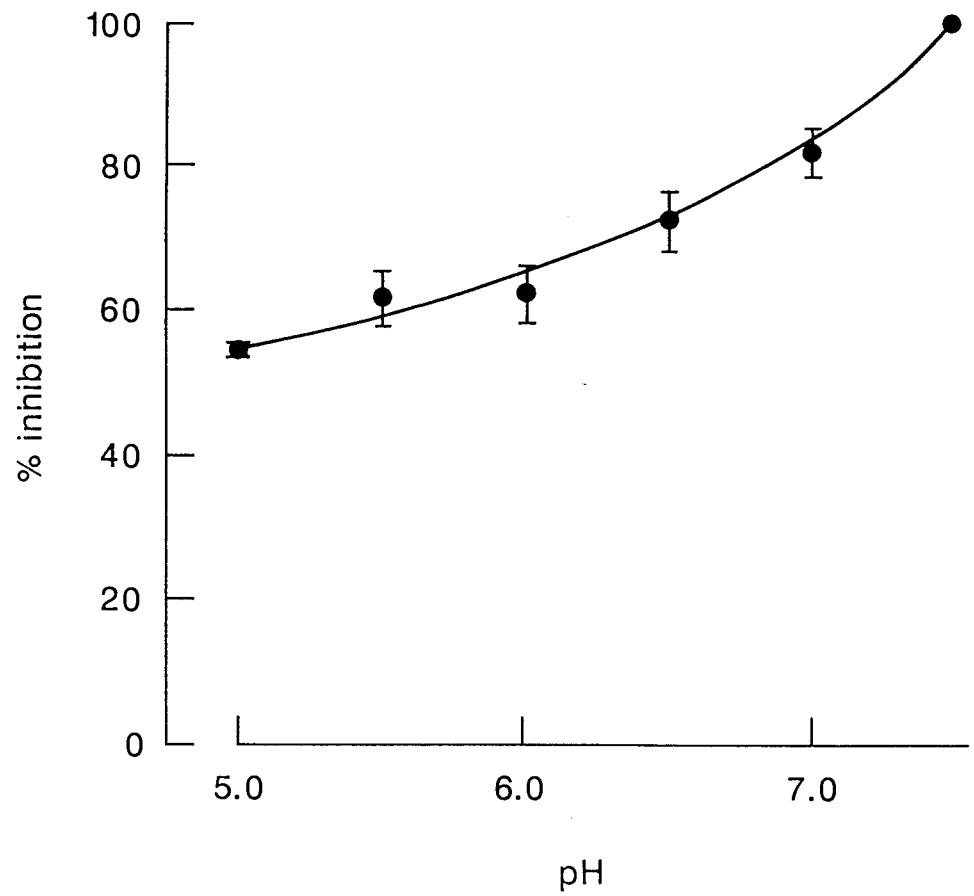


Figure 4.7: The effect of pH on inhibition by sodium vanadate (0.1 mol $m^{-3}$) of ATPase activity from $U_3$ after partitioning in a 6.5% phase system. Results are from two experiments and points are expressed as mean % inhibition ± standard error (n≥3).

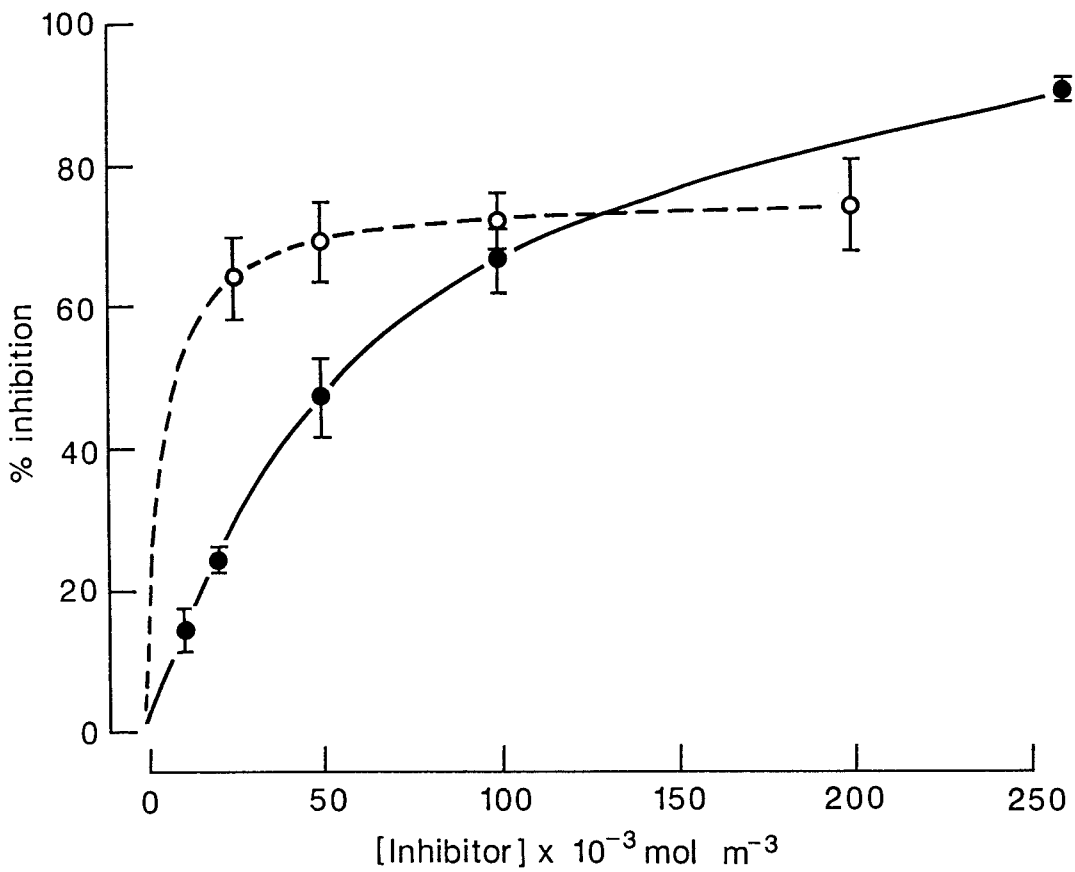


Figure 4.8: The effect of sodium vanadate (●) and SW26 (○) concentration on ATPase activity from $U_3$ after partitioning in a 6.5% phase system. Results are from two experiments and points are expressed as mean % inhibition ± 95% confidence interval (n≥4).

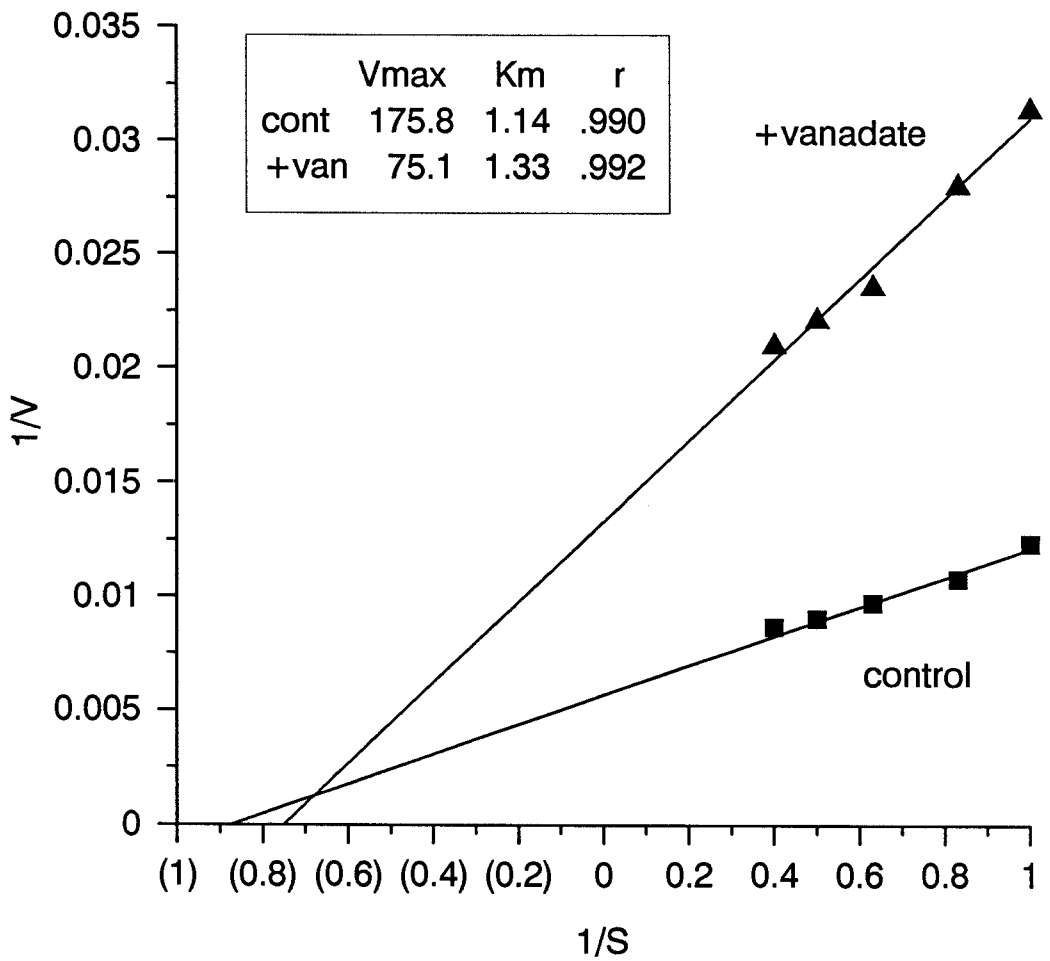


Figure 4.9: Lineweaver–Burk plot of ATPase activity from $U_3$, after partitioning in a 6.5% phase system, measured in the absence and presence of $50x10^{-3}$ mol $m^{-3}$ sodium vanadate. Negative values on the X axis are shown in brackets. Results are from one of three experiments (n=3) and kinetic parameters were calculated by linear regression.

activity. Inhibition was maximal at a concentration of $50x10^{-3}$ mol $m^{-3}$ and no more than 70% of the total activity was inhibited even using SW26 concentrations of $200x10^{-3}$ mol $m^{-3}$. Figure 4.10 shows that SW26 is a competitive inhibitor with a $K_i$ value of $4.7x10^{-3}$ mol $m^{-3}$. Interestingly, DMSO, the solvent in which SW26 was added, inhibited the ATPase non-competitively.

### 4.3.10. The effect of erythrosin B.

Erythrosin B was a potent inhibitor of the plasma membrane $H^+$-ATPase with $25x10^{-3}$ mol $m^{-3}$ sufficient for almost complete inhibition (Figure 4.11). Figure 4.12 shows a Lineweaver-Burk plot for inhibition by erythrosin B which was also of the competitive type with a $K_i$ of $2.7x10^{-3}$ mol $m^{-3}$.

### 4.3.11. The effect of $Ca^{2+}$ ions.

It has previously been shown that, in crude membrane preparations, $Ca^{2+}$ ions stimulate wheat leaf ATPase activity (see Chapter 3). In view of this it was a little surprising that $Ca^{2+}$ ions were found to inhibit wheat leaf plasma membrane ATPase activity, although inhibition by $Ca^{2+}$ is common in other species (Sze 1985). Figure 4.11 shows the effect of increasing $Ca^{2+}$ ion concentration on plasma membrane ATPase activity. $Ca^{2+}$ inhibition was saturated at a concentration of 1 mol $m^{-3}$ ($I_{50} \simeq 80x10^{-3}$ mol $m^{-3}$) and inhibited approximately 70% of the total ATPase activity. Inhibition was competitive with a $K_i$ value of $46.0x10^{-3}$ mol $m^{-3}$ (Figure 4.12).

Although $Ca^{2+}$ ions were inhibitory to $Mg^{2+}$-dependent ATPase activity there was also some $Ca^{2+}$-dependent activity present in this preparation. This activity was not stimulated by $K^+$ ions (see 4.3.3.). Figure 4.13 shows the pH profile of $Ca^{2+}$-dependent ATPase activity. The small peak of activity at pH 5.5 probably represents non-specific activity still associated with the membranes as it coincides with the pH of the maximum activity in the absence of a divalent cation (Figure 4.1). Activity was otherwise constant between pH 5.0 and 8.0 after which it fell sharply to zero at pH 8.5.

## 4.4. DISCUSSION

The plasma membrane preparation described in this chapter is purer than that isolated previously (see 3.3.7.3.). A comparison of the data in Tables 4.1 and 3.14 demonstrates that both mitochondrial (cytochrome c oxidase) and

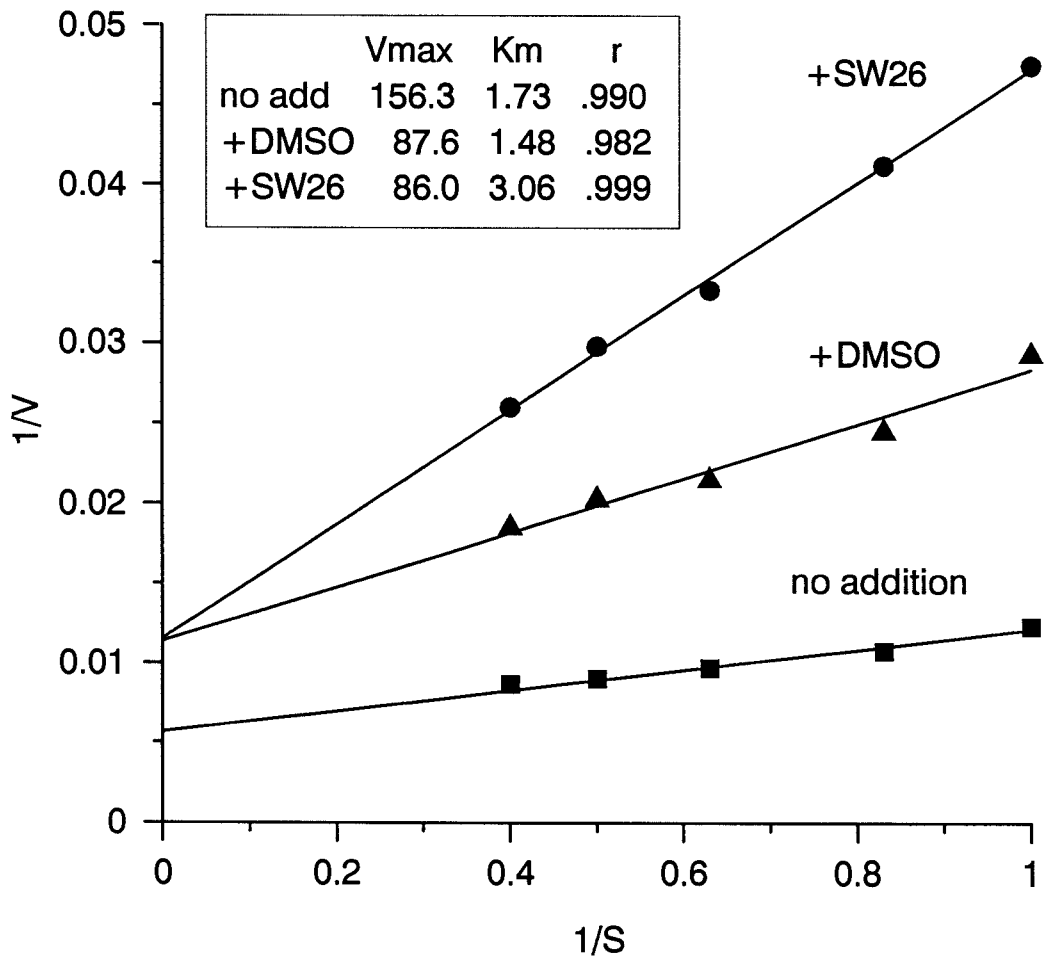


Figure 4.10: Lineweaver–Burk plot of ATPase activity from $U_3$, after partitioning in a 6.5% phase system, measured in the absence of an inhibitor or in the presence of 10% (v/v) DMSO and $5x10^{-3}$ mol $m^{-3}$ SW26 in 10% (v/v) DMSO. Results are from one of two experiments and kinetic parameters were calculated by linear regression (n=3).

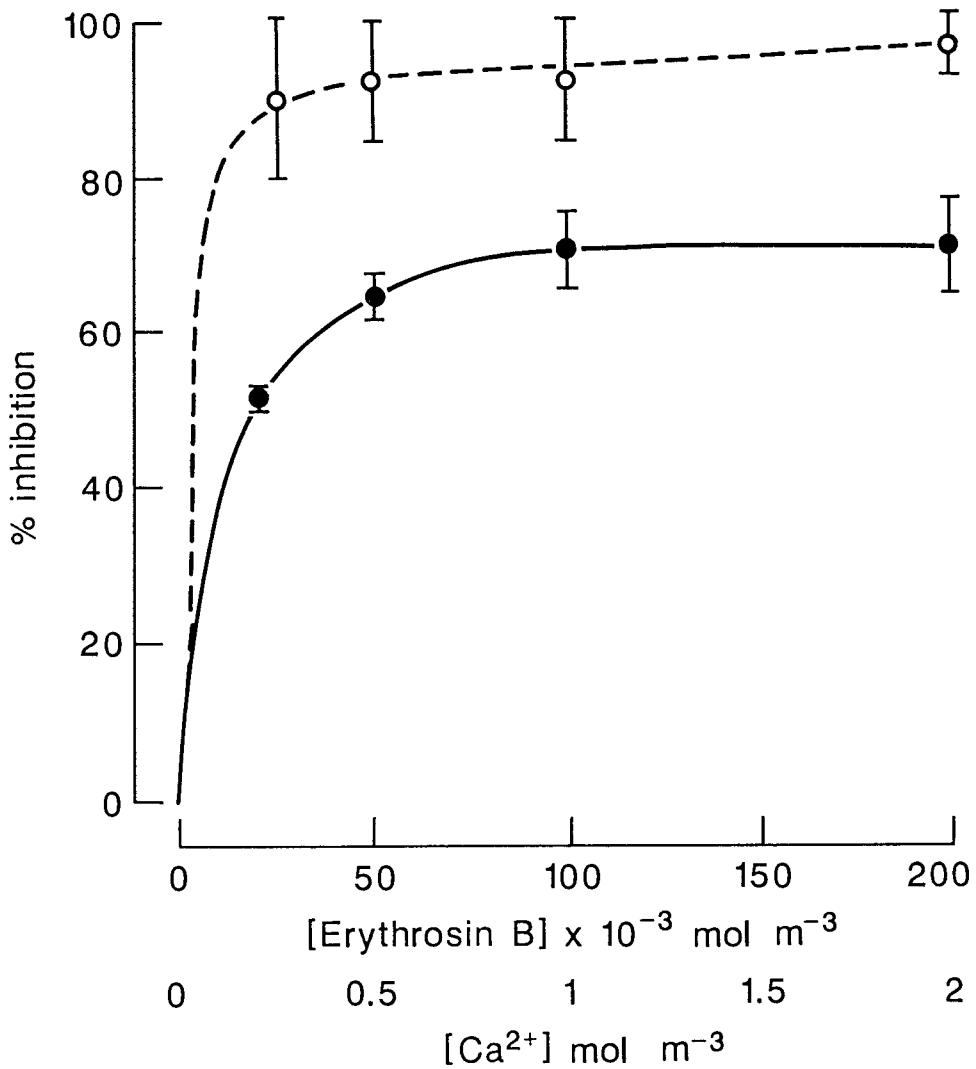


Figure 4.11: The effect of $Ca^{2+}$ ions (●) and erythrosin B (○) concentration on ATPase activity from $U_3$ after partitioning in a 6.5% phase system. Results are from two experiments and points are expressed as mean % inhibition ± 95% confidence interval (n=6).

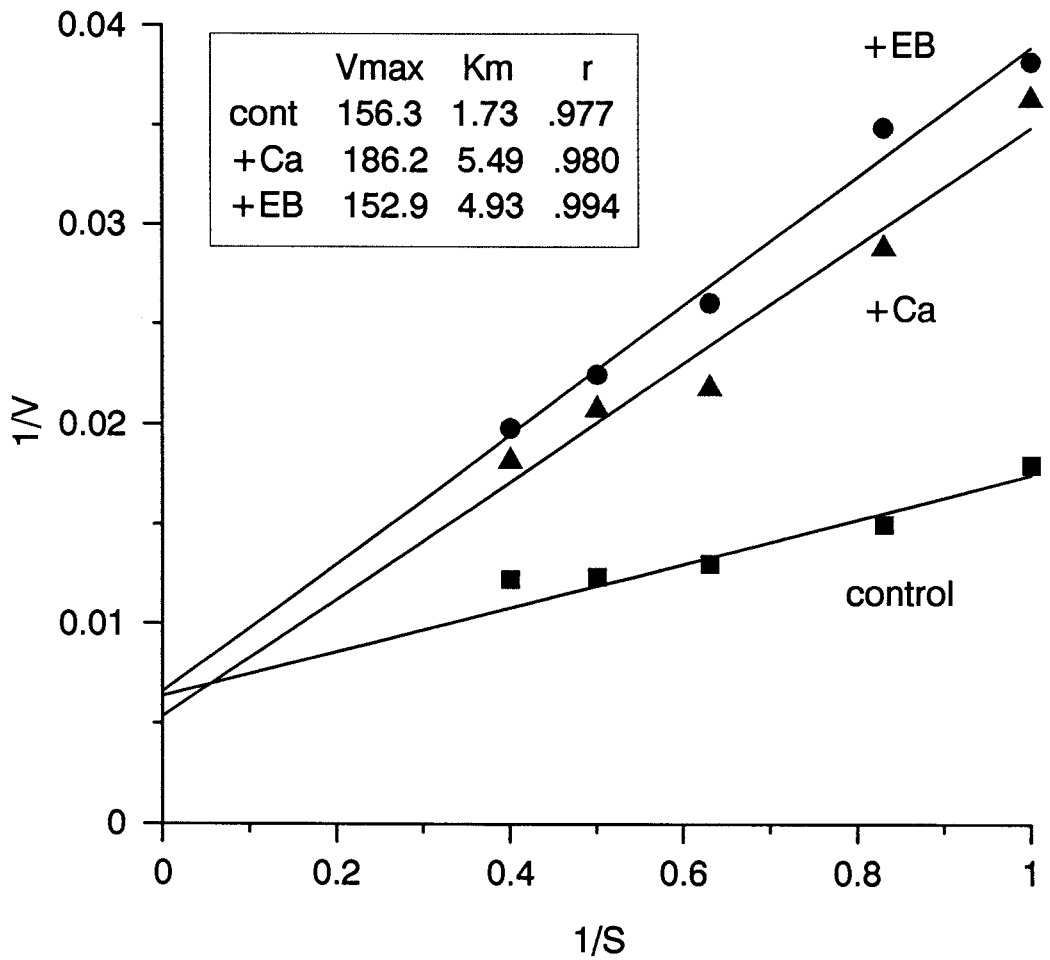


Figure 4.12: Lineweaver–Burk plot of ATPase activity from $U_3$, after partitioning in a 6.5% phase system, measured in the absence of an inhibitor or in the presence of 0.1 mol $m^{-3}$ $Ca^{2+}$ ions and $5x10^{-3}$ mol $m^{-3}$ erythrosin B. Results are from a single experiment and kinetic parameters were calculated by linear regression (n=3).

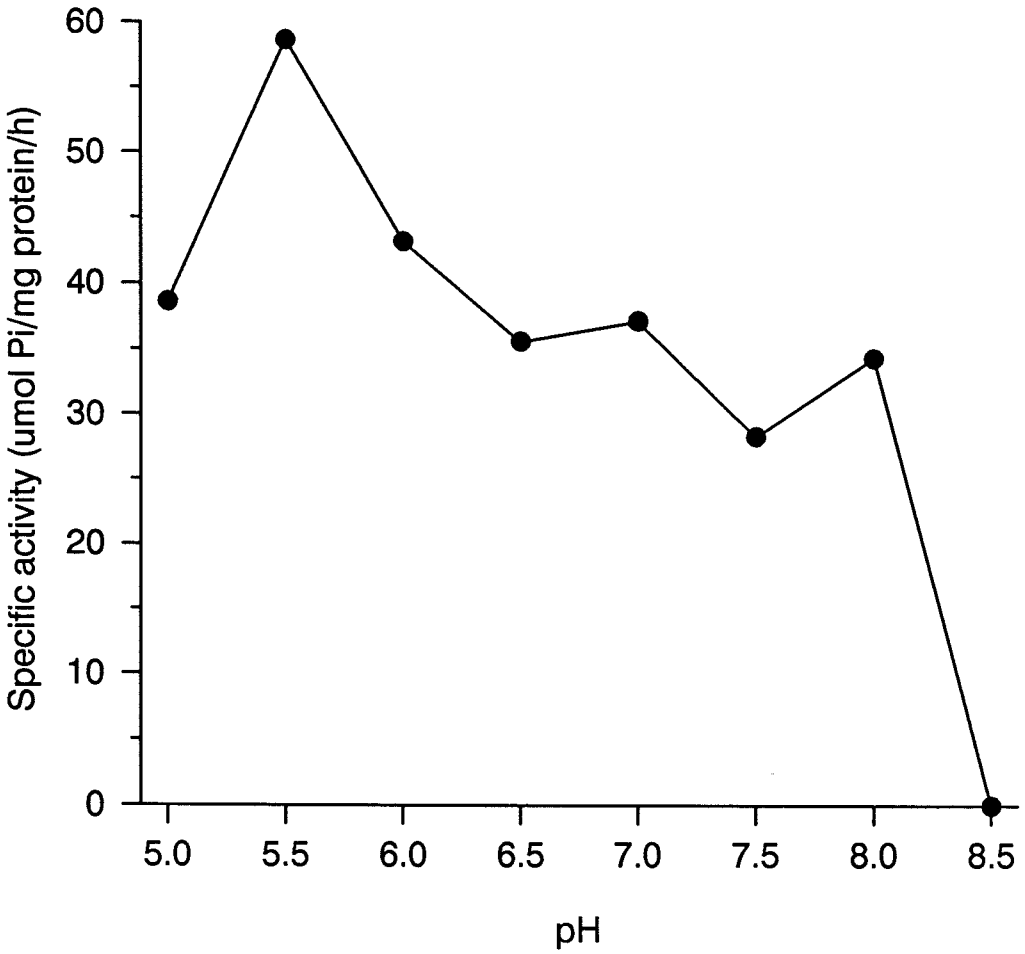


Figure 4.13: The effect of pH on ATPase activity from $U_3$, after partitioning in a 6.5% phase system, measured in the presence of 2 mol $m^{-3}$ $CaCl_2$ and in the absence of KCl. Results are from a single experiment and points are expressed as mean specific activity (n=3).

Golgi (latent IDPase) contamination has now been greatly reduced. NADH cytochrome c reductase activity is also reduced although endoplasmic reticulum is probably still the major contaminant. There is some evidence in the literature that a proportion of the activity measured for this enzyme might be associated with the plasma membrane. Sandelius *et al* (1986a) found a reduction of cytochrome c of 21 nmol/mg protein/min in 95% pure plasma membranes from soybean hypocotyls and Widell and Larsson (1983) reported NADH–cytochrome c reductase in plasma membrane preparations from a number of tissues. Larsson (1985) concluded that, because of the presence of cytochrome P–450/420 in the plasma membrane, the apparent cytochrome c reductase activity in these preparations was artificially high and that contamination by endoplasmic reticulum was probably of a similar value to that of the mitochondrial membranes. However, it is also possible that the NADH cytochrome c reductase activity is associated solely with endoplasmic reticulum as some co–purification of endoplasmic reticulum with plasma membranes is likely.

The enrichment of plasma membrane in this fraction can be seen clearly in both the increased glucan synthetase II activity and in the properties of the associated ATPase activity which are now consistent with those of the $K^+$,$Mg^{2+}$–ATPase activity found in plasma membrane preparations of other species (Sze 1985). The reason for this improved separation is not clear as essentially the same procedure was used for both preparations. However, the improved separation did coincide with a change in laboratories and this is the unexplained but likely cause.

The pH optimum of 6.0 for both the $Mg^{2+}$–dependent and the $K^+$–stimulated ATPase activity (Figure 4.1) is slightly lower than the pH optima reported for most species which are in the pH range 6.5–7.0. For example, ATPase activities in plasma membranes of corn (Leonard and Hotchkiss 1976, De Michelis and Spanswick 1986), oat (Sommarin *et al* 1985) and red beet (O'Neill and Spanswick 1984a) all have pH optima of 6.5. The lower pH optimum found in this study is in agreement with that found in wheat roots (Sommarin *et al* 1985) but is lower than that from wheat shoots (Pomeroy and McMurchie 1982, Aleshina *et al* 1988) where the pH optima was also 6.5. There are two possible reasons for this reported difference. In both the above studies on wheat shoots, plants were harvested much earlier and the tissue used would therefore be predominantly coleoptile. It is also probable that these two plasma membrane preparations were less pure. The properties of the plasma membranes isolated by Pomeroy and McMurchie (1982) have already been discussed (see 3.1.). The more recent study by Aleshina *et al* (1988) used a two–step phase partition technique and, although plasma membrane enriched, was also less pure as judged by the

presence of ATPase activity in the absence of divalent cations, lower substrate specificity and less vanadate sensitivity.

The specificity for monovalent cations is in general agreement with the expected rank order (Sze 1985) with the exception that $Rb^+$ ions stimulated $Mg^{2+}$-dependent ATPase activity more than $K^+$ ions. This was not found by Aleshina *et al* (1988) where $K^+$ ions were most effective. The substrate specificity of the plasma membrane $H^+$-ATPase is normally very high. For example in oat, all other substrates give less than 10% of the activity with ATP (Sommarin *et al* 1985). The wheat leaf plasma membrane preparation isolated in this study also exhibits high specificity and this contrasts with other wheat plasma membrane preparations where considerable activity is present with all nucleoside triphosphates and many diphosphates (Sommarin *et al* 1985, Aleshina *et al* 1988).

In the presence of $K^+$ ions the $K_m$ of the ATPase was 0.54 mol $m^{-3}$. There is no previous estimate for wheat but this value is similar to that found in other species. In plasma membrane preparations of red beet, $K_m$ values for the ATPase activity of 0.33 mol $m^{-3}$ (O'Neill and Spanswick 1984a) and 0.52 mol $m^{-3}$ (Briskin and Poole 1983) have been obtained. Higher $K_m$ values have also been found using plasma membranes from corn roots (0.89 mol $m^{-3}$, Gallagher and Leonard 1982) and mung bean roots (1.6 mol $m^{-3}$, Kasamo 1986). In the absence of $K^+$ ions the $K_m$ was higher while the $V_{max}$ was reduced. This result is consistent with the reported effect of $K^+$ ions on the plasma membrane ATPase activity of corn roots (Leonard and Hotchkiss 1976) and both oat and *Atriplex* roots (Mills and Hodges 1988). It is also consistent with $K^+$ ions increasing the rate of breakdown of the phosphorylated intermediate of the red beet $H^+$-ATPase (Briskin 1986). The degree of $K^+$ stimulation reported varies greatly, from over 200% in oat roots (Hodges *et al* 1972) to being absent completely in sugar beet (Briskin and Thornley 1985). The $K^+$ stimulation measured in wheat leaves is quite low (35% with 50 mol $m^{-3}$ KCl) and similarly low values have been found in plasma membrane preparations from wheat shoots (34%, Pomeroy and McMurchie 1982 and 18%, Aleshina *et al* 1988). Stimulation is maximal at pH 5.5 and this low pH optimum has also been widely reported (*e.g.* Vara and Serrano 1982, Sommarin *et al* 1985, Anthon and Spanswick 1986). Furthermore, at physiological pH values, there is no $K^+$ stimulation of the wheat leaf plasma membrane ATPase and these two findings raise doubts about the role of $K^+$ ions *in vivo*.

The $E_1/E_2$ type ATPases (see 1.2.) which form a covalent phosphorylated intermediate are inhibited by vanadate in the +5 oxidation state (Macara 1980) as it competes with phosphate for this binding site (Cantley *et al* 1978). Because of the specificity of vanadate for this type of transport ATPase over those with alternative mechanisms, it has been used widely as a specific

marker for the presence of the plasma membrane $H^+$–ATPase of higher plants (Hall 1983). Inhibition by vanadate was greater in this preparation (70% inhibition with 0.1 mol $m^{-3}$) than in previously published wheat plasma membrane preparations. Using wheat roots Sommarin *et al* (1985) obtained 66% inhibition with 0.25 mol $m^{-3}$ and Berczi and Moller (1986) obtained 36% inhibition of ATPase activity with 1 mol $m^{-3}$ vanadate. In wheat shoots, Aleshina *et al* (1988) found that 0.1 mol $m^{-3}$ vanadate inhibited 50% of the ATPase activity measured.

In addition to these differences in the vanadate sensitivity of the wheat plasma membrane ATPases there is also a wide variation in the reported $K_i$ values from other tissues. O'Neill and Spanswick (1984b) reported values of between $6x10^{-3}$ and $15x10^{-3}$ mol $m^{-3}$ vanadate for roots of corn and red beet. A similarly low value for corn roots was reported by Gibrat *et al* (1989). However, a $K_i$ value of $233x10^{-3}$ mol $m^{-3}$ vanadate was determined by Gallagher and Leonard (1982) also in corn roots and Perlin and Spanswick (1981) reported half maximum inhibition of plasma membrane ATPase activity with $500x10^{-3}$ mol $m^{-3}$ vanadate. These values are far in excess of a typical value given by Sze (1985) of $3–10x10^{-3}$ mol $m^{-3}$ vanadate, although inhibition of the yeast $H^+$–ATPase is in this range (Dufour *et al* 1980). In addition, both the *Neurospora* $H^+$–ATPase (Bowman and Slayman 1979) and the mammalian $Na^+$,$K^+$–ATPase (Cantley *et al* 1978) are inhibited by vanadate with $K_i$ values of less than $1x10^{-3}$ mol $m^{-3}$. In conclusion, the $K_i$ value of $37.3x10^{-3}$ mol $m^{-3}$ vanadate for the inhibition of the wheat leaf plasma membrane ATPase appears to be well within the range for inhibition of plasma membrane ATPases from other higher plants.

There are three possible explanations for the observed discrepancies in vanadate sensitivity. Firstly, variation in the purity of the ATPase preparations is important as the presence of a second ATP–hydrolysing enzyme with a different sensitivity to vanadate, such as the plasma membrane $Ca^{2+}$–ATPase (Butcher and Evans 1987b) or associated phosphatase activity (Gallagher and Leonard 1982), would hinder the measurement of the vanadate sensitivity of the $H^+$–ATPase when assayed by the release of free phosphate. Vanadate sensitivity is also altered by changes in the ATPase assay medium. Gibrat *et al* (1989) measured a 10–fold variation in the apparent $K_i$ value depending on the addition of $Mg^{2+}$ or $K^+$ ions, both of which reduce the apparent inhibition constant. This is achieved by reducing the net negative charge around the enzyme so allowing the vanadate anion to bind more easily. However, $K^+$ ions also seem to interact more directly indicating a possible explanation of $K^+$ stimulation (Gibrat *et al* 1989). The third reason is that the vanadate itself may exist in different forms. Although there was no reported difference in the inhibition of ATPase activity when vanadate was supplied as $Na_3VO_4$ or $V_2O_5$ (O'Neill and Spanswick 1984b), in solution vanadate

can assume multiple oxidation states and degrees of polymerization (Pick 1981). Therefore differences in source or preparation may effect the activity of the vanadate solution. Recent work by Gibrat *et al* (1989) suggests that the active species is probably $H_2VO_4^{1-}$ (or $HVO_4^{2-}$) and differences in the pH of the assay medium will also change the relative proportions of these vanadate species.

The nature of vanadate inhibition is also uncertain. Analysis using a Lineweaver–Burk plot indicates that the wheat leaf enzyme is inhibited non–competitively. With this type of inhibition $I_{50}$ and $K_i$ values should be equal. In this study these values were $38x10^{-3}$ and $37.3x10^{-3}$ mol $m^{-3}$ respectively which supports the proposal of a non–competitive mode of inhibition. The uncertainty about the mechanism is highlighted in studies on the corn root plasma membrane ATPase. In this tissue there are two reports of non–competitive inhibition (Gallagher and Leonard 1982, Tu and Sliwinski 1985) and two of uncompetitive inhibition (O'Neill and Spanswick 1984b, Gibrat *et al* 1989). In addition, Kasamo (1986) has reported a competitive inhibition of the mung bean root $H^+$–ATPase. For vanadate inhibition of fungal $H^+$–ATPases the picture is more clear. In both yeast (Dufour *et al* 1980) and *Schizosaccharomyces pombe* (Dufour and Goffeau 1980) inhibition appears to be non–competitive, although a more recent paper reports that inhibition of the yeast $H^+$–ATPase is only non–competitive at $Mg^{2+}$ ion concentrations above 0.1 mol $m^{-3}$ and below this concentration is a mixture of uncompetitive and non–competitive inhibition (Borst–Pauwels and Peters 1981).

An uncompetitive inhibitor is one which only binds to the enzyme–substrate complex. As vanadate can inhibit the formation of the phosphorylated intermediate of the red beet $H^+$–ATPase an uncompetitive mode of inhibition is unlikely (Briskin and Poole 1983). However, phosphorylation appears to be less sensitive to vanadate than ATPase activity from the same preparation indicating that a more complex pattern of vanadate inhibition may exist (Briskin and Leonard 1982). The mammalian $Na^+$,$K^+$–ATPase has two binding sites for vanadate, one of high affinity (the phosphate binding site itself) and one of low affinity which is only filled after the enzyme has been phosphorylated (Cantley *et al* 1978). The presence of vanadium in mammalian tissues led to the suggestion that it might regulate the activity of the $Na^+$,$K^+$–ATPase *in vivo* (Cantley 1981) and it is interesting that vanadium can also be found in higher plant tissues (Marschner 1986).

The carbanilate derivative, SW26, was first demonstrated to inhibit the plasma membrane $H^+$–ATPase in sycamore cell cultures by Blein *et al* (1986). It has subsequently been shown to inhibit the plasma membrane $H^+$–ATPase of corn shoots and yeast (Blein *et al* 1987) and of *Ricinus* cotyledons and cucumber hypocotyls (Ball *et al* 1987). The $K_i$ value determined for the inhibition of the

wheat leaf plasma membrane ATPase of $4.7x10^{-3}$ mol $m^{-3}$ was slightly lower than that reported in sycamore of $14.7x10^{-3}$ mol $m^{-3}$ SW26 (Blein *et al* 1986). There also appears to be a different mechanism of action in the two tissues. In sycamore, SW26 inhibits non-competitively below $20x10^{-3}$ mol $m^{-3}$ but above this concentration Michaelis-Menten kinetics were no longer observed. In wheat, $5x10^{-3}$ mol $m^{-3}$ SW26 inhibited ATPase activity competitively although the solvent, DMSO, in which the SW26 was added, inhibited non-competitively.

The colour additive erythrosin B (tetraiodofluorescein) has been shown to be an inhibitor of the plasma membrane $H^+$-ATPase both *in vitro* and *in vivo* (Coccuci 1986, Ball *et al* 1987). However, erythrosin B is not a specific inhibitor of this enzyme and also inhibits the tonoplast $H^+$-ATPase (Coccuci 1986), numerous metabolic enzymes (Gimmler 1988) and other ion transporters of the $E_1/E_2$ type, such as the $Na^+,K^+$-ATPase of rat brain (Silbergeld 1981) and the sarcoplasmic $Ca^{2+}$-ATPase of rabbit muscle (Morris *et al* 1982). In wheat leaves, erythrosin B was a very potent inhibitor of plasma membrane ATPase activity with a $K_i$ of $2.7x10^{-3}$ mol $m^{-3}$. This compares favourably to inhibition of the radish $H^+$-ATPase ($I_{50}=10x10^{-3}$ mol $m^{-3}$, Coccuci and Marre 1984) and the rabbit $Ca^{2+}$-ATPase ($I_{50}=1x10^{-3}$ mol $m^{-3}$, Morris *et al* 1982). When the radish enzyme was partially purified the $I_{50}$ was shifted to $0.1x10^{-3}$ mol $m^{-3}$ erythrosin B (Coccuci 1986). Inhibition of the wheat leaf plasma membrane ATPase is competitive and again this is in contrast to a previous result. Coccuci (1986) showed that the radish $H^+$-ATPase was inhibited by erythrosin B non-competitively. However, erythrosin B is clearly related to fluorescein 5'-isothiocyanate which has been used to label the ATP binding site of animal ATPases (Farley and Faller 1985, Serrano 1988) and which suggests that erythrosin B may compete with ATP for the substrate binding site.

$Ca^{2+}$ ions have been shown to inhibit $Mg^{2+}$-dependent, plasma membrane ATPase activity from many tissues including corn (Leonard and Hotchkiss 1976) and wheat roots (Klotz *et al* 1989). In this latter paper $300x10^{-3}$ mol $m^{-3}$ $Ca^{2+}$ inhibited 38% of the $Mg^{2+}$-dependent ATPase activity. The wheat leaf enzyme is more sensitive to $Ca^{2+}$ ions, with a $K_i$ of $46x10^{-3}$ mol $m^{-3}$ and an $I_{50}$ of approximately $80x10^{-3}$ mol $m^{-3}$ $Ca^{2+}$. The inhibition is competitive and this is in agreement with the $Ca^{2+}$ inhibition of the corn root plasma membrane ATPase activity (Tu and Brouillette 1987) and of $K^+$ influx into corn root cells (Leonard *et al* 1975) but not with the $Ca^{2+}$ inhibition of oat root plasma membrane ATPase activity which was uncompetitive (Balke *et al* 1974). The mechanism of $Ca^{2+}$ inhibition is unknown. $Ca^{2+}$ ions could be competing with $Mg^{2+}$ ions to form a $CaATP^{2-}$ complex, thereby reducing the true substrate concentration (Balke *et al* 1974), but the low concentrations of $Ca^{2+}$ ions required

for half inhibition suggests that this is unlikely to be the only explanation. Furthermore, Bennett *et al* (1985) demonstrated that $Ca^{2+}$ ions inhibited plasma membrane but not tonoplast ATPase activity suggesting a direct interaction with the plasma membrane ATPase.

In addition to the presence of $Ca^{2+}$–sensitive, $Mg^{2+}$–dependent ATPase activity there was also some $Ca^{2+}$–dependent ATPase activity associated with the plasma membrane. This activity was not stimulated by $K^+$ ions (Table 4.3) suggesting that the $Ca^{2+}$ was not substituting for $Mg^{2+}$ ions. The pH profile gives an indication of the origin of this $Ca^{2+}$–dependent activity. The peak at pH 5.5 coincides with the peak of ATPase activity measured in the absence of divalent cations and shown in Figure 4.1. It is likely that this activity is attributable to some remaining non–specific ATPase which has been described in Chapter 3. The $Ca^{2+}$–dependent activity measured at higher pH values may possibly represent ATPase activity associated with the calmodulin–stimulated, $Ca^{2+}$–transporting ATPase which has a pH optimum between 7.0 and 8.0 (Gross and Marme 1978, Butcher and Evans 1987a, Robinson *et al* 1988). The activity of this enzyme is reduced above pH 8.0 and although it is considered to be $Mg^{2+}$–dependent, Carter and Tipton (1987) have demonstrated that a similar enzyme from *Zea mays* has a $Mg^{2+}$ requirement that is met by traces of $Mg^{2+}$ ions in the enzyme and substrate preparations. The proposal that a proportion of the remaining $Ca^{2+}$–dependent ATPase activity is due to the activity of the plasma membrane $Ca^{2+}$–ATPase is supported by the observation that while both $Ca^{2+}$ ions and SW26 inhibit 70% of the total ATPase activity, erythrosin B and vanadate inhibit activity completely. Vanadate (Butcher and Evans 1987b) and erythrosin B (D.E. Evans pers. comm.) have both been shown to inhibit the $Ca^{2+}$–ATPase but there is no evidence that SW26 does.

In conclusion, the work in this chapter demonstrates that a highly enriched plasma membrane preparation can be isolated from wheat leaf tissue. The ATPase associated with this preparation has properties similar to those normally associated with the plasma membrane $H^+$–ATPase of higher plants (Sze 1985), and therefore this plasma membrane preparation is suitable for studying the effect of light treatments on this enzyme.

# CHAPTER FIVE

# THE EFFECTS OF LIGHT AND PHYTOCHROME ON PLASMA MEMBRANE ATPASE ACTIVITY

## 5.1. INTRODUCTION

Evidence that effects of phytochrome on plasma membrane-related processes are mediated through the $H^+$-ATPase is limited. The possible role of this enzyme in the R-induced protoplast swelling response (Blakeley *et al* 1983) has already been discussed (1.3.4.3.). In addition, there are a number of reported instances of a net efflux of protons to the external medium after R irradiation. These include Yunghans and Jaffe (1972) in mung bean roots, Roth-Bejerano and Hall (1986b) in cucumber hypocotyls, and Brownlee and Kendrick (1979) in apical hook segments of mung bean, although there was no rapid proton extrusion. One approach taken to confirm the involvement of the $H^+$-ATPase in this response has been to examine the photoregulation of ATPase activity in isolated membranes.

An initial investigation (Yunghans and Jaffe 1972) concluded that there was no effect of R or FR irradiation, given *in vivo*, on ATPase activity isolated from root tips of mung bean. Since then there have been a number of contradictory papers on this subject. De Greef *et al* (1976) also found that R *in vivo* had no effect on ATPase activity from cotyledons or primary leaves of mung bean seedlings but did find that ATPase activity was reduced in hypocotyl hooks to 10% of the dark level within 2 min. It was not, however, shown that this response was under phytochrome control. Subsequently, Jose (1977) also reported a R-induced inhibition of ATPase activity in hypocotyl hooks and this response required 60 to 90 s R to be detected. The effect of *in vitro* R irradiation was also investigated. R *in vitro* without a prior R *in vivo* treatment had no effect on activity but if an *in vivo* treatment was given the level of activity depended on the final form of the phytochrome whether established *in vivo* or *in vitro*. Interestingly, the regulation of ATPase activity in this system was shown to be subject to a light-sensitive oscillator with a period of about 60 s, similar to the oscillation time of the growth response (Klein 1980). It should be noted, perhaps, that in the paper by Jose and a subsequent one (Jose and Schafer 1979) the level of enzyme activity after R *in vivo* is the same with or without R *in vitro* but is stimulated by FR. The second investigation also reported an *in vitro* effect on the linear phase of the ATPase reaction, with R stimulating the reaction rate after FR pre-irradiation *in vitro*.

The requirement for an *in vivo* treatment to see an effect from an *in*

*vitro* treatment was also found in cucumber hypocotyls by Thomas and Tull (1981) and Roth-Bejerano and Hall (1986a). In contrast to the earlier work both groups demonstrated an increase in activity after R *in vitro*. It is possible that this anomaly is due either to the different plants or to different regions of the hypocotyls used. This view is supported by a more in-depth study by Ball (1988). Of 6 hypocotyl regions examined only the apical hook (used by Roth-Bejerano and Hall 1986a,b) showed R-induced stimulation of ATPase activity. This stimulation had been masked in experiments with the whole hypocotyl. Additionally, Ball (1988) was unable to detect a light effect on ATPase activity from any enriched membrane fraction prepared by sucrose density gradient centrifugation, nor in proton pumping experiments. Blakeley *et al* (1987) were also unable to see any light effects on microsomal ATPase activity in wheat leaves. However, the absence, in this study, of a R pre-treatment means that this result is not unexpected in the light of previous papers.

Phytochrome regulation of the ATPase may manifest itself more clearly as a change in the kinetics of the enzyme. The first investigation of this was by Thomas and Tull (1981). They found that, after 2 min R *in vivo*, FR increased the $K_m$ for ATP and that this increase was R reversible. There was little change in $V_{max}$ and the effect on the $K_m$ could be removed by FR reversal of the R *in vivo* treatment. Similar results were reported by Roth-Bejerano and Hall (1986a), but Ball (1988) was unable to detect any consistent effects on $K_m$ values in the same system.

The aim of the present study is to examine thoroughly the possibility that light directly regulates plasma membrane $H^+$-ATPase activity as measured *in vitro*. By using a highly purified, well characterized system and with the availability of purified phytochrome it is hoped that some of the confusion that has resulted from this line of investigation can be dispelled. All experiments were performed at Littlehampton.

## 5.2. EXPERIMENTAL

The duration of the ATPase assay was extended to 40 min with the pH of the assay as given in the appropriate figure legends. *In vivo* light treatments were uniformly 5 min R and 10 min FR. *In vitro* light treatments were 2 min R and 10 min for FR and other wavelengths used. All work was performed under dim, green safe light.

The data from the experiments where two or more treatments were applied, were analysed by one-way analysis of variance. This statistical method tests the hypothesis that there is no difference between a number of treatments.

The total variation is calculated by measuring the variation both between and within treatment groups. This variation is represented by an estimate for the standard error of the difference (S.E.D.) between experimental values. The difference between two treatment means is statistically significant at the 5% level of the $t$–distribution if it is greater than the S.E.D. multiplied by the $t$–value. The $t$–value at the 5% level of the $t$–distribution is obtained from the residual number of degrees of freedom taken from the analysis of variance and approximates to 2 as $v$ increases. The degrees of freedom (df) for each analysis are given in the appropriate figure.

## 5.3. RESULTS

### 5.3.1. Light Sources.

Figure 5.1 shows the spectral emission of the R and FR light sources in the light box used. The R source has a peak at 660nm (the measurements are taken at 5nm intervals) and falls to zero before 700nm. The FR source rises sharply after 700nm peaking above 780nm. The fluence rates are 1.19 $Wm^{-2}$ (6.47 $\mu$mol $m^{-2}$ $s^{-1}$) between 600 and 700nm and 0.88 $Wm^{-2}$ (5.45 $\mu$mol $m^{-2}$ $s^{-1}$) between 700 and 780nm respectively.

The difference spectrum for wheat leaf phytochrome is shown in Figure 5.2. The sample used was spun at 10,000g for 10 min to remove particulate material and so prevent light scattering. The difference maxima are 659nm for $P_r$ and 729nm for $P_{fr}$.

### 5.3.2. The effects of *in vivo* light treatments.

The possibility that an *in vivo* light treatment affected the plasma membrane ATPase in such a way as to be detected by an *in vitro* assay was investigated. No consistent effects were found on either the $K_m$ or $V_{max}$ of the ATPase after a 5 min R treatment. A typical result is shown in Figure 5.3. In addition, in 17 experiments during the course of the present study where ATPase activity was measured with and without a 5 min R *in vivo* treatment, there was no difference in specific activity (Table 5.1).

### 5.3.3. The effects of *in vitro* light treatments.

The effect of *in vitro* light treatments on plasma membrane ATPase activity was examined. Figures 5.4 and 5.5 demonstrate an *in vitro* R–dependent

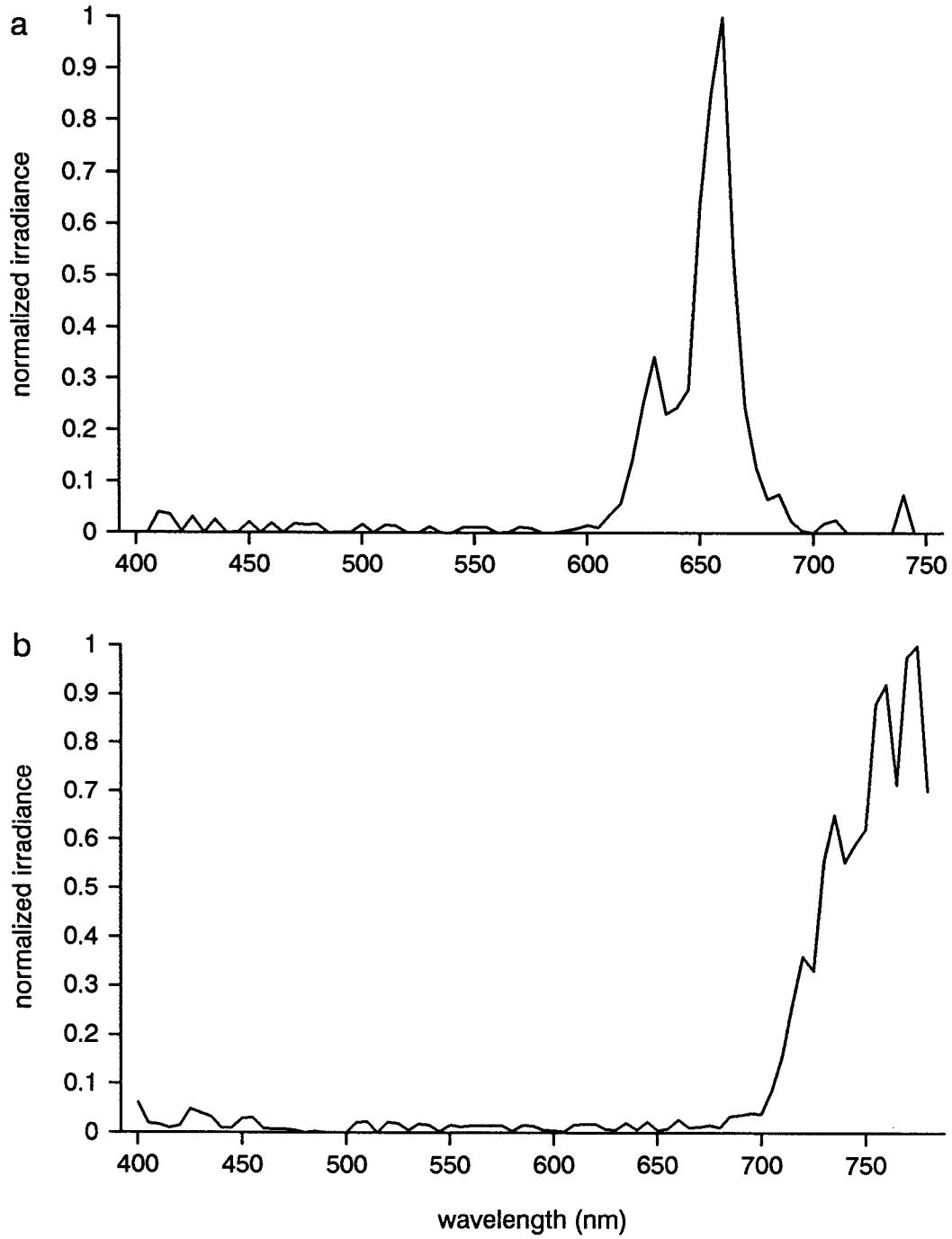


Figure 5.1: Spectral emission of R (Figure 5.1a) and FR (Figure 5.1b) light sources measured by spectroradiometry.

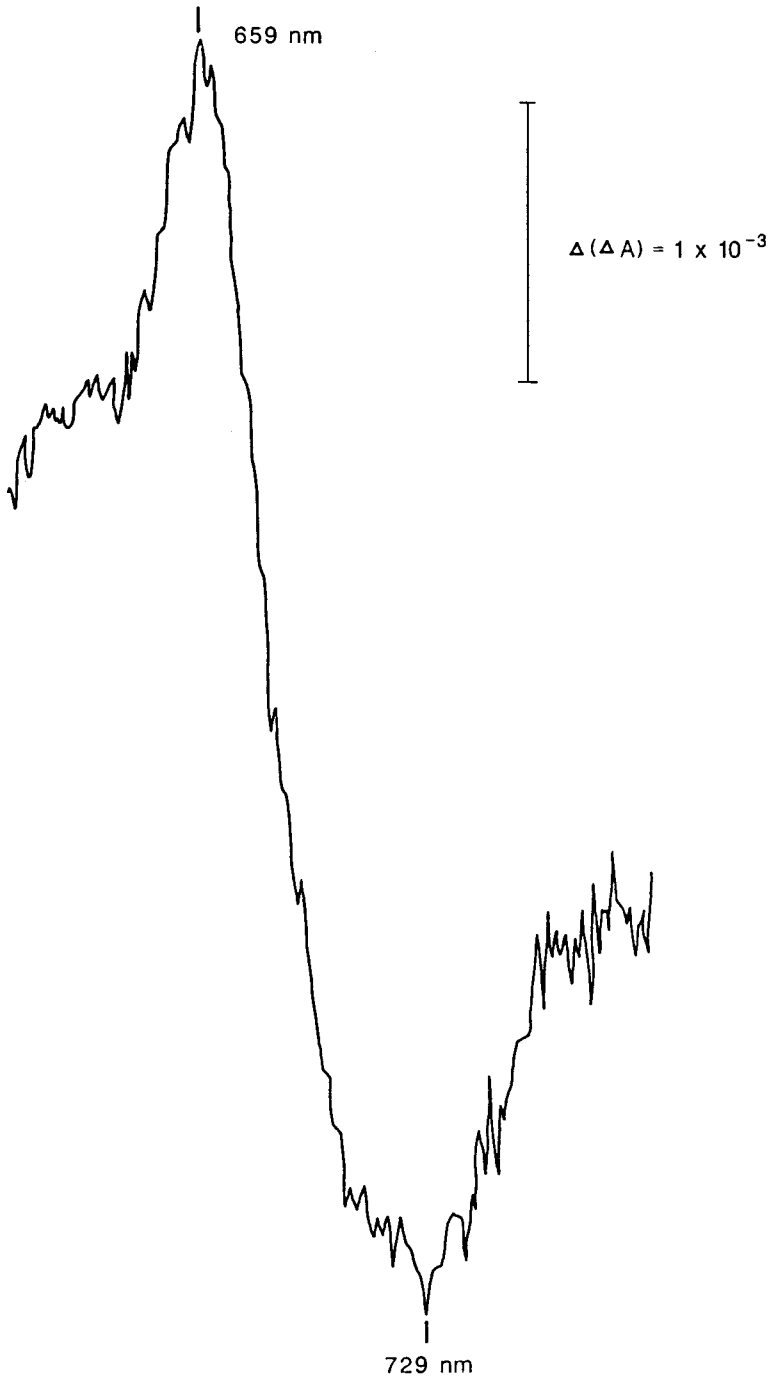


Figure 5.2: Difference spectrum of wheat leaf phytochrome present in the supernatant after centrifugation of a crude homogenate at 10,000g. Absorbance maxima are 659 nm for $P_r$ and 729 nm for $P_{fr}$.

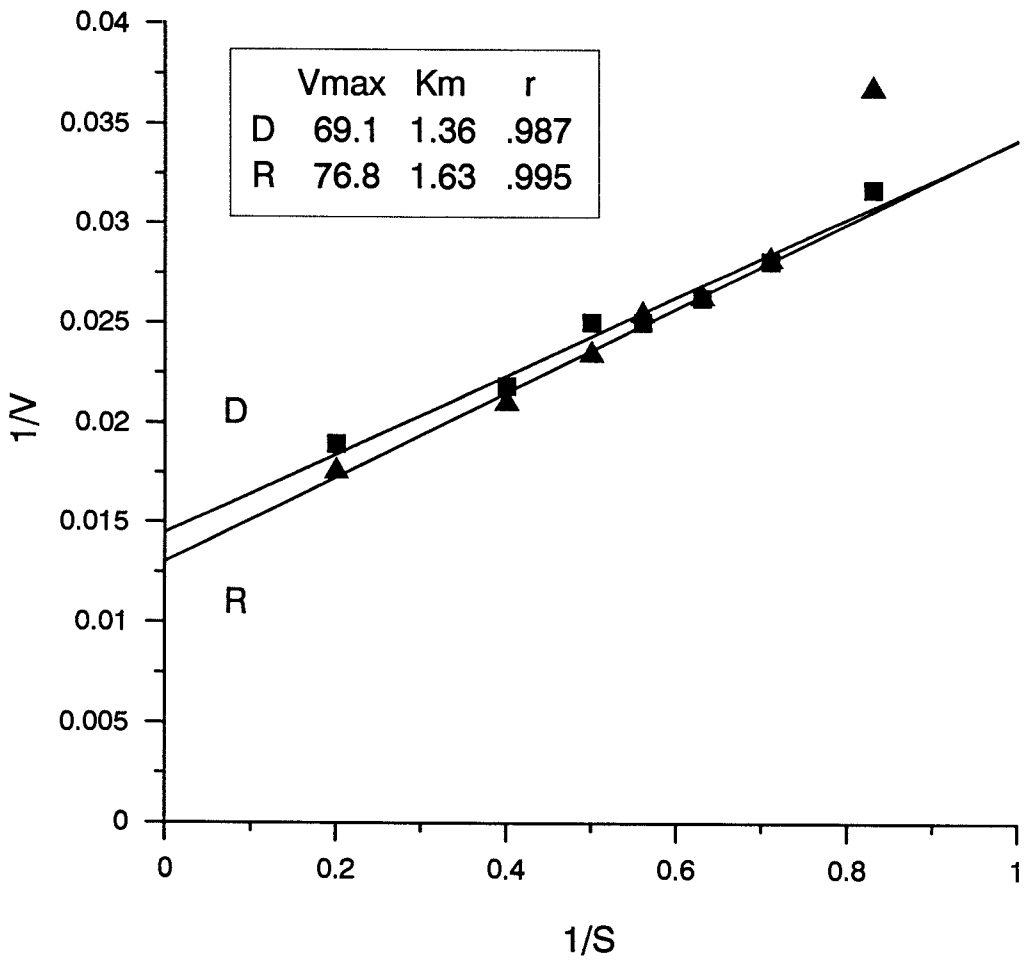


Figure 5.3: Lineweaver–Burk plot of plasma membrane ATPase activity assayed at pH 6.0 after R *in vivo* (R) and no light treatment (D). Results are from a single representative experiment (n=3) and kinetic parameters were calculated by linear regression.

| Treatment | Specific Activity ($\mu$mol $P_i$/mg protein/h) |
|---|---|
| D | 95.14 ± 12.09 |
| R | 95.79 ± 10.23 |

Table 5.1: The effect of 5 min R *in vivo* on plasma membrane ATPase activity. Values are from seventeen experiments (n=17) and are expressed as mean specific activity ± standard error.

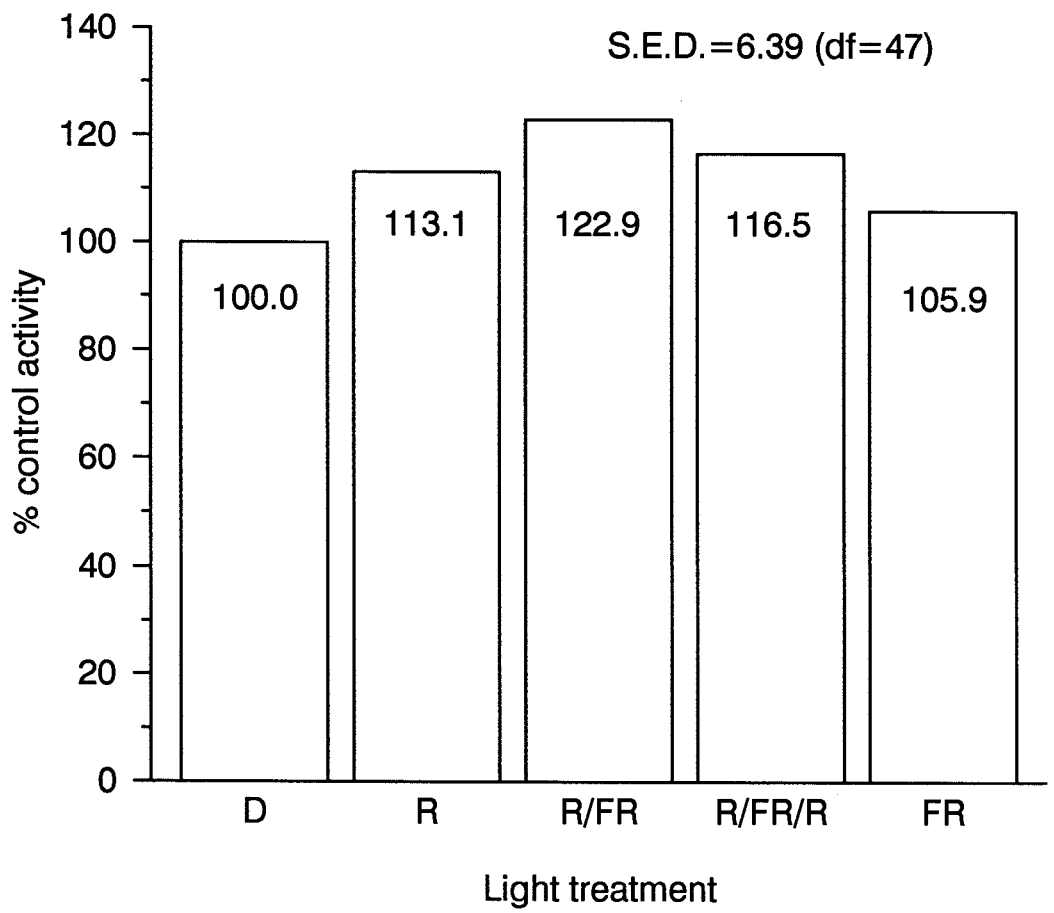


Figure 5.4: The effect of different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 6.0 in the absence of an *in vivo* light treatment. Results are from four experiments and are expressed as mean % control activity (D).

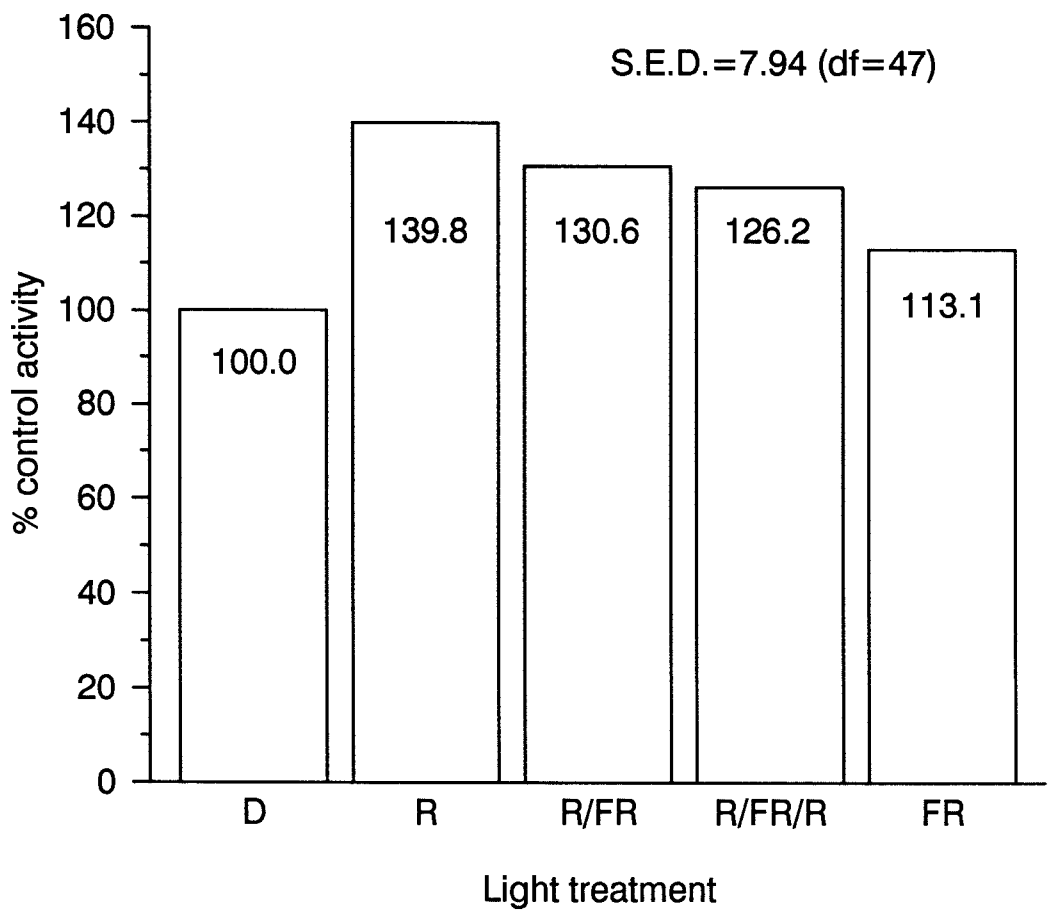


Figure 5.5: The effect of different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 6.0 after 5 min R *in vivo*. Results are from four experiments and are expressed as mean % control activity (D).

increase in ATPase activity. With no *in vivo* treatment there was a significant increase in ATPase activity in all *in vitro* light treatments with an initial R, but not with FR alone. A similar pattern is seen with an additional 5 min R *in vivo* treatment but in this case the stimulation of activity was approximately two-fold greater. There is no apparent FR reversal of the response on the time scale used with either set of treatments.

The pH profile and $K^+$ dependence of this response were examined (Figure 5.6). R stimulation of the $K^+$ dependent component of the ATPase activity was greatest at pH 6.5, although total stimulation was highest at pH 6.0. Problems with a low level of measurable ATPase activity at this time made reliable measurement of this response more difficult at pH 6.0 than at higher pH values and ATPase activity was subsequently assayed at pH 6.5.

A dose-response curve was constructed for the R-induced response (Figure 5.7). There is no increase in ATPase activity until after 40s R and the response appears to saturate between 60 and 90s. The data presented in this figure are from 2 experiments. In 3 subsequent experiments the response was no longer seen (Figure 5.8). The reason for this is not clear and attempts were made to optimize the system.

### 5.3.4. Factors affecting response stability.

It has previously been demonstrated that the presence of boron in the homogenization medium enhances R stimulation of ATPase activity (Roth-Bejerano and Hall 1986a). Figures 5.9 and 5.10 show the results from a single experiment where 5 mol $m^{-3}$ $H_3BO_3$ was included. There is a significant difference, with or without an *in vivo* treatment, between the R and R/FR *in vitro* treatments and between the R/FR and R/FR/R treatments. The interpretation of these results, though, is difficult. With no *in vivo* treatment ATPase activity is stimulated by $P_{fr}$ and this is reversible. After 5 min R *in vivo* $P_r$ appears to stimulate activity reversibly. In 3 further experiments there were no light effects under these conditions (Figures 5.11 and 5.12). The reason for the inclusion of this data from a single experiment is that the changes in activity are large and that the pattern of response, although not easily explainable, does bear considerable resemblance to that expected of a phytochrome-mediated response. It is unlikely that this pattern would occur by chance, particularly as the dark controls were assayed at the beginning and at the end of the light treatment series.

Triton X-100 is routinely included in these ATPase assays to allow the substrate access to the substrate binding site of the ATPase (see 4.3.5.). There are two possibilities for Triton affecting the stability of the response. It could either be

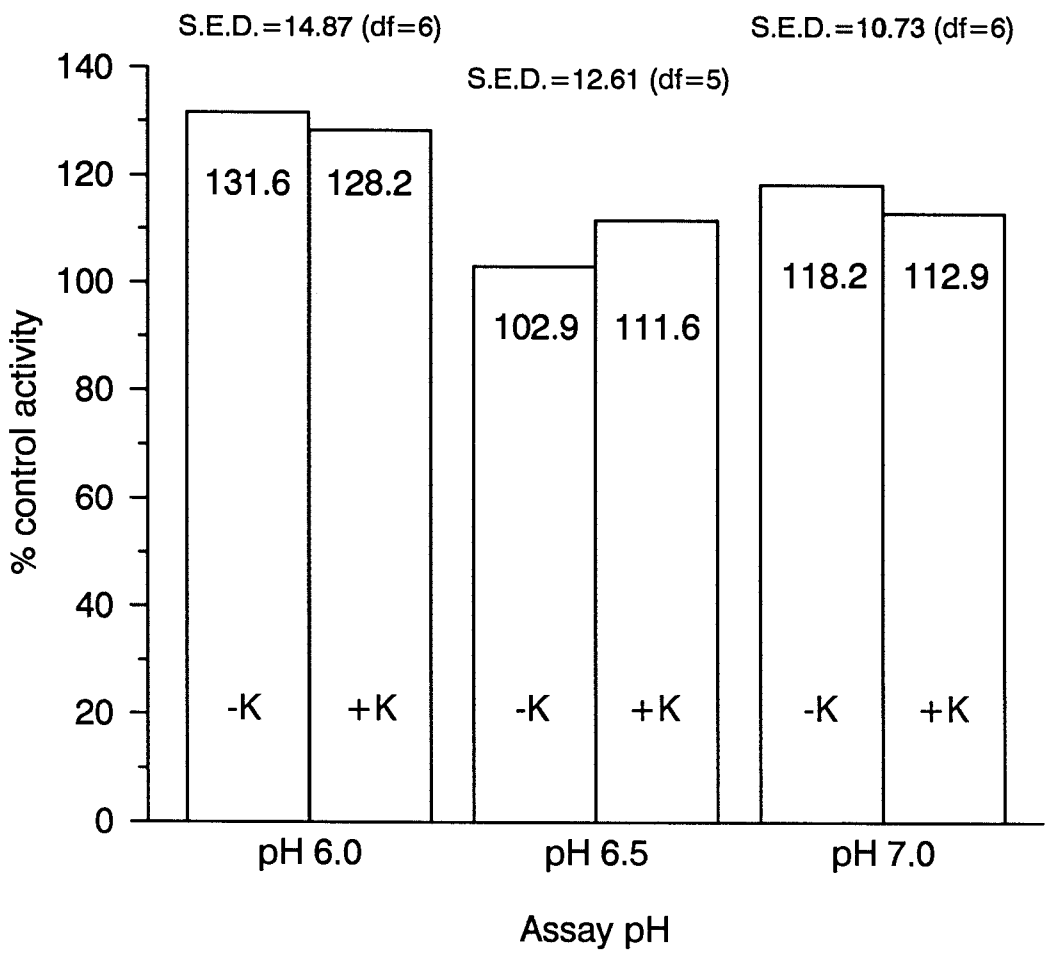


Figure 5.6: The effect of assay pH and the addition of 50 mol $m^{-3}$ KCl on the stimulation of plasma membrane ATPase activity by R *in vitro* after 5 min R *in vivo*. Results are from two experiments and are expressed as mean % of control activity (D).

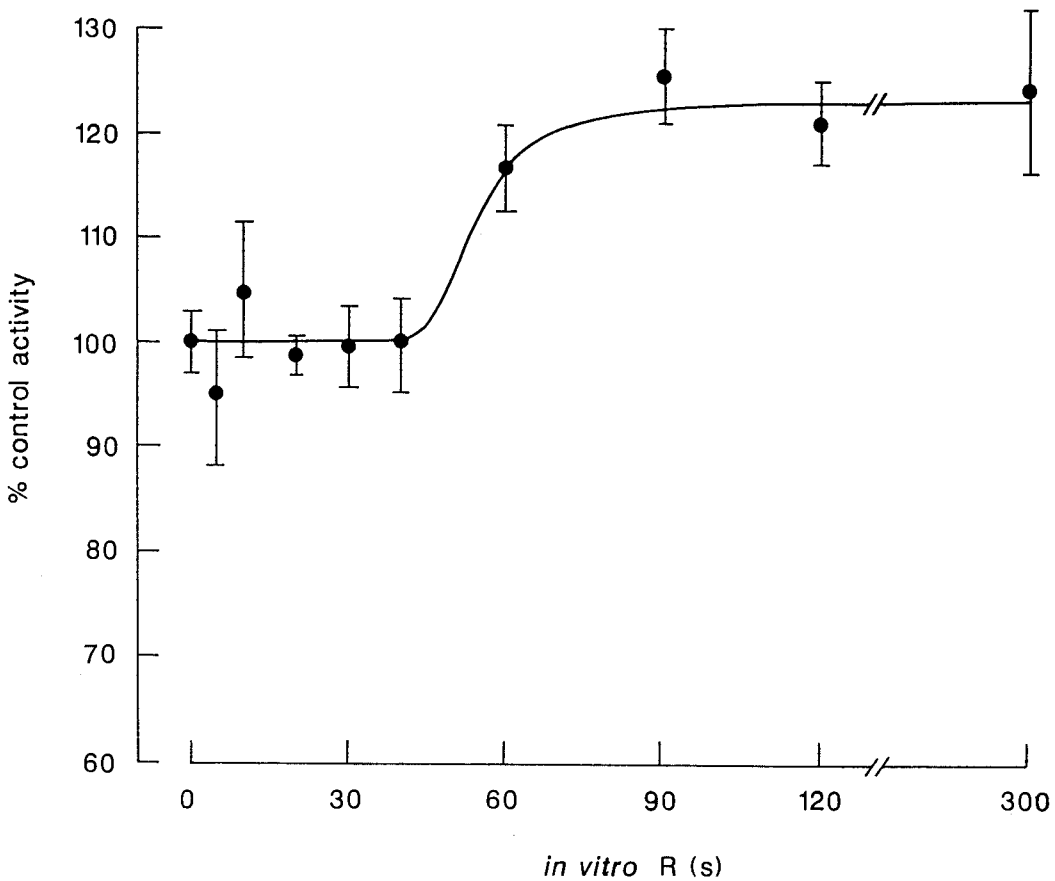


Figure 5.7: Dose–response curve for the effect of R *in vitro* on plasma membrane ATPase activity assayed at pH 6.5 after 5 min R *in vivo*. Results are from two experiments and points are expressed as mean % of control activity (D) ± standard error (n=6).

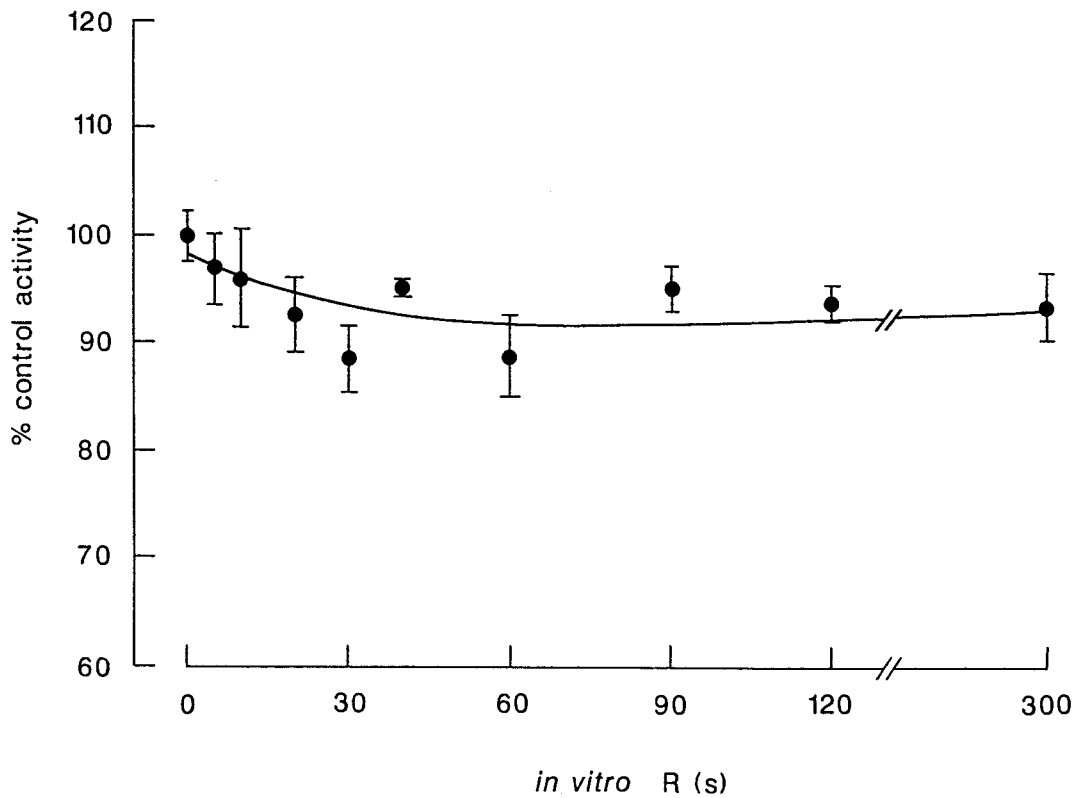


Figure 5.8: Dose–response curve for the effect of R *in vitro* on plasma membrane ATPase activity assayed at pH 6.5 after 5 min R *in vivo*. Results are from three experiments and points are expressed as mean % of control activity (D) ± standard error (n=9).

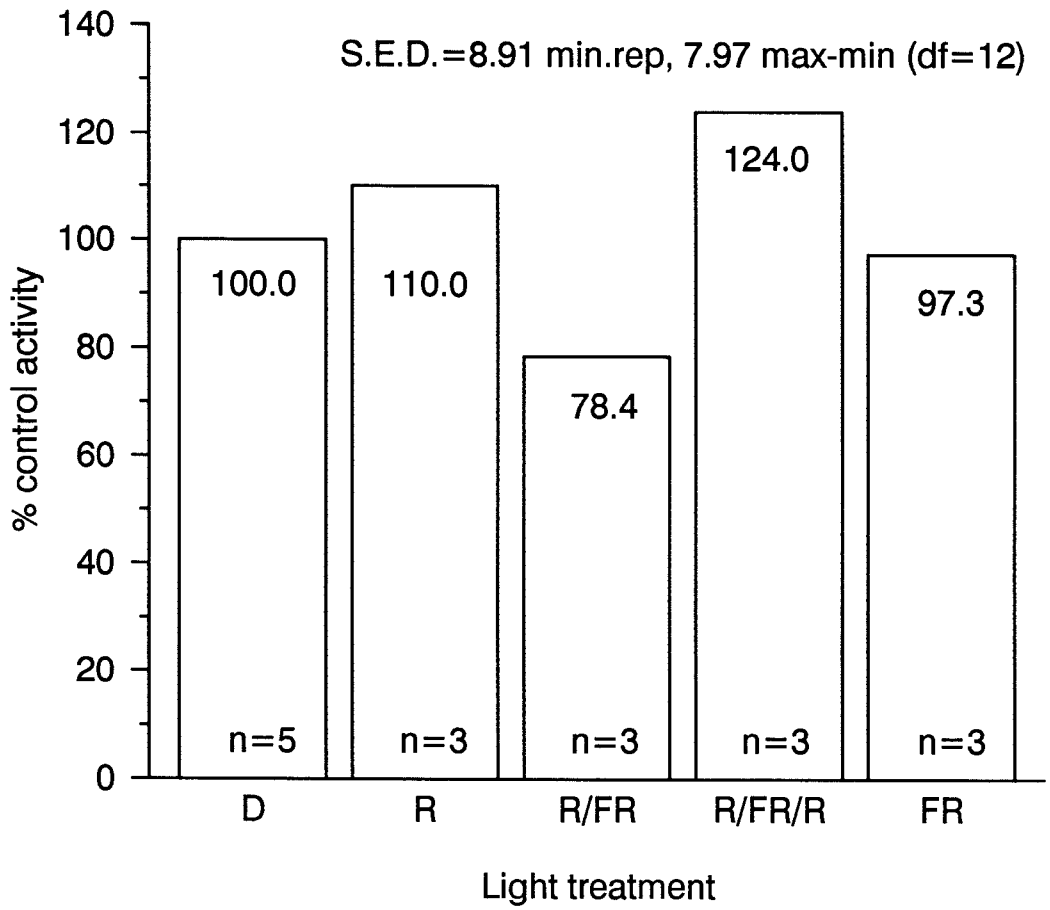


Figure 5.9: The effect of different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 6.5 after homogenization in the presence of 5 mol $m^{-3}$ $H_3BO_3$ and in the absence of an *in vivo* light treatment. Results are from a single experiment and are expressed as mean % of control activity (D).

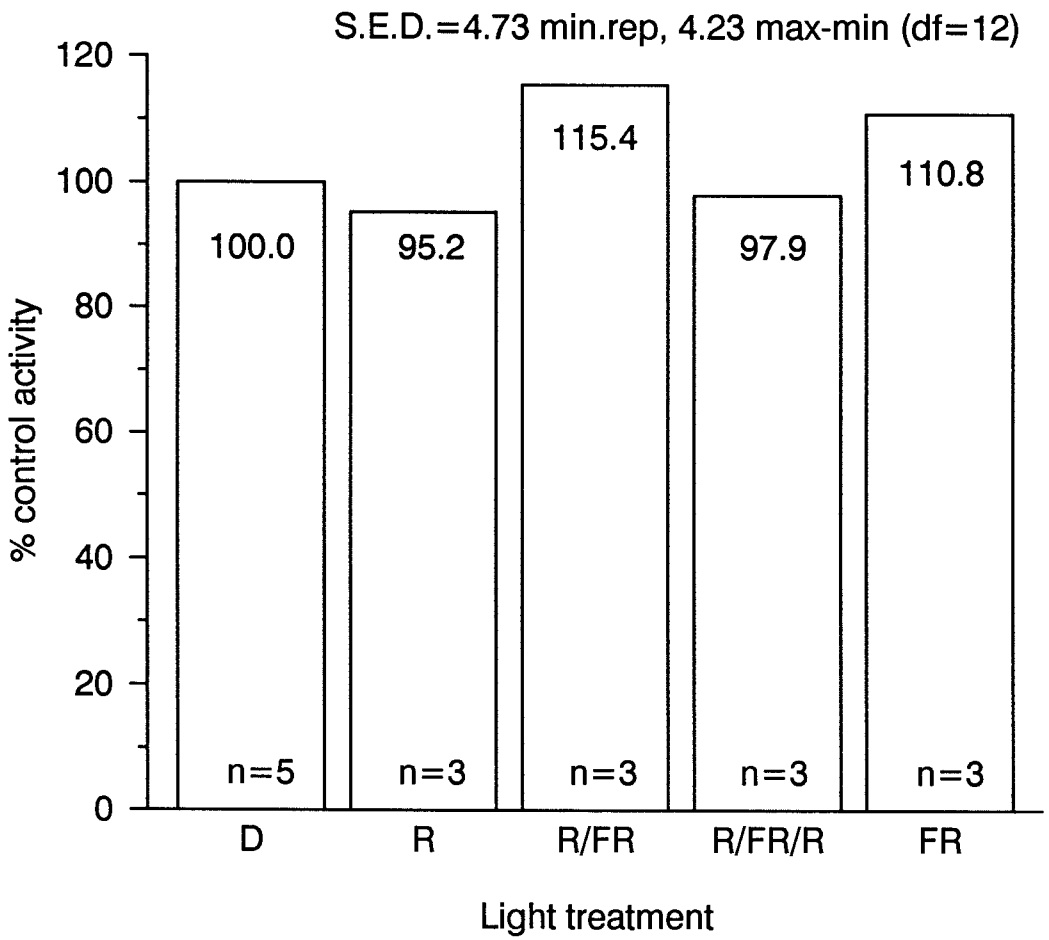


Figure 5.10: The effect of different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 6.5 after homogenization in the presence of 5 mol $m^{-3}$ $H_3BO_3$ and after 5 min R *in vivo*. Results are from a single experiment and are expressed as mean % of control activity (D).

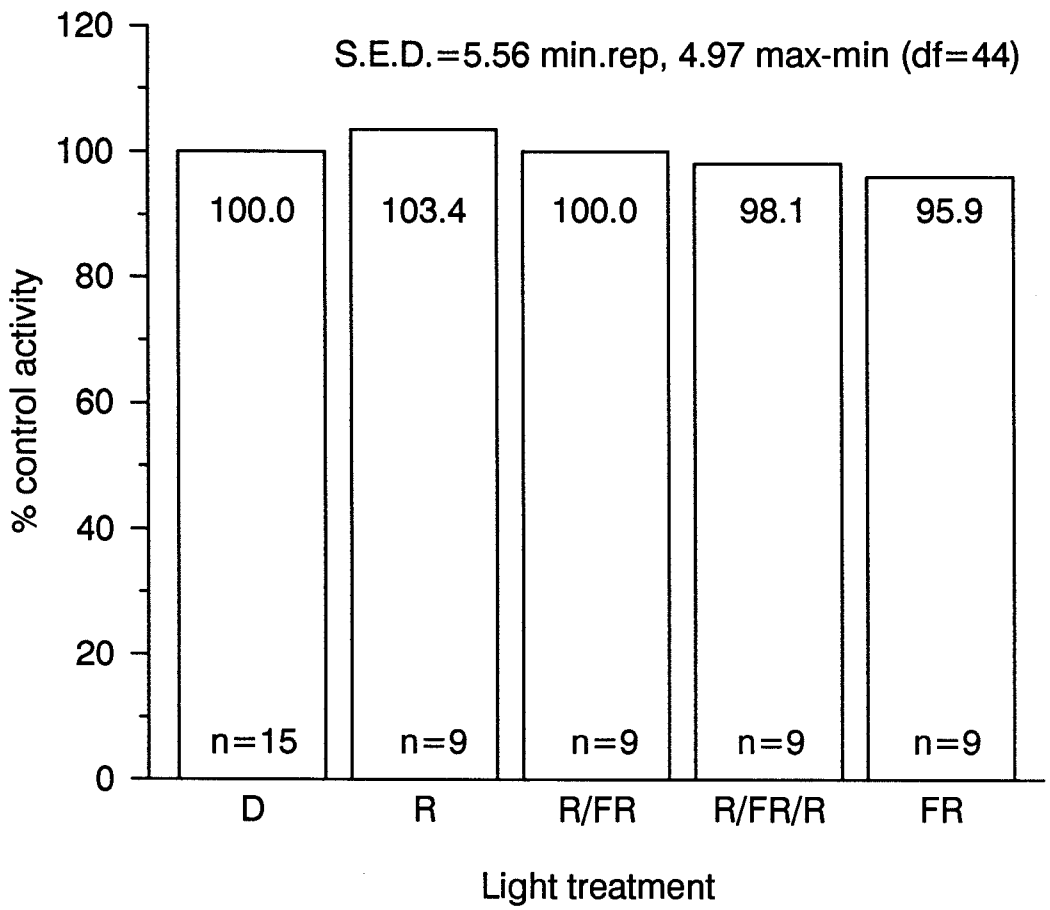


Figure 5.11: The effect of different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 6.5 after homogenization in the presence of 5 mol $m^{-3}$ $H_3BO_3$ and in the absence of an *in vivo* light treatment. Results are from three experiments and are expressed as mean % of control activity (D).

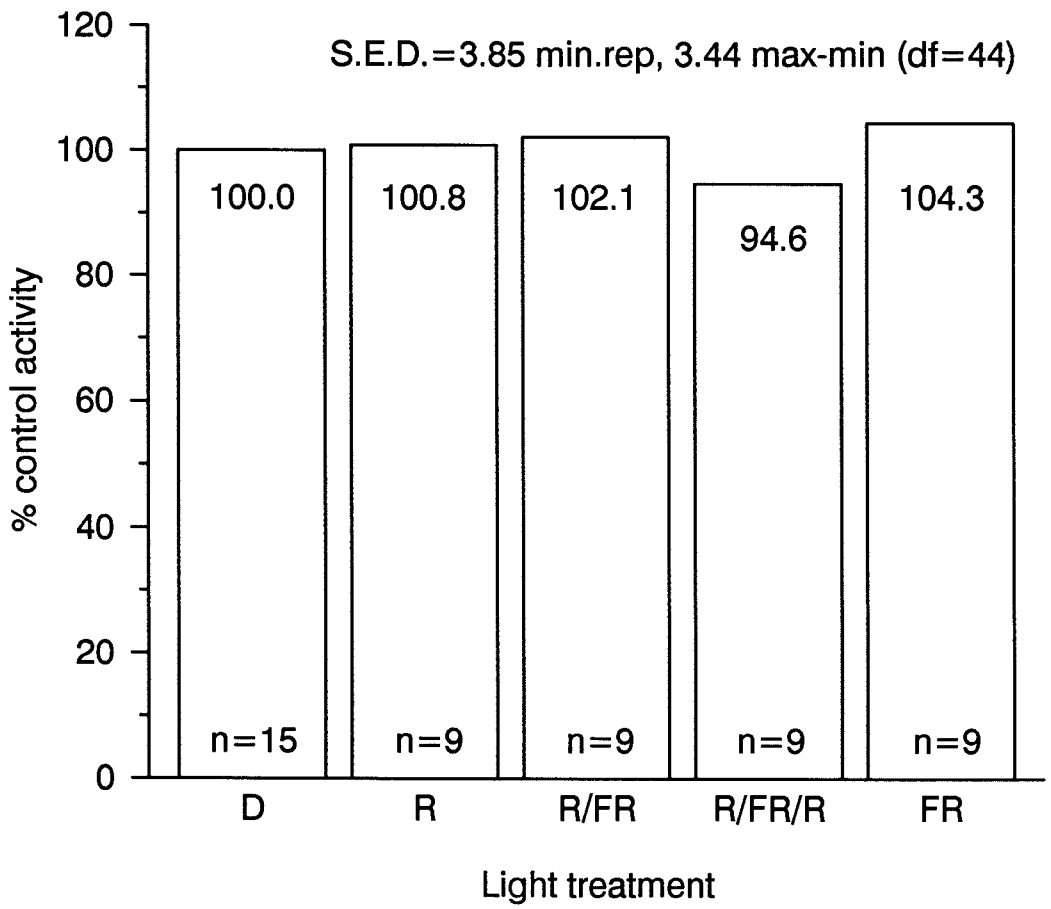


Figure 5.12: The effect of different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 6.5 after homogenization in the presence of 5 mol $m^{-3}$ $H_3BO_3$ and after 5 min R *in vivo*. Results are from three experiments and are expressed as mean % of control activity (D).

stripping the phytochrome away from the membrane or peroxide contaminants found in commercial grade Triton could be bleaching the phytochrome chromophore. To test these possibilities the R-stimulated response was re-examined at lower Triton:protein (t:p) ratios than previously, using purified Triton X-100 with low peroxide contamination. Figure 5.13 demonstrates that neither factor was responsible for the loss of the response. Indeed the average t:p ratio in all ATPase assays in which light effects were examined was 10.36 ± 0.57 below the 12.32 ± 1.70 for the experiments where the response was seen. The effect of assay temperature was also investigated. Although 37°C is not a particularly high temperature for an *in vitro* enzyme assay it may be that the control mechanism for the R stimulation is more temperature sensitive. The effect of an *in vitro* R treatment after 5 min R-irradiation *in vivo* was examined at 25°C (Figure 5.14) but no stimulation was found.

A further possibility was that the R treatment was leading to a change in the kinetics of the enzyme. As for the experiments with *in vivo* treatments no consistent effects were detected. This was true when comparing either dark versus *in vivo* and *in vitro* R (Figure 5.15 is a typical example) or *in vitro* R and FR treatments after an *in vivo* R treatment (data not shown).

### 5.3.5. Is the response due to a photoreceptor other than phytochrome?

Figure 5.16 demonstrates the effect of different light wavelengths on ATPase activity after an *in vivo* R treatment. Although some values were significantly lower than the control value, no neighbouring treatments were different and the drift seen was probably an artifact of the experimental method. Each light treatment was of 10 min duration to ensure sufficient fluence and the time difference between the lower and higher wavelengths was considerable.

An alternative explanation for the response is that it is mediated through the photodynamic action of a photosynthetic pigment such as protochlorophyll (Sundqvist 1974, Virgin 1981) or chlorophyll itself. To investigate this, chlorophyll extracts from etiolated and green wheat leaves were added to the plasma membrane preparations prior to an *in vitro* R treatment. There was no change in ATPase activity with either extract (Figure 5.17). The possibility that there is protochlorophyll-mediated photodamage which interferes with the manifestation of the R response cannot be ruled out but these data suggest that there is no direct damage to the ATPase itself.

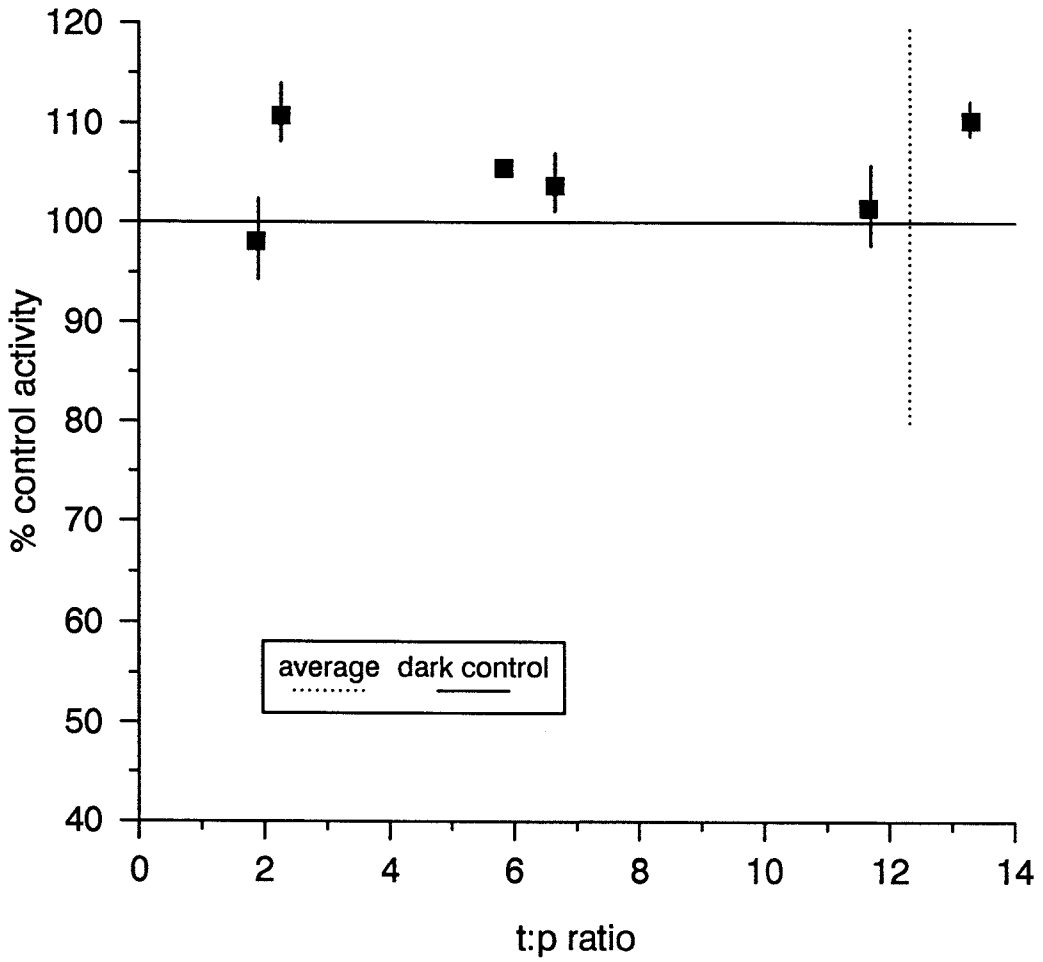


Figure 5.13: The effect of R *in vitro* after 5 min R *in vivo* on plasma membrane ATPase activity assayed at pH 6.5 and at different Triton:protein (t:p) ratios. The Triton X-100 used was specially pure with low peroxide contamination. The vertical dotted line (t:p = 12.32±1.70) represents the mean t:p ratio in all experiments where a stimulation of ATPase activity by R *in vitro* was detected. Results are from four experiments and points are expressed as mean % of control activity (D) ± standard error (n≥3).

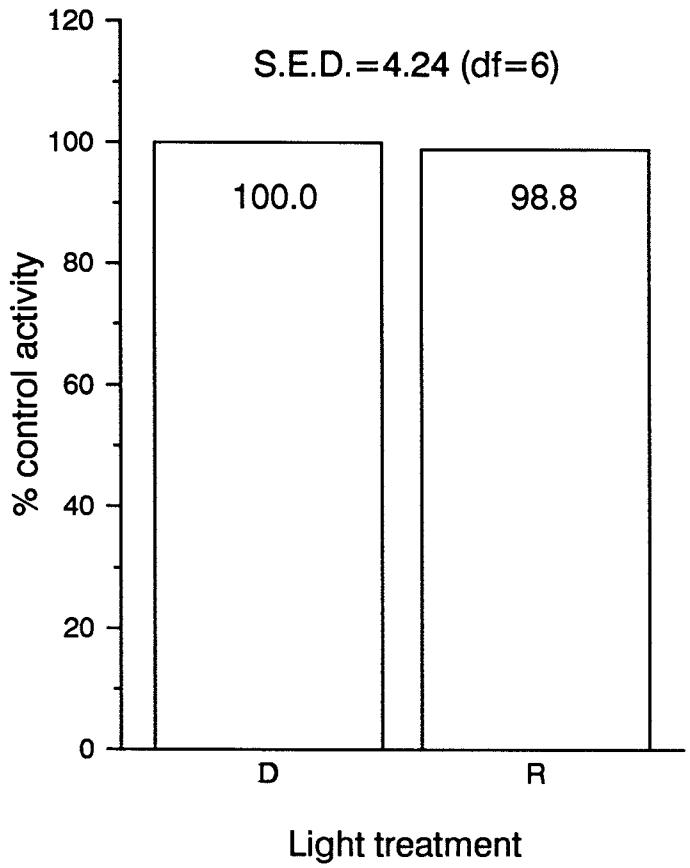


Figure 5.14: The effect of R *in vitro* after 5 min R *in vivo* on plasma membrane ATPase activity assayed at pH 6.5 and at 25°C. Results are from a single experiment and are expressed as mean % of control activity (D).

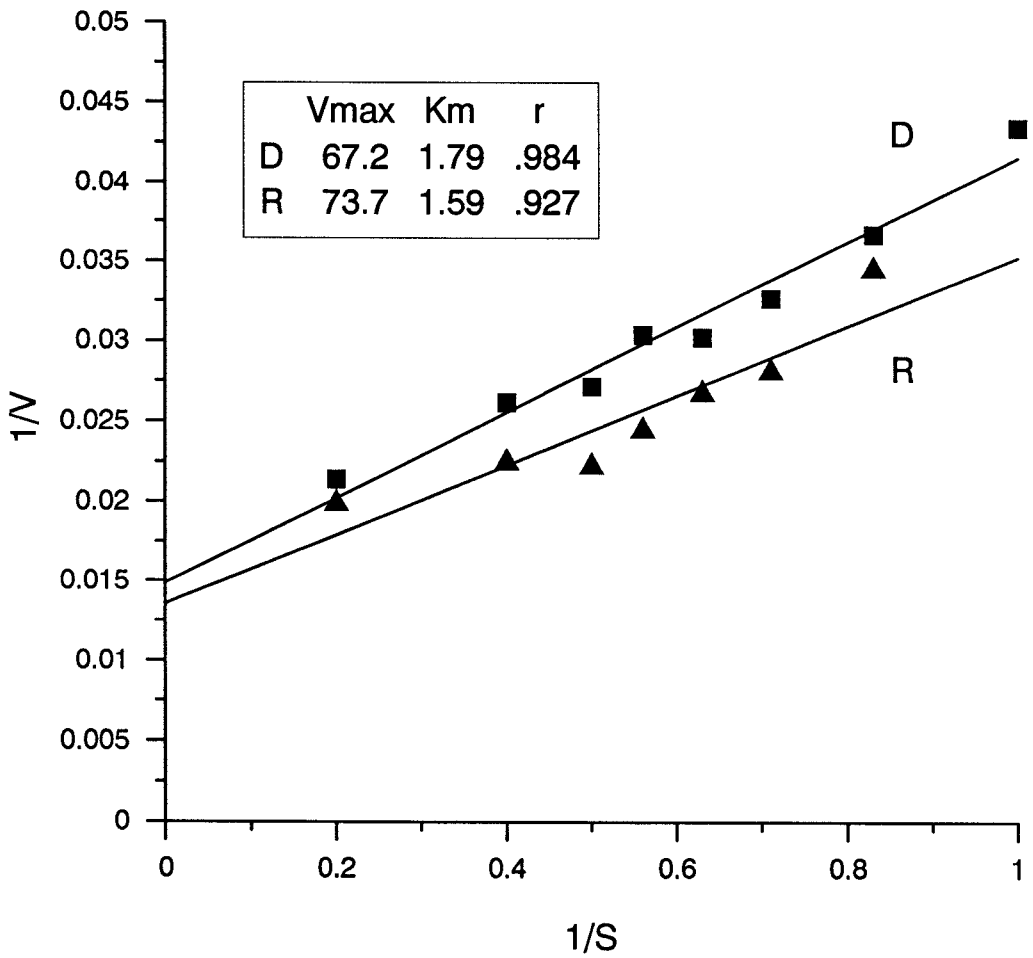


Figure 5.15: Lineweaver–Burk plot of plasma membrane ATPase activity assayed at pH 6.0 after R *in vivo* and *in vitro* (R) and no light treatment (D). Results are from a single representative experiment (n=3) and kinetic parameters were calculated by linear regression.

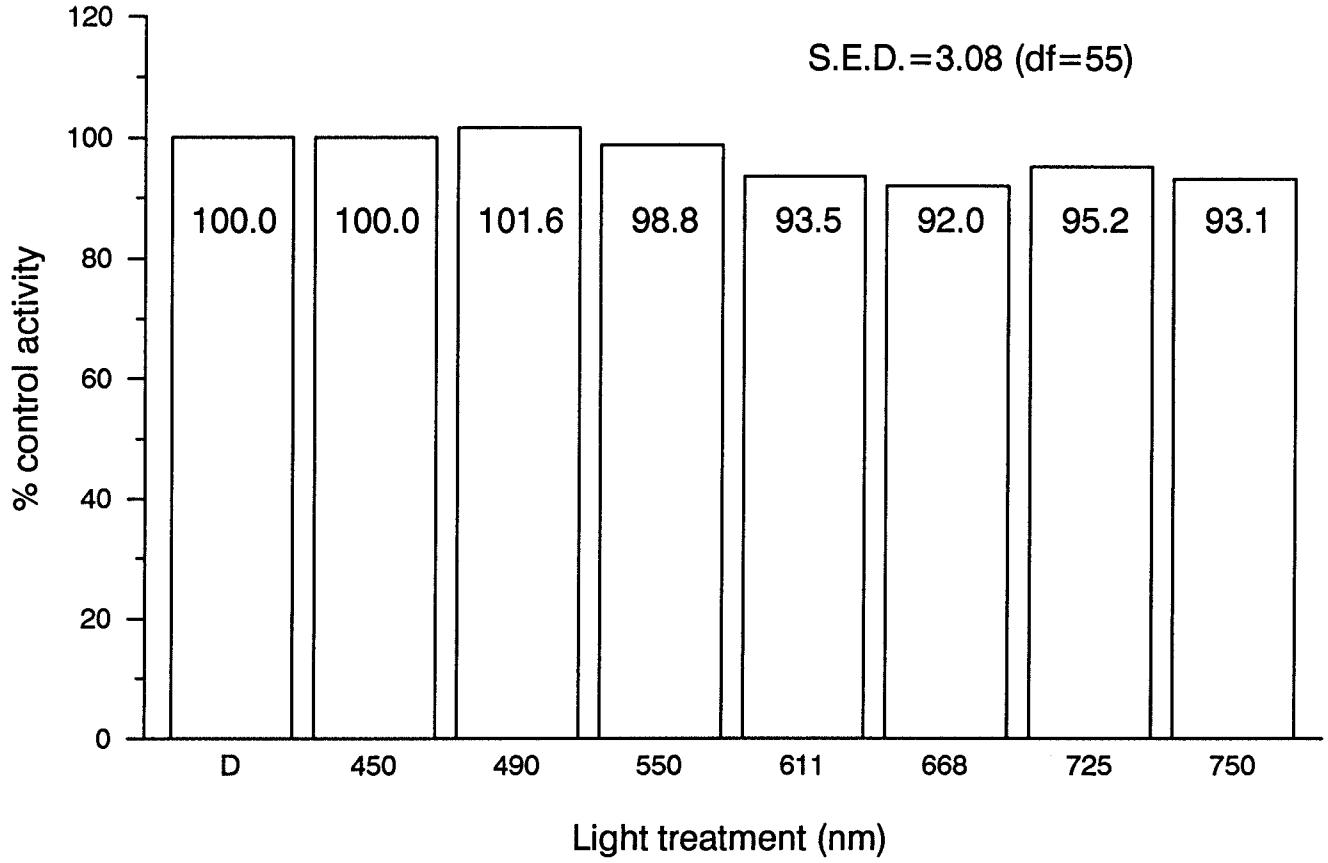


Figure 5.16: The effect of different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 6.5 after 5 min R *in vivo*. Results are from two experiments and are expressed as mean % of control activity (D).

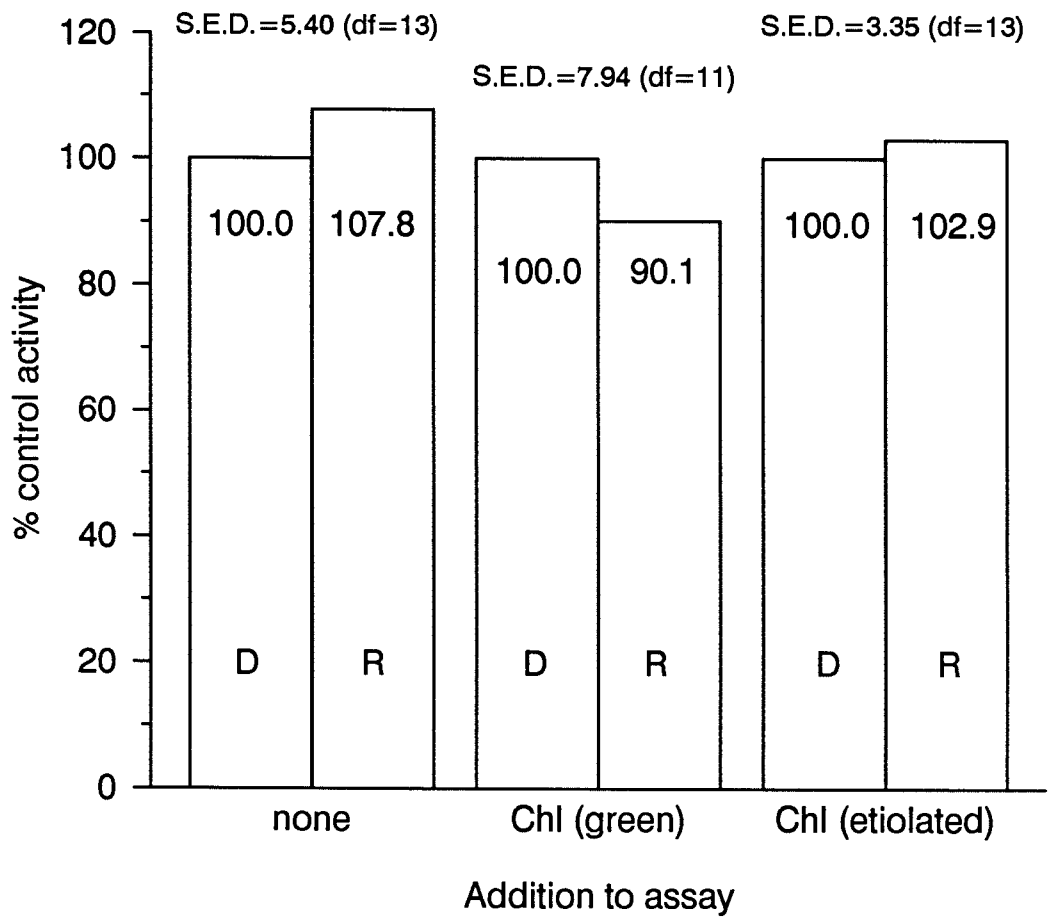


Figure 5.17: The effect of adding chlorophyll extracts from etiolated (5 $mm^3$ of 0.89 $\mu g/cm^3$) and green (5 $mm^3$ of 46.3 $\mu g/cm^3$) tissue prior to R *in vitro* on plasma membrane ATPase activity assayed at pH 6.5 after 5 min R *in vivo*. Results are from two experiments and are expressed as mean % of control activity (D).

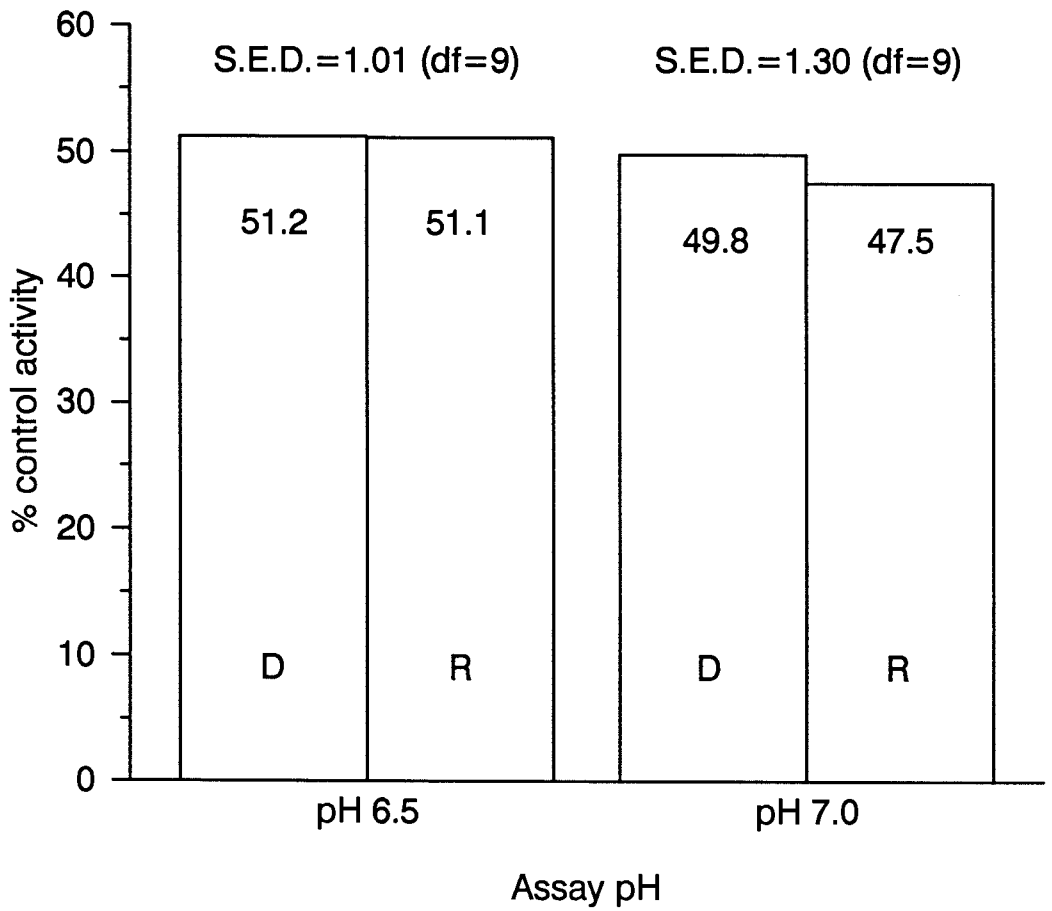


Figure 5.18: The effect of R *in vitro* on $Ca^{2+}$-inhibition of plasma membrane ATPase activity assayed at pH 6.5 and pH 7.0 in the absence of an *in vivo* light treatment. Results are from two experiments and are expressed as mean % of control activity (activity in the absence of $Ca^{2+}$ ions).

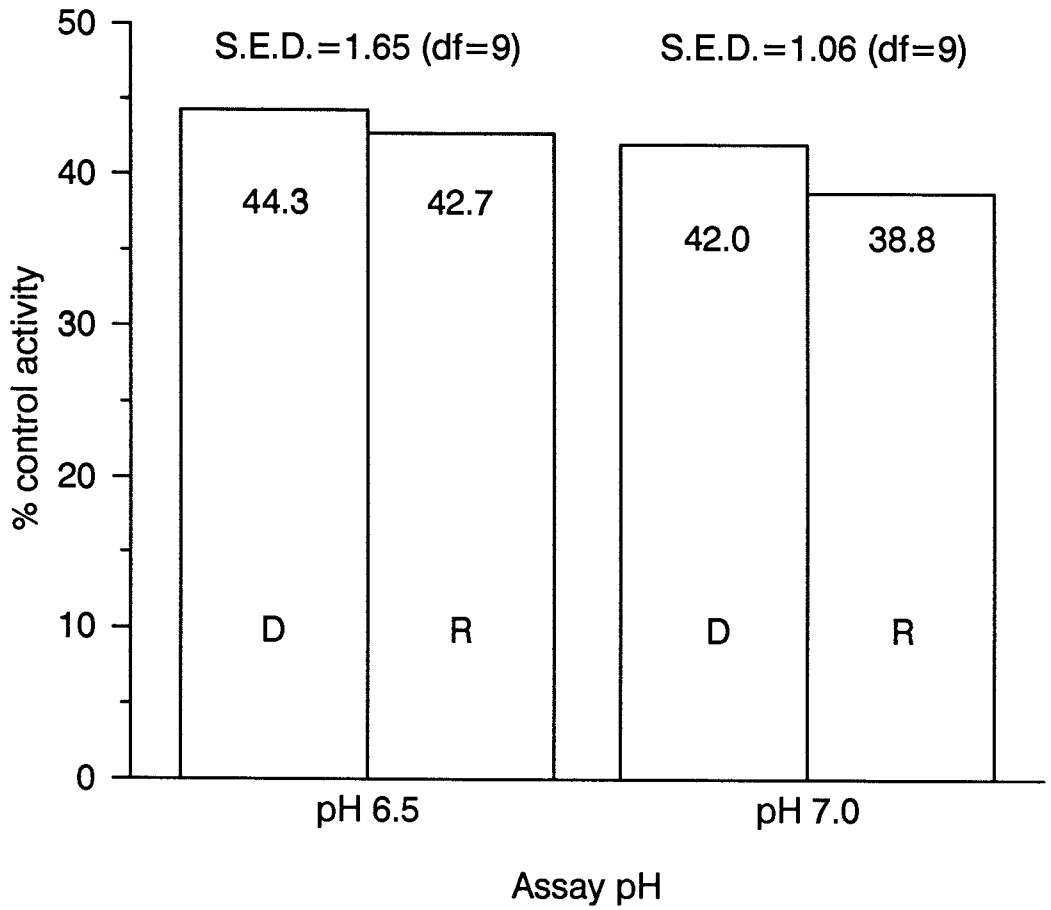


Figure 5.19: The effect of R *in vitro* on $Ca^{2+}$-inhibition of plasma membrane ATPase activity assayed at pH 6.5 and pH 7.0 after 5 min R *in vivo*. Results are from two experiments and are expressed as mean % of control activity (activity in the absence of $Ca^{2+}$ ions).

### 5.3.6. The effect of light treatments on $Ca^{2+}$-inhibition of the plasma membrane ATPase.

As $Ca^{2+}$ ions are the most abundant inhibitor of the plasma membrane ATPase *in vivo*, the possibility that phytochrome acts by changing the sensitivity of the enzyme to $Ca^{2+}$ was tested. Figures 5.18 and 5.19 show inhibition by 0.1 mol $m^{-3}$ $Ca^{2+}$ after different *in vivo* and *in vitro* light treatments at pH 6.5 and 7.0. Only with an *in vitro* and *in vivo* R measured at pH 7.0 was a difference seen. This was further investigated with a range of light treatments (Figure 5.20) but no effect was found. Over all 3 experiments there was no significant difference in any treatment.

### 5.3.7. Addition of partially purified wheat phytochrome to plasma membranes.

Partially purified wheat phytochrome was treated on a spin column of Sephadex G25 to overcome the problems of (a) resuspension in a phosphate buffer and (b) the presence of phosphatase activity in the preparation (41% inhibition of activity with 0.1 mol $m^{-3}$ ammonium molybdate). A 0.2 $cm^3$ sample of partially purified phytochrome was added to the column and the phytochrome was eluted with successive 0.2 $cm^3$ volumes of buffer. Figure 5.21 demonstrates that nearly 90% of the recoverable phytochrome (determined spectrophotometrically) was found in fraction 2. In contrast, most of the free phosphate was recovered in fraction 1 as was over half the phoshatase activity. Fraction 2 was used for all adding-in experiments.

The properties of the partially purified wheat phytochrome are shown in Figure 5.22. The difference spectrum has absorption maxima for $P_r$ of 660nm and for $P_{fr}$ of 730nm. Figure 5.22b shows a western blot of the partially purified phytochrome and confirms that it was free from proteolysis with an apparent molecular mass of 124 kDa.

Phytochrome was added at a final concentration of ~40ng phytochrome/$\mu$g protein in assay. At this concentration there were still problems of interference by phosphatase activity and after an initial experiment assaying at pH 6.5 (Figure 5.23) subsequent assays were performed at pH 7.0. At this pH phosphatase activity was proportionally lower. There was no difference in ATPase activity with a variety of light treatments measured at pH 6.5 but at pH 7.0 there appears to be a small inhibition by R (Figure 5.24). There is, however, no difference between successive treatments and not all light treatments with an initial R irradiation have lower activity. With an *in vivo* R treatment there was no effect on ATPase activity by *in vitro* light treatments measured at pH 7.0 (Figure 5.25).

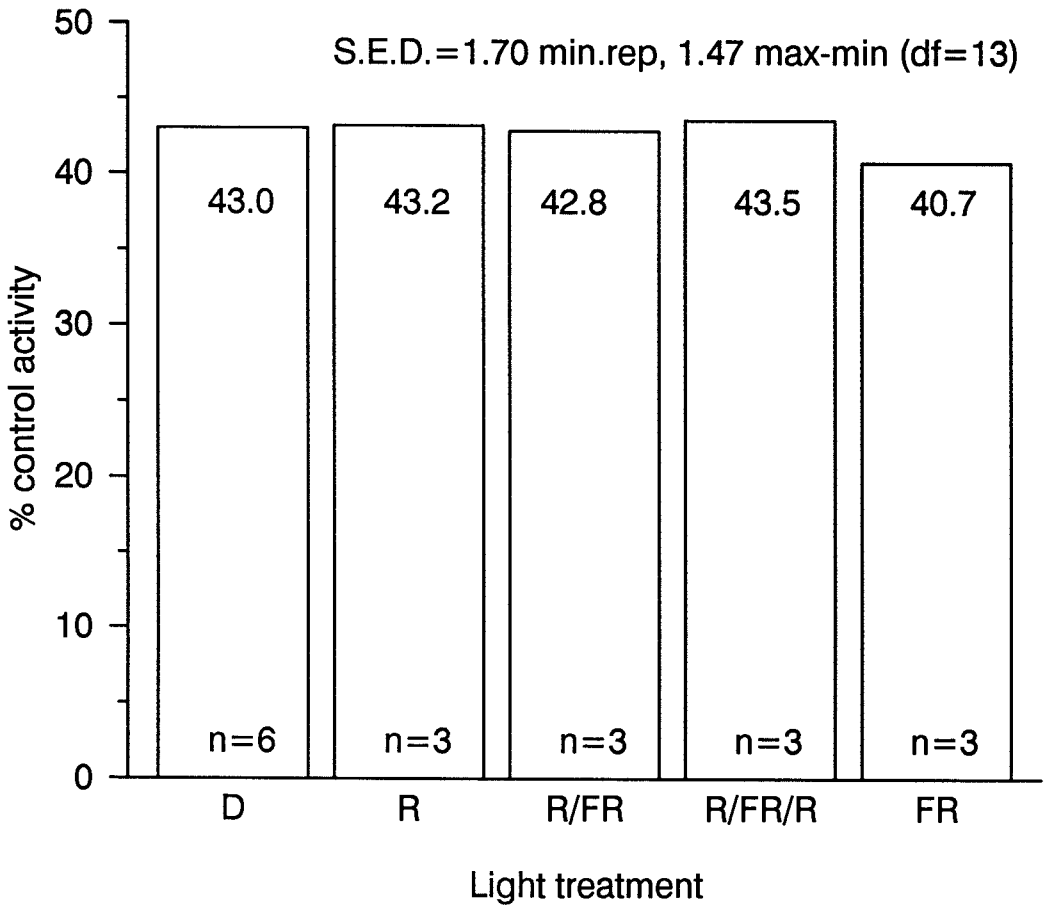


Figure 5.20: The effect of different *in vitro* light treatments on $Ca^{2+}$-inhibition of plasma membrane ATPase activity assayed at pH 7.0 after 5 min R *in vivo*. Results are from a single experiment and are expressed as mean % of control activity (activity in the absence of $Ca^{2+}$ ions).

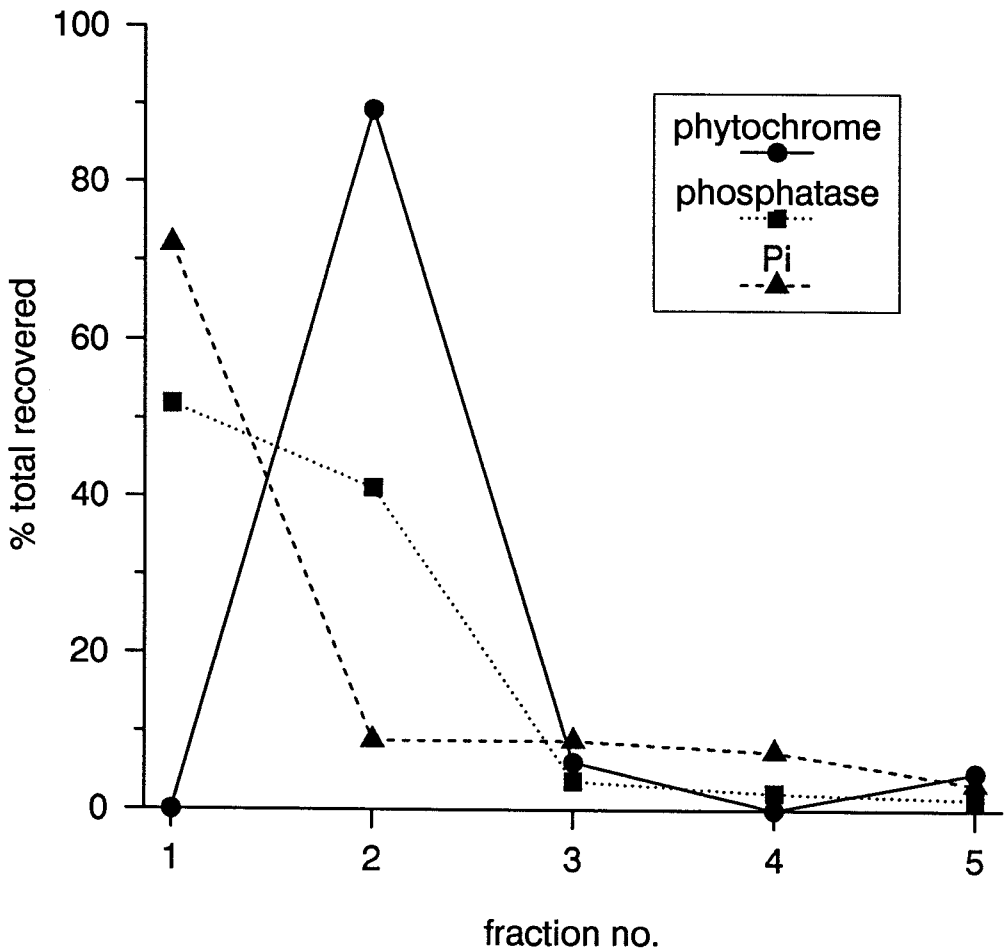


Figure 5.21: The distribution of components of a partially purified wheat phytochrome preparation in 0.2 $cm^3$ fractions from a Sephadex G25 spin column. Results are from a single representative experiment and points are expressed as mean % of total recovered. Phytochrome was assayed spectrophotometrically (n=1), phosphatase activity was assayed at pH 6.5 as for ATPase activity (n=3) and $P_i$ was also determined by the method used for ATPase assays (n=3).

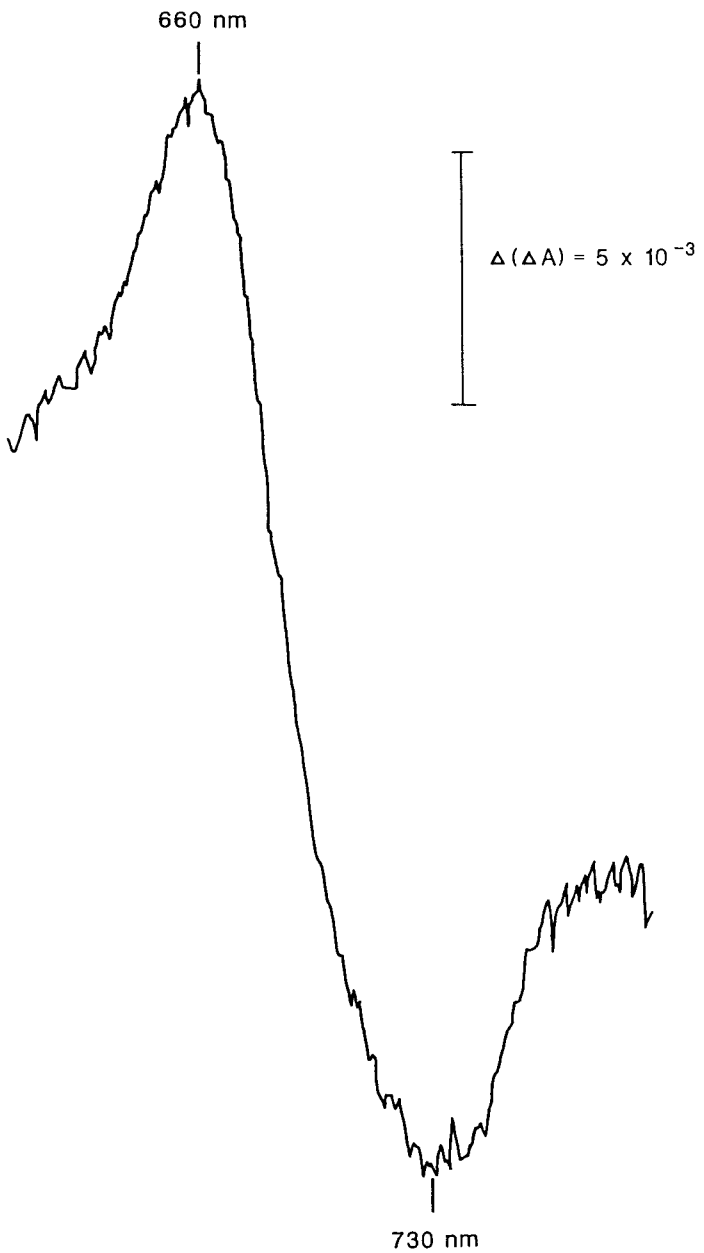


Figure 5.22a: Difference spectrum of partially purified wheat phytochrome. Absorbance maxima are 660 nm for $P_r$ and 730 nm for $P_{fr}$.

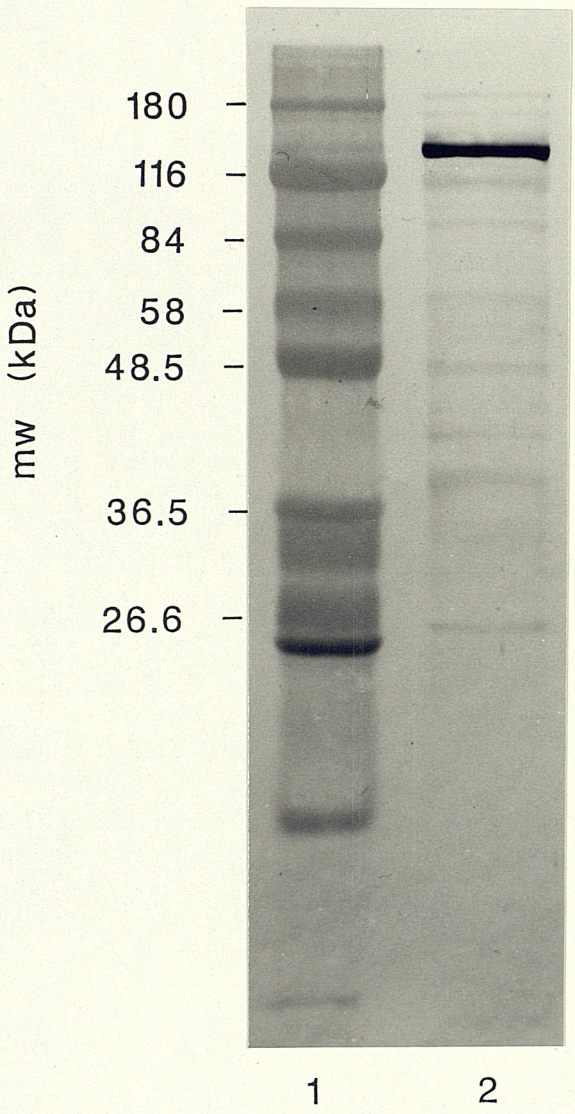


Figure 5.22b: Western blot of partially purified wheat phytochrome stained using anti-phytochrome monoclonal antibodies MAC 52, MAC 197 and MAC 199 (2 $\mu g/cm^3$) and anti-rat IgG HRP conjugate. Lane 1: pre-stained molecular weight markers; lane 2: ~30 ng partially purified wheat phytochrome.

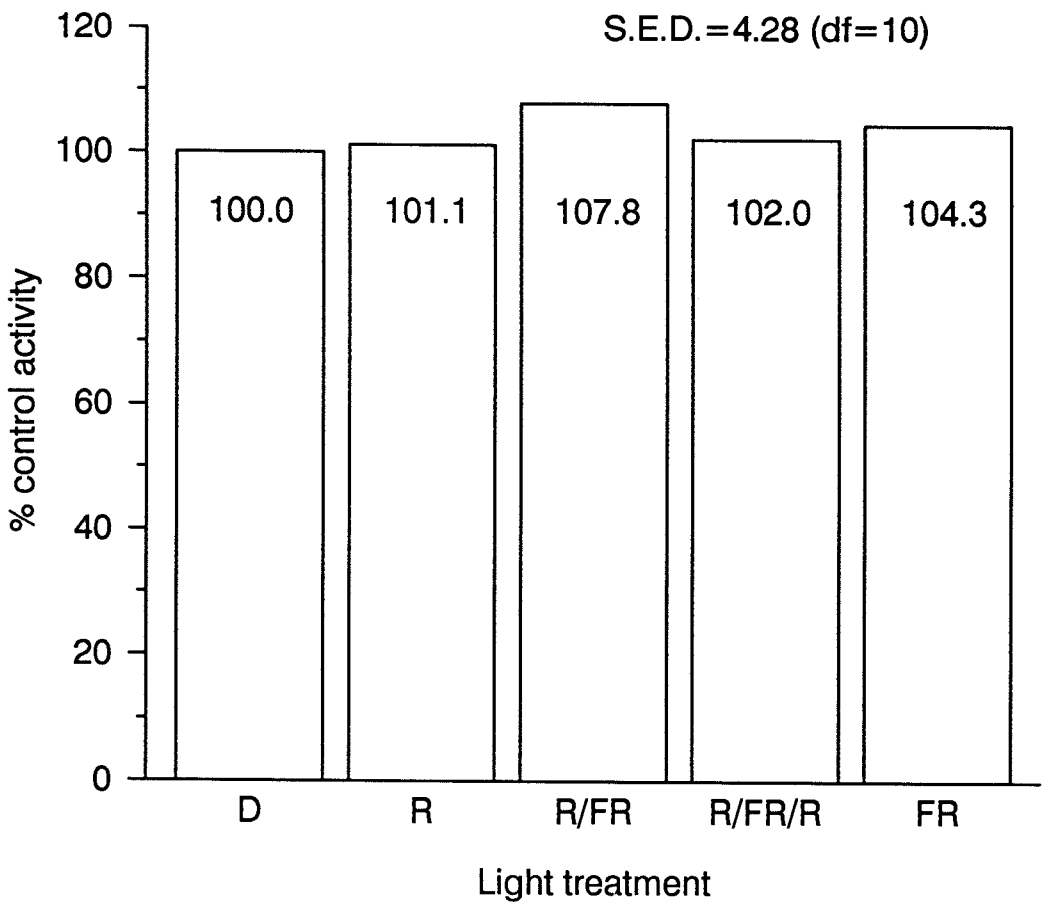


Figure 5.23: The effect of adding partially purified wheat phytochrome (2.95 $\mu g/cm^3$ plasma membranes) prior to different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 6.5 in the absence of an *in vivo* light treatment. Results are from a single experiment and are expressed as mean % of control activity (D).

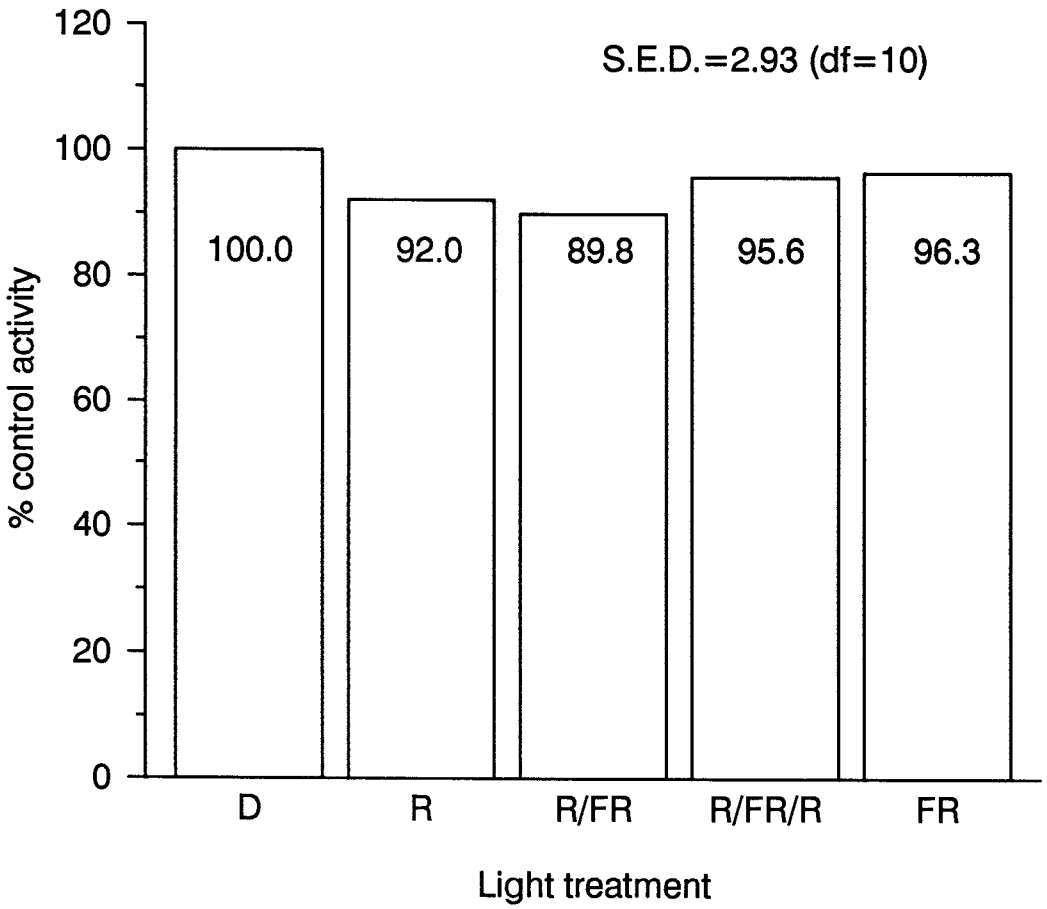


Figure 5.24: The effect of adding partially purified wheat phytochrome (2.95 $\mu g/cm^3$ plasma membranes) prior to different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 7.0 in the absence of an *in vivo* light treatment. Results are from a single experiment and are expressed as mean % of control activity (D).

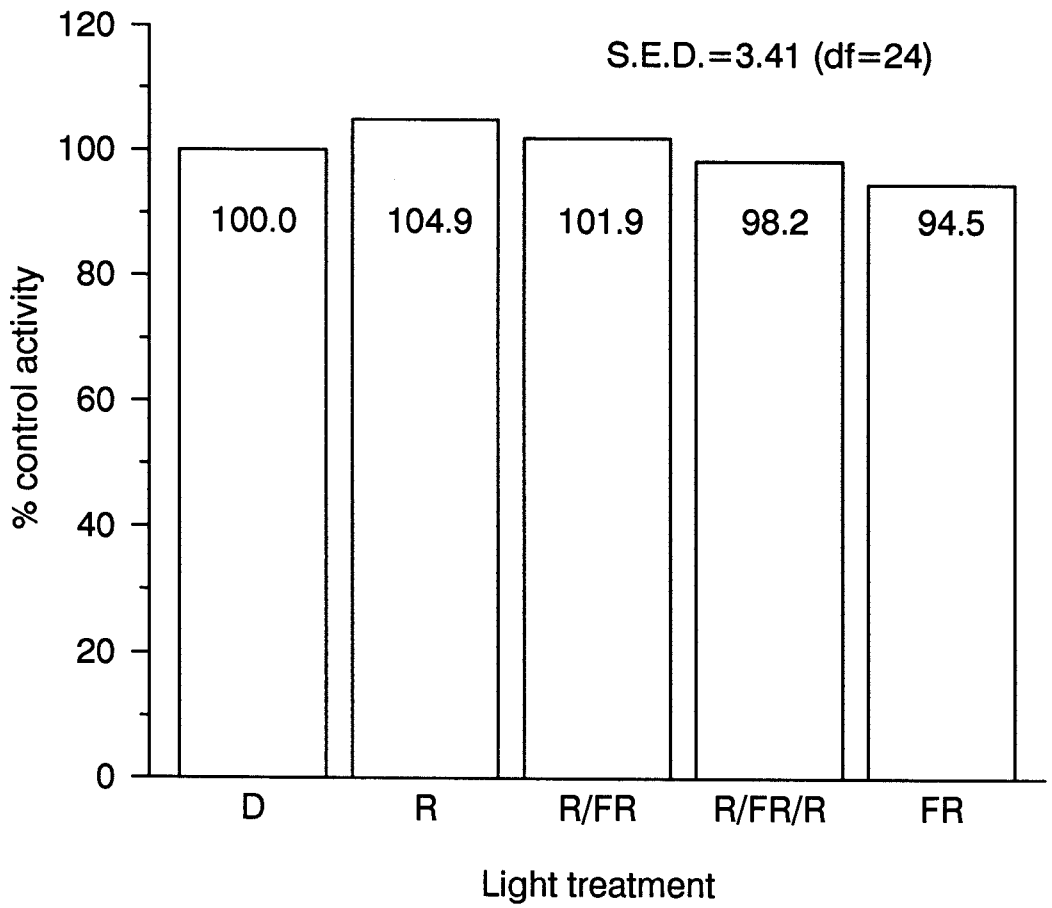


Figure 5.25: The effect of adding partially purified wheat phytochrome (2.95 $\mu g/cm^3$ plasma membranes) prior to different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 7.0 after 5 min R *in vivo*. Results are from two experiments and are expressed as mean % of control activity (D).

In summary, these data suggest that light treatments in the presence of high concentrations of wheat phytochrome do not alter ATPase activity assayed *in vitro*.

### 5.3.8. Addition of purified oat phytochrome to plasma membranes.

Purified oat phytochrome (a gift from Dr. M.D. Partis) was added to the plasma membranes to see if a higher concentration of phytochrome could induce a response. The phytochrome added was 124 kDa with difference maxima of 665nm for $P_r$ and 730nm for $P_{fr}$ (M.D. Partis pers. comm.) and a concentration of ~250ng phytochrome/$\mu$g protein was used in the assay. The results from these experiments are shown in Figure 5.26. There was no difference in ATPase activity with any initial light treatment but the R/FR/R treatment results in lower activity than the other treatments.

## 5.4. DISCUSSION

The mechanisms by which phytochrome could, in theory, alter ATPase activity *in vivo* can be classified into three groups. There may be a direct interaction of $P_{fr}$ with a membrane (or soluble) component, including the ATPase itself, which would lead to changes in the properties of the enzyme. For example, the binding of an regulatory polypeptide or the stimulation of protein kinase/phosphatase activity. Secondly, the amount of the active enzyme present on the plasma membrane could be changed through the regulation of *de novo* synthesis, destruction or the balance between active and inactive pools. Finally, ATPase activity could be altered as a secondary consequence of a different primary phytochrome action, such as a response to a change in cytosolic pH.

The present investigation is concerned with the first of these possibilities. Does phytochrome have a direct effect on plasma membrane-associated ATPase activity? The scope of this study, however, is limited by the use an *in vitro* ATPase assay where a soluble component of the control mechanism would, most likely, be absent. Is phytochrome a soluble or a membrane-bound component of this system? In Chapter 6 the presence of plasma membrane-bound phytochrome (1-2ng phytochrome/$\mu$g membrane protein) is demonstrated. Furthermore, this phytochrome was not removed by the concentrations of Triton X-100 used in the ATPase assay and there was also no reduction in binding with 50 mol $m^{-3}$ KCl. 10 mol $m^{-3}$ $MgCl_2$ was found to have a limited effect but this was at concentrations 5-fold higher than were present in the ATPase assay.

The results presented here show conclusively that there is no direct effect of an *in vivo* R-irradiation on ATPase activity measured *in vitro*. This does

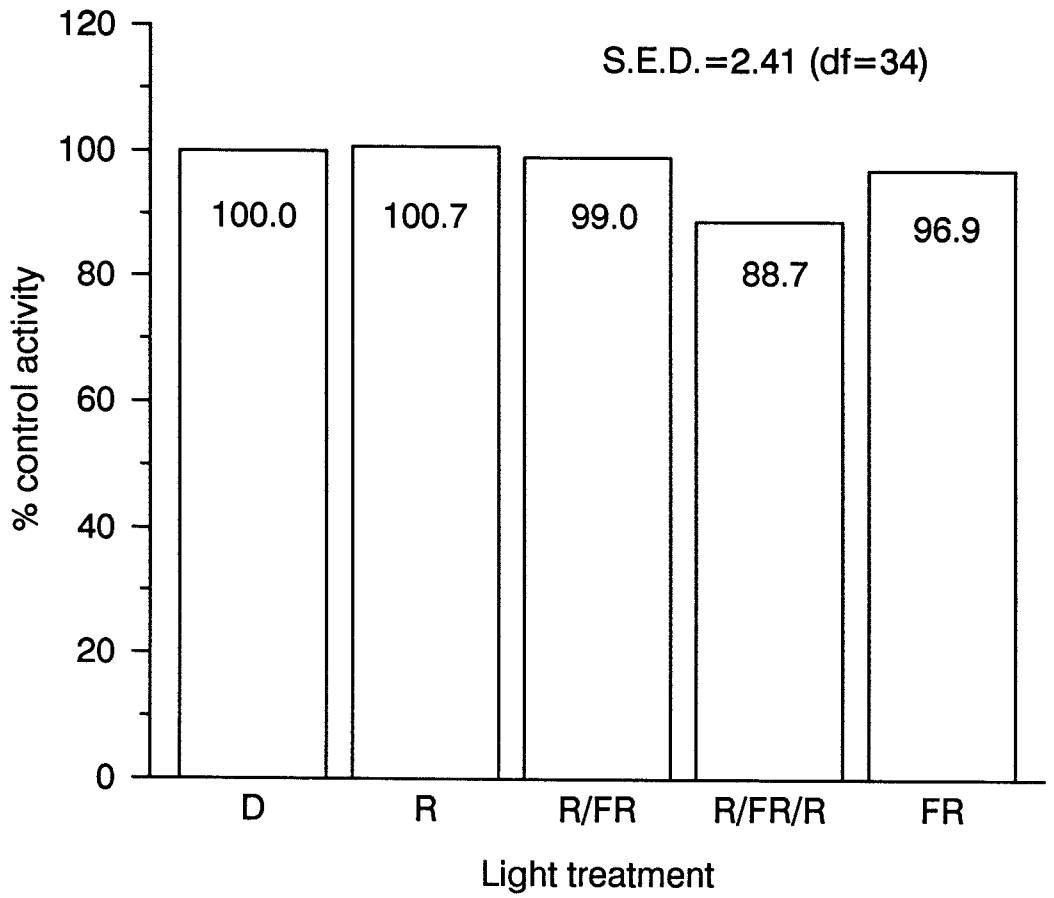


Figure 5.26: The effect of adding purified oat phytochrome (18.4 $\mu g/cm^3$ plasma membranes) prior to different *in vitro* light treatments on plasma membrane ATPase activity assayed at pH 7.0 after 5 min R *in vivo*. Results are from two experiments and are expressed as mean % of control activity (D).

not rule out the possibility that R-irradiation causes a direct but transient change in ATPase properties that are no longer apparent after membrane isolation. The only *in vivo* measurement of $H^+$-ATPase activity comes from $H^+$ extrusion experiments where the involvement, but not the direct stimulation of the plasma membrane $H^+$-ATPase, can be demonstrated.

In contrast to the results obtained after *in vivo* light treatments, there is more doubt when interpreting results after *in vitro* treatments. The R-stimulation of ATPase activity (28%) was repeatable for 8 out of 9 consecutive experiments. This effect is unlikely to be caused by a time factor as FR treatments or D samples (data not shown) assayed at the end of the light series did not show a significant increase in ATPase activity. There is a possibility that the response may be due to an effect of temperature but this is also unlikely as successive treatments were not additive and the assays themselves were carried out at constant temperature for 40 minutes.

Although this response appears to be a light-dependent one, it has not been demonstrated that phytochrome is the photoreceptor involved. That there is no FR reversibility is not in itself evidence against the involvement of phytochrome. The escape from reversibility could be more rapid than the 3 minutes between the R and FR treatments. Blakeley *et al* (1983) found that reversibility of the R-induced protoplast swelling response was lost between 2 and 5 minutes at 4°C. The escape time would be expected to be more rapid at room temperature. There is no evidence that another photoreceptor is involved. Both the addition of (proto)chlorophyll and blue light were unable to elicit a response.

To help determine whether the response was phytochrome mediated the dose-response relationship was investigated (Figure 5.7). The shape of the curve is very different to the hyperbolic curve normally associated with phytochrome responses, where the magnitude of the response is a function of $P_{fr}$ present. For this response there appears to be an all or nothing effect more consistent with the opening of an ion channel than the stimulation of enzyme activity. Despite this, the shape of the curve does bear a striking resemblance to the dose-response curve for the R-induced inhibition of ATPase activity detected in hypocotyl hooks of *Phaseolus* (Jose 1977). In this instance there was no response after 45 seconds but the response was saturated within 90 seconds. Unfortunately, in the wheat leaf system further investigation was impossible due to the disappearence of the response.

The reason for the loss of the response is not known. Blakeley *et al* (1987) suggested that their failure to detect R-induced changes in wheat leaf microsomal ATPase activity might be due to proteolytic degradation of phytochrome. In the plasma membrane preparations used here, however, 124 kDa

phytochrome is the predominant species on a western blot (Figure 6.3) even in the absence of additional protease inhibitors (EGTA is routinely present in the homogenization medium). Another possibility for the disappearence of the R-stimulated response is that a component in a regulatory chain is either very labile or easily removed from the membrane. The presence, however, of 124 kDa phytochrome suggests that proteolysis is not a problem and both homogenization by pestle and mortar and membrane purification by phase partition are considered to be relatively mild treatments. However, removal from the membrane cannot be discounted. In an attempt to further enhance stability, boron (as $H_3BO_3$) was added to the homogenization medium. It is thought to interact directly with membranes by binding to glycoproteins and glycolipids (Parr and Loughman 1983) and has previously been used to increase the magnitude of phytochrome-mediated responses (Tanada 1978, Roth-Bejerano and Hall 1986a). Despite one promising experiment it can be concluded that this treatment was unsuccessful in stabilizing the response.

The overall conclusion that must be drawn from this study is that phytochrome does not directly regulate plasma membrane ATPase activity assayed *in vitro*, although the possibility that a labile signal transduction mechanism is mediating the response cannot be ignored. This result does not necessarily disagree with those already in the literature (see 5.1). The only in-depth study on plasma membrane $H^+$-ATPase activity (Ball 1988) reached the same conclusion, as did the only previous investigation using wheat leaves (Blakeley *et al* 1987). It does, however, cast doubt on the origin of the light-regulated ATPase activity in numerous other reports. The most likely explanation for this discrepancy is that only some ATPases from some plant tissues are photoregulated. For example, it has been shown in cucumber that the microsomal ATPase activity of apical hooks, but not of the hypocotyl, is stimulated by R *in vitro* (Ball 1988).

There are many examples of light-regulated enzymes in both plants and animals (Hug *et al* 1980, Hug 1981). Perhaps the most relevant to the present study is the stimulation of $H^+$-ATPase activity in guard cell protoplasts by blue light (Assmann *et al* 1985). A combination of $H^+$ extrusion and patch clamping experiments have demonstrated that a rapid blue light-induced hyperpolarization is caused by activation of a proton pump. The kinetics of this response show that blue light is acting as an activator of, not as an energy source for, the $H^+$-pumping. Interestingly, this response can also be produced by R, but in this case the stimulation is mediated by an unknown photosynthetic product (Serrano *et al* 1988). In contrast to the blue light stimulation, when the red light is turned off, $H^+$ extrusion stops. This R-induced $H^+$ extrusion appears to be common to many photosynthetic tissues. The mechanism is not known but Marre *et al* (1989)

have shown that this response and the stimulation of the plasma membrane $H^+$–ATPase by the plant growth stimulator, fusicoccin, are not additive, indicating a common stimulatory pathway.

The general mechanisms by which the plasma membrane $H^+$–ATPase is regulated are still not known (Sussman and Surowy 1987). One possible control mechanism is through changes in cytosolic pH to which the plasma membrane $H^+$–ATPase is very sensitive. A fall in cytosolic pH increases $H^+$–ATPase activity. If this is the only mechanism how do stimulators such as fusicoccin and the growth regulator, auxin cause a decrease in cytosolic pH? One answer is that they may directly affect proton cotransporters, for example a $Na^+/H^+$ antiport. Another is that they activate the plasma membrane–bound redox system which stimulates the metabolic generation of acids *via* NADPH oxidation (Rubinstein and Stern 1986), although such a simple activating mechanism would not be a sensible strategy for the plant because nutrient accumulation could not be stimulated (Serrano 1988).

An alternative to the above hypothesis is that plasma membrane $H^+$–ATPase activity is modified more directly. Examples could include an allosteric regulation by a metabolite (of photosynthesis?) or covalent modification by a phosphorylation/dephosphorylation mechanism. It has been demonstrated that the plasma membrane $H^+$–ATPase is phosphorylated *in vivo* and can also be phosphorylated at multiple sites *in vitro* by a plasma membrane–associated protein kinase (Schaller and Sussman 1988). This protein kinase activity is sensitive to both protons and $Ca^{2+}$ ions and this would provide a link to other possible control mechanisms. There is, however, no conclusive proof at present that phosphorylation leads to a change in activity or indeed how the activity might be altered. Zocchi (1985) found an inhibition of $H^+$–pumping associated with an increase in phosphorylation but, in contrast, Bidwai and Takemoto (1987) were able to correlate a stimulation of the $H^+$–ATPase by syringomycin to a syringomycin–induced stimulation of plasma membrane protein kinase activity. It is possible that the $H^+$–ATPase is controlled through a complicated pattern of phosphorylation.

This study does not provide conclusive proof that phytochrome is not involved in the regulation of plasma membrane $H^+$–ATPase activity and further experiments should be undertaken. Measurement of proton transport in plasma membrane vesicles is a more accurate technique for assessing $H^+$–ATPase activity than free $P_i$ determination. Unfortunately, there are difficulties obtaining sealed, inside–out plasma membrane vesicles. The phase partition methods used here produce predominantly right–side–out vesicles (see 4.3.5.) and a more recent method in which the tonoplast and mitochondrial $H^+$–ATPases are denatured by a

KI treatment (Giannini *et al* 1987) would also remove membrane-bound phytochrome. Perhaps the most suitable method to demonstrate the presence of a direct interaction would be the whole-cell configuration patch clamp technique which has been so successful in studies with guard cell protoplasts. Attempts, however, to use this method to investigate light-induced changes in membrane potential with wheat leaf protoplasts have proved difficult (M.E. Bossen pers. comm.).

This investigation has addressed the question of whether phytochrome has a direct interaction with the $H^+$-ATPase, but what of the other possible mechanisms outlined in the opening paragraph. There is no evidence that phytochrome controls the amount of active enzyme in the plasma membrane but it is highly likely that that it's cellular action(s) does impinge upon the complex mechanisms of $H^+$-ATPase regulation described, in part, above (see Chapter 7).

# CHAPTER SIX

# PHYTOCHROME BINDING TO PLASMA MEMBRANES

## 6.1. INTRODUCTION.

There appears from the literature to be two distinct types of phytochrome–membrane associations. One involves a small proportion of the total measureable phytochrome (2.4%, Napier and Smith 1987b) which is associated with crude membrane preparations either in the dark or after a FR treatment. This association is stimulated three–fold by R irradiation *in vivo* and is FR reversible after binding. The second type of phytochrome–membrane association is detected when the tissue is extracted in conditions of low pH and/or divalent cations. Under these conditions R–irradiation *in vivo* results in binding of up to 60% of the total measurable phytochrome (Quail and Briggs 1978) and is not FR reversible after binding. This association is termed pelletability and can also be induced by R irradiation given *in vitro*.

### 6.1.1. Pelletability.

The majority of the investigations of phytochrome binding to membranes have concerned the study of pelletability (Marme 1977, Pratt 1978 for reviews) although this is not neccessarily a membrane–binding event (see below). The association of $P_{fr}$ with membrane fractions after R irradiation *in vitro* has been demonstrated in only a small number of tissues; for example, in dark–grown *Zucchini* where 80% of the total measurable phytochrome was pelleted (Marme *et al* 1976) and in mustard where 35% pelleted. The *in vitro* association in *Zucchini* was not dependent on divalent cations, but was stimulated by their addition and was also FR reversible before binding. Quail (1974) found 37% of phytochrome pelleted after R–irradiation of corn homogenate but none was found by Marme *et al* (1976) using the same system although the observed difference may be due to the light treatments being given at different temperatures. In addition, Yamamoto and Furuya (1975) found a membrane association in homogenates of dark–grown peas that was $Ca^{2+}$ dependent, with $Mg^{2+}$ ions unable to substitute.

In contrast to the *in vitro* association, pelleting after R irradiation *in vivo* has been reported in a wide variety of tissues including corn, oat, rye, pea, *Zucchini*, radish and bean (Pratt and Marme 1976, Marme 1977) and seems to be a general phenomenon. About 60% of the total measurable phytochrome is pelleted and this response shows greater consistency than has been reported for the

*in vitro* association. There is a requirement for a divalent cation (usually 10 mol $m^{-3}$ $Mg^{2+}$ is included) and the association is completely FR reversible if the treatment is given before binding. After binding there is no FR reversibility. The pelletability response in oats and maize has a half-time of 2s at 25°C (Quail and Briggs 1978) and there is considerable similarity between the kinetics of pelletability and those of sequestering, indicating that they may be different manifestations of the same event.

Sequestering is the term given to the redistribution of phytochrome after R irradiation to discrete 1 $\mu$m sites within the cell (Verbelen *et al* 1982). These sites do not seem to be associated with a particular organelle or structure and there is no evidence that they are membrane bound, although the absence of inhibition by colchicine or cytochalasin B suggests that neither microtubules nor microfilaments are involved (McCurdy and Pratt 1986). The physiological role of sequestering is also in doubt; it may be an early event in the primary mode of action of $P_{fr}$ but it has also been suggested that this process is involved in phytochrome destruction (MacKenzie *et al* 1975). It has been demonstrated that the amount of phytochrome associated with membrane preparations is very sensitive to the pH of the homogenization medium as well as to the concentration of divalent cations (Napier and Smith 1987a). Under conditions of low pH or high $Mg^{2+}$ the net negative charge on the membranes is reduced and this increases the likelihood of phytochrome binding electrostatically (Quail 1978a,b, Furuya *et al* 1981). It may be that the pelletability of phytochrome is purely an artifactual response with no physiological significance.

### 6.1.2. Hydrophobic binding of phytochrome.

#### 6.1.2.1. Binding to microsomal membranes.

The R/FR reversible association between phytochrome and membranes in the absence of divalent cations (association I, Napier and Smith 1987a, see below) has not been intensively studied. Early workers routinely included divalent cations in their extraction media and, in the experiments where these were not included (Rubinstein *et al* 1969, Evans and Smith 1976, Hilton and Smith 1980), the small amount recovered in membrane preparations (5% of total measurable phytochrome in a 20,000g pellet, Rubinstein *et al* 1969) was thought to arise from co-precipitation of soluble phytochrome and therefore to be an artifact (Marme *et al* 1974).

Evidence against this interpretation was provided in a more detailed study of this interaction in oat shoots by Watson and Smith (1982b,c). They used

a series of washes and harsh resuspension conditions to eliminate the possibility of vesicular entrapment or electrostatic interactions and went on to investigate the nature of the phytochrome binding, concluding that it was hydrophobic. The amount of phytochrome associated with the microsomal fraction was a little less than 6% and this could be increased to 9% by R irradiation *in vivo*. This phytochrome was FR reversible after binding and was as strongly bound as the phytochrome in the etiolated tissue. The properties of this membrane-bound phytochrome were also found to be kinetically different to those of the bulk phytochrome pool in the supernatant. Total phytochrome loss in membrane fractions during a 5h dark period after R irradiation was only 50% compared to 70% loss for the whole tissue. However, $P_{fr}$ was lost from the membrane fraction at a more rapid rate than total phytochrome, while $P_r$ only decreased a small amount. This contrasted sharply with the results from the supernatant where both $P_r$ and $P_{fr}$ were lost at similar rates to those of the total cell phytochrome and it was proposed that membrane-bound $P_{fr}$ was undergoing dark reversal to $P_r$ but that supernatant $P_{fr}$ was not.

This phytochrome-membrane association in oat shoots was examined more thoroughly by Napier and Smith (Napier and Smith 1987a,b). They found that 3% of total measurable phytochrome was pelleted from etiolated or FR-treated tissue, with R irradiation increasing this FR reversibly to 8%. The probable explanation for the discrepancy between this binding data and that of Watson and Smith (1982b) was that in the earlier work, the pH of the homogenate ranged from pH 7.6 to 7.8. Napier and Smith (1987a) subsequently showed that below pH 7.75 electrostatic interactions would alter the stoichiometry of the binding.

Napier and Smith (1987a) distinguished this small R/FR reversible binding (association I) from the electrostatic interaction (association II, see pelletability), concluding that association I was pH and divalent cation independent but that, under conditions of low pH and the presence of divalent cations, this binding would be obscured by association II. In addition, this binding response obeyed the law of reciprocity and was found to have the properties of an inductive, low-fluence response (Napier and Smith 1987b). The stoichiometry was found to be exactly 2 molecules of $P_{fr}$ bound for each molecule of $P_{fr}$ present on the membrane and a model was proposed whereby a membrane-bound phytochrome molecule regulated 2 phytochrome binding sites adjacent to it.

### 6.1.2.2. Binding to plasma membranes.

The majority of studies on both types of phytochrome binding have

been undertaken with heterogeneous membrane preparations and there are very few studies of hydrophobic binding to purified membrane preparations. Widell and Sundqvist (1984) examined phytochrome binding *in vivo* to microsomal and plasma membranes from oat shoots. In the absence of $Mg^{2+}$ ions, microsomal membranes from etiolated tissue contained 1.8% of the total measurable phytochrome. This value could be increased to 2.1% by R irradiation *in vivo*, an effect that was FR reversible. In the presence of 10 mol $m^{-3}$ $Mg^{2+}$ phytochrome binding was increased and this binding had similar properties to those of pelletable phytochrome.

In plasma membranes prepared by phase partition there was a different binding pattern observed. In the absence of $Mg^{2+}$ there was a small, FR reversible increase in binding after R irradiation. The addition of 10 mol $m^{-3}$ $Mg^{2+}$ produced a much larger stimulation by R and this stimulation was almost completely FR reversible. However, it should be noted that the 'no $Mg^{2+}$ treatment' contained 0.1 mol $m^{-3}$ $Mg^{2+}$ and homogenization was performed at pH 7.0 in all experiments so some pelleting of phytochrome would be expected (Napier and Smith 1987a). Despite this, an investigation of the nature of this plasma membrane association under high $Mg^{2+}$ conditions showed that only KI and to a lesser extent Triton X-100 removed bound phytochrome suggesting that the association was hydrophobic. An interaction between plasma membranes and phytochrome added *in vitro* was also reported (Sundqvist and Widell 1983). The nature of this association largely resembled that for the *in vivo* phytochrome binding described above.

### 6.1.2.3. Binding to organelles.

Isolated organelles represent a source of membranes which are relatively easy to obtain in high yield and purity. For this type of study they also have the advantage that $P_{fr}$ *in vitro* has been shown to alter physiological properties in mitochondria, etioplasts and chloroplasts, and nuclei (Roux 1987 for review) making an association of phytochrome with organelle membranes a likely possibility.

Manabe and Furuya (1975) demonstrated R/FR reversible phytochrome binding *in vivo* in mitochondria from pea shoots and Cedel and Roux (1980) showed that mitochondria from oat seedlings bound added phytochrome *in vitro* at distinct specific and non-specific sites. More significantly perhaps, Serlin and Roux (1986) showed that $P_{fr}$ bound *in vivo* to mitochondria was protected from proteolytic degradation, indicating that the phytochrome molecule had been imported into the organelle. Phytochrome has also been detected in barley etioplasts (Evans and Smith 1976, Hilton and Smith 1980) and more recently Singh *et al* (1989) demonstrated that 124 kDa phytochrome was bound hydrophobically to

oat chloroplasts. Similarly, 125 kDa phytochrome was found to be associated with pea nuclei (Nagatini *et al* 1988) although the possibility that this association was purely non-specific was indicated.

### 6.1.3. Immunological detection of membrane-bound phytochrome.

The limitations inherent in using a spectrophotometric assay for phytochrome have already been discussed (1.3.3.1.). These problems, however, make the study of membrane-bound phytochrome particularly difficult. The presence, in the assay, of high concentrations of membranous material can accentuate problems of light scattering and, in addition, the measurement of very low quantities of phytochrome is often required. For example, the assay of plasma membrane-bound phytochrome (Widell and Sundqvist 1984) relied on signals barely above background levels. The study of membrane-bound phytochrome is therefore highly suitable for the application of immunological techniques.

Immunocytochemical methods have been used in an attempt to detect *in vivo* associations of phytochrome with a particular membrane or membranes but these results have, up to now, been equivocal (Roux 1986, Thomas *et al* 1986). Unfortunately, the use of an ELISA for phytochrome quantitation has been limited to an examination of crude membrane fractions from oat seedlings (Jordan *et al* 1984). In this study, it was also demonstrated that the phytochrome bound to microsomal membranes was full size, 124 kDa phytochrome and, in addition to the phytochrome-organelle associations described above, full size phytochrome has also been detected on pea shoot plasma membranes purified by sucrose gradient centrifugation (Gallagher *et al* 1988).

## 6.2. EXPERIMENTAL.

96-well microtitre plates from both Nunc and Dynatech were examined for uniformity. Contour maps were constructed and an estimate of the variability between wells was determined using a Genstat computer program (data not shown). The plates from Nunc were more uniform than those from Dynatech and were therefore chosen for the phytochrome quantitation experiments. However, there was still variability between wells and to account for this samples were randomized within plates, with $\geq 3$ samples per plate and at least two plates for each sample set. For each plate a phytochrome standard curve was fitted (see Figure 6.2) by a different Genstat program which also read off the individual sample values. *In vivo* light treatments were 5 min R and 10 min FR unless given otherwise in the appropriate figure legend.

## 6.3. RESULTS.

### 6.3.1. Plasma membrane purity.

A homogenization medium of pH 8.2 was used to minimise the amount of non-specific, electrostatic binding of phytochrome to membranes that has been widely reported in the literature (see 6.1). This gave a final homogenate pH of between 7.8 and 8.0. In the absence of $Mg^{2+}$ ions it has been shown that there is no 'pelleting' of phytochrome above pH 7.75 in oat seedlings (Napier and Smith 1987a) or above pH 7.0 in corn coleoptiles (Marme *et al* 1976) and these results indicated that wheat leaf membranes might also be free from non-specific phytochrome binding. Changing the pH of the homogenization medium to pH 8.2 affects the surface charge of membrane vesicles, making them more negative. Because the purification of plasma membrane vesicles by phase partition is by surface (charge) properties the separartion of the vesicles and therefore the purity of the plasma membrane preparation will be altered.

Table 6.1 shows the distribution of marker enzymes after homogenization at pH 8.2. Compared with the distribution after homogenization at pH 7.0 there was little change in mitochondrial or endoplasmic reticulum contamination but latent IDPase activity was increased indicating the presence of more Golgi membranes. Table 6.2 shows the inhibition of plasma membrane-associated $K^+$,$Mg^{2+}$-ATPase activity. Although inhibition by $NO_3^-$ ions was reduced slightly, vanadate inhibition was down from 70.5% previously to 52.0% which suggested that the purity of the plasma membrane was reduced. This conclusion was supported by a small reduction in $K^+$ stimulation from 20.4% to 17.9% and an increase in ATPase activity with either no added cation or $Ca^{2+}$ ions replacing $Mg^{2+}$ ions (data not shown). The membrane preparation isolated under these conditions is therefore still predominantly of plasma membrane origin but is slightly less pure than that described previously (Terry *et al* 1989).

### 6.3.2. Preliminary ELISA experiments.

Preliminary experiments were conducted to ascertain whether the plasma membranes interfered with the measurement of phytochrome by an ELISA and also to find a suitable concentration range over which to measure membrane-bound phytochrome. Figure 6.1 shows the addition of different dilutions of plasma membranes to a standard curve of partially purified phytochrome. The membranes only affected the standard curve at low concentrations of phytochrome. This was because the assay also detected the phytochrome which was bound to the

| Enzyme | Specific activity | |
|---|---|---|
| | $U_3$ | $L_3$ |
| Cytochrome c Oxidase ($\mu$mol/mg protein/min) | 0.029 ± 0.004 | 0.926 ± 0.021 |
| NADH Cytochrome c Reductase ($\mu$mol/mg protein/min) | 0.055 ± 0.001 | 0.197 ± 0.003 |
| Latent IDPase ($\mu$mol $P_i$/mg protein/h) | 5.12 ± 0.43 | 15.05 ± 3.43 |

Table 6.1: The distribution of marker enzyme activities between $U_3$ and $L_3$ after homogenization at pH 8.2. Results are from a single experiment and values are expressed as mean specific activity ± standard error ($n \geq 2$).

| Inhibitor | % of control |
|---|---|
| none | 100.00 ± 0.65 |
| vanadate | 47.96 ± 2.44 |
| nitrate | 90.46 ± 1.56 |

Table 6.2: The effects of inhibitors on plasma membrane ATPase activity after homogenization at pH 8.2. Inhibitors used were sodium vanadate (0.1 mol $m^{-3}$) and potassium nitrate (50 mol $m^{-3}$). Results are from five experiments and values are expressed as mean % of control ± standard error ($n \geq 14$).

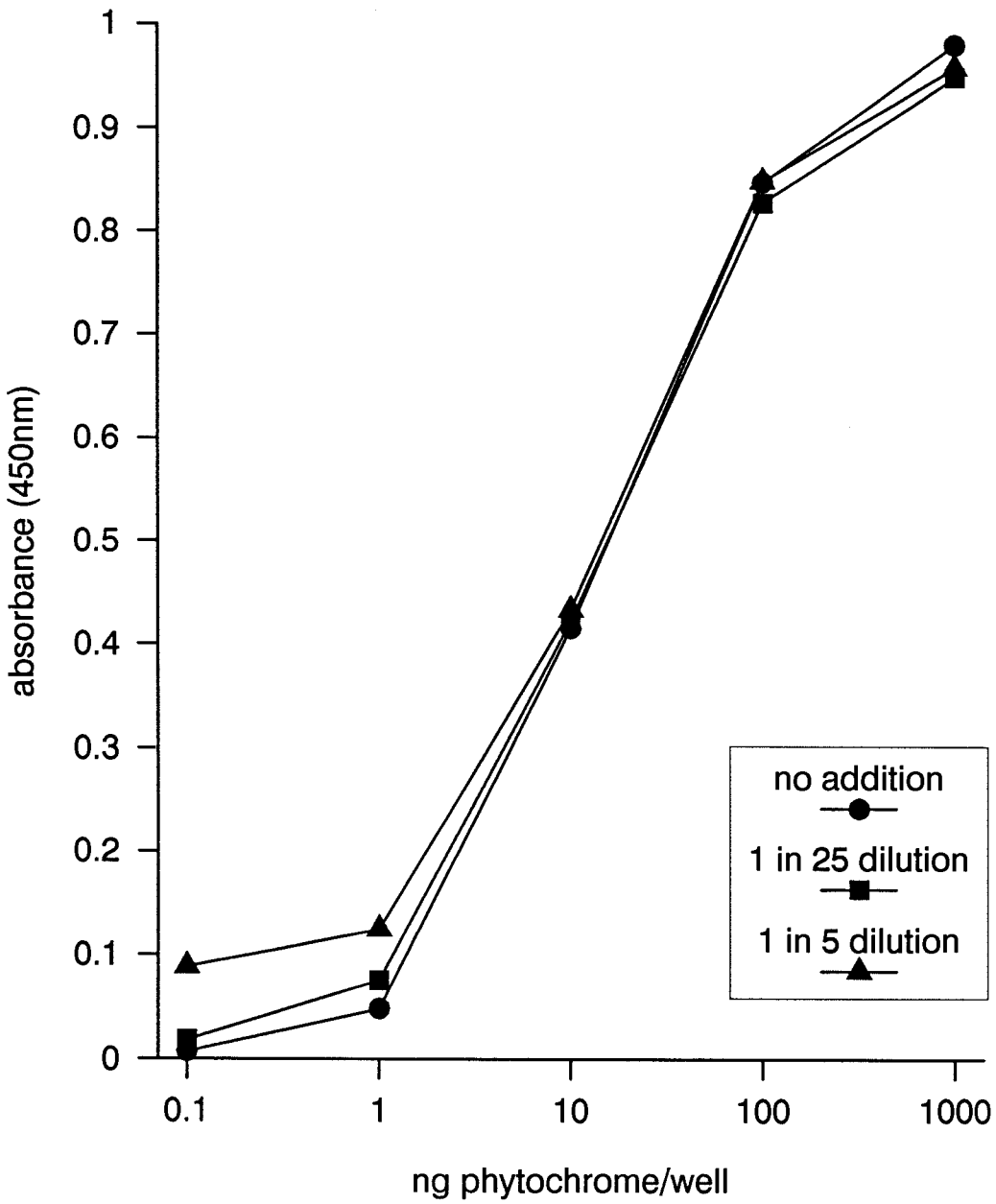


Figure 6.1: The effect of adding dilutions of a plasma membrane preparation to standard concentrations of partially purified wheat phytochrome measured by ELISA. Results are from one of two experiments (n=2).

plasma membranes. At higher phytochrome concentrations, where the amount of membrane-bound phytochrome was negligible, there was no interference in phytochrome measurement. A similar result was obtained when the phytochrome standard curve was examined in the presence of samples of extracted tissue (data not shown) which were used later for the determination of total type I phytochrome. For subsequent experiments standard curves for phytochrome of 0.1-10 ng/well were used. A typical standard curve is shown in Figure 6.2.

To demonstrate that the phytochrome being measured was intact, dark- and *in vivo* R-treated plasma membranes were prepared, run on SDS-gels and transferred to nitrocellulose. Figure 6.3 shows 124 kDa phytochrome stained using secondary antibodies conjugated to horseradish peroxidase (HRP) or alkaline phosphatase. Although alkaline phosphatase is supposedly more sensitive (Oberfelder 1989) this did not appear to be the case here. As the phosphatase staining procedure was considerably more complicated, the peroxidase stain was used on all subsequent western blots.

### 6.3.3. The effects of light treatments on phytochrome binding.

Figure 6.4 shows the effects of different *in vivo* light treatments on the amount of phytochrome present in the plasma membrane fraction as a function of the total protein in each sample. Analysis of variance between these treatments gave an SED of 5.03. This means that at the 5% level of the t-distribution the only significantly different treatments are the R and R/FR. If the errors from within the experiments were also included in this analysis the SED was 1.997 and the increase in bound phytochrome after the final R treatment is significant at the 5% level.

The absence of R-enhanced phytochrome binding under these homogenization conditions can also be demonstrated in the 10,000g and 35,000g pellets (Figure 6.5). These results were in contrast to those when homogenization was performed at pH 7.0 and in the presence of 10 mol $m^{-3}$ $Mg^{2+}$ (Figure 6.6). At this pH there was a small but significant R-induced increase in bound phytochrome in the 10,000g and the 35,000g pellets and a doubling of the amount bound to the plasma membrane. The total phytochrome recovered in the microsomal fraction (35,000g pellet) after homogenization at pH 8.2 represented ~0.7% of the total type I phytochrome.

### 6.3.4. The nature of the phytochrome-membrane association.

The effect of Triton X-100 treatments on the plasma membrane-bound

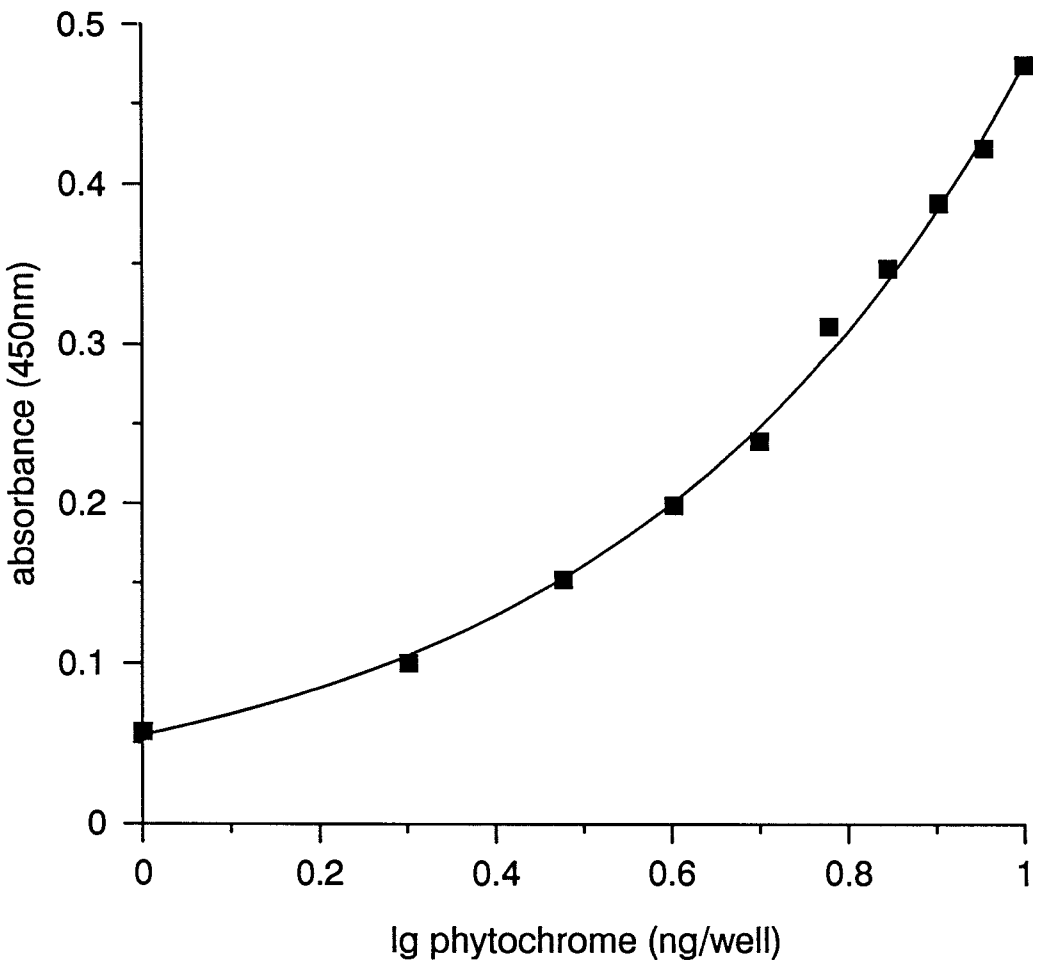


Figure 6.2: Standard curve for partially purified wheat phytochrome (1 to 10 ng/well) as measured by ELISA. Results are from a single representative experiment (n=3).

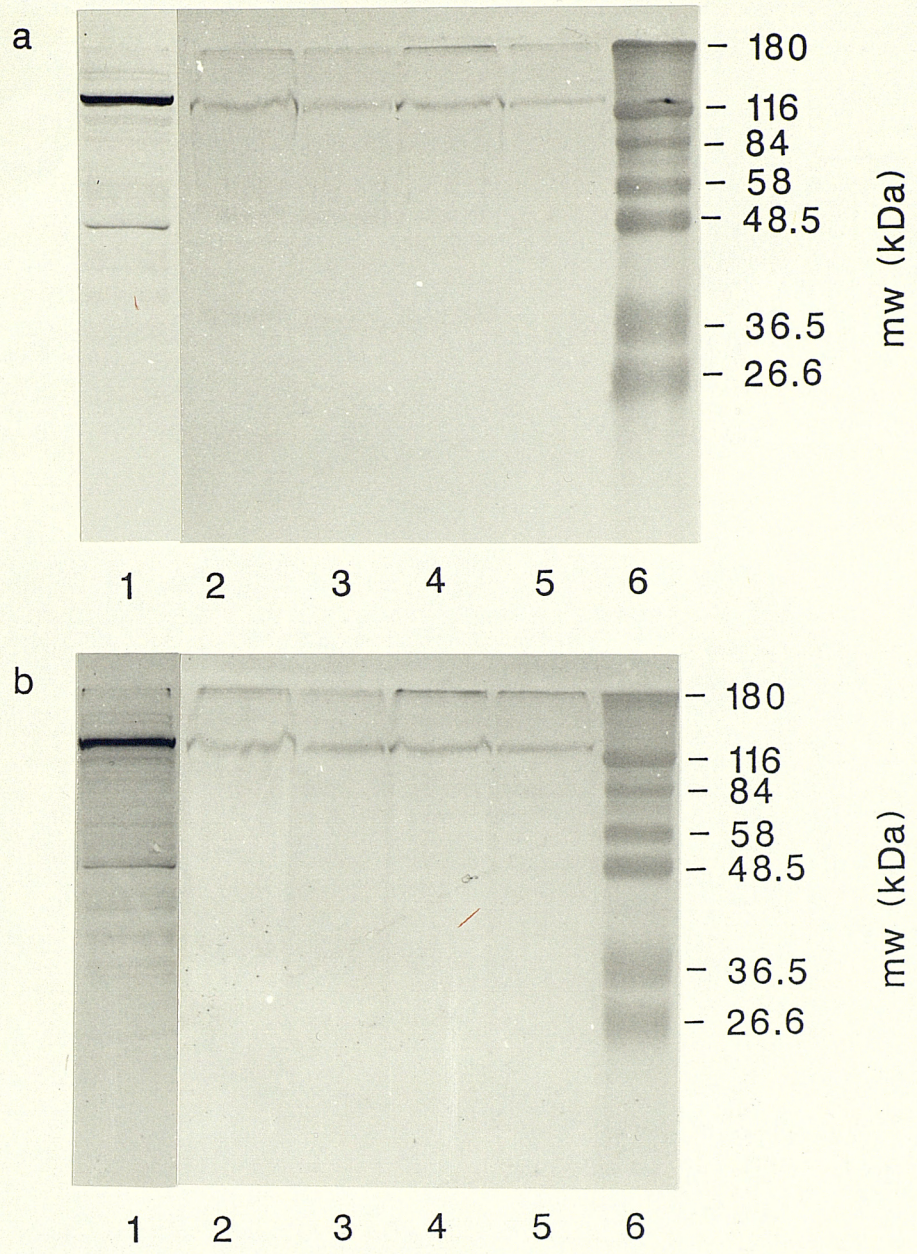


Figure 6.3: Western blot of plasma membrane-bound phytochrome stained using anti-phytochrome monoclonal antibodies MAC 52, MAC 197, MAC 199 and MAC 200 (2 $\mu g/cm^3$ each) and anti-rat IgG horse radish peroxidase (HRP) conjugate (Figure 6.3a) or anti-rat IgG alkaline phosphatase conjugate (Figure 6.3b). Plasma membranes were purified with no *in vivo* light treatment (lanes 2 and 3) or after 5 min R *in vivo* (lanes 4 and 5) and were either loaded at ~25 $\mu g$ protein/lane (lanes 2 and 4) or ~12.5 $\mu g$ protein/lane (lanes 3 and 5). Pre-stained molecular weight markers (lane 6) and ~50 ng protein/lane of partially purified wheat phytochrome (lane 1) are also included.

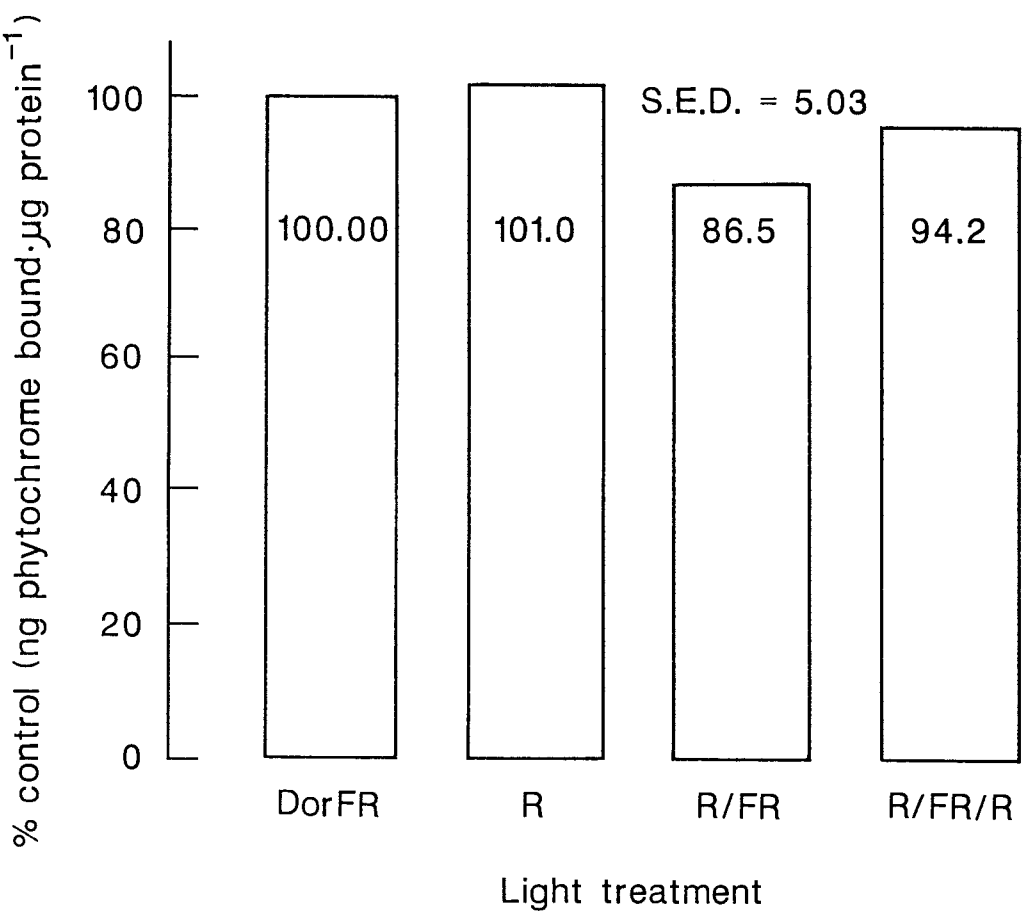


Figure 6.4: The effect of different *in vivo* light treatments on the binding of phytochrome to plasma membranes purified after homogenization at pH 8.2 in the absence of $Mg^{2+}$ ions. Results are from four experiments and are expressed as mean % of control (D or FR).

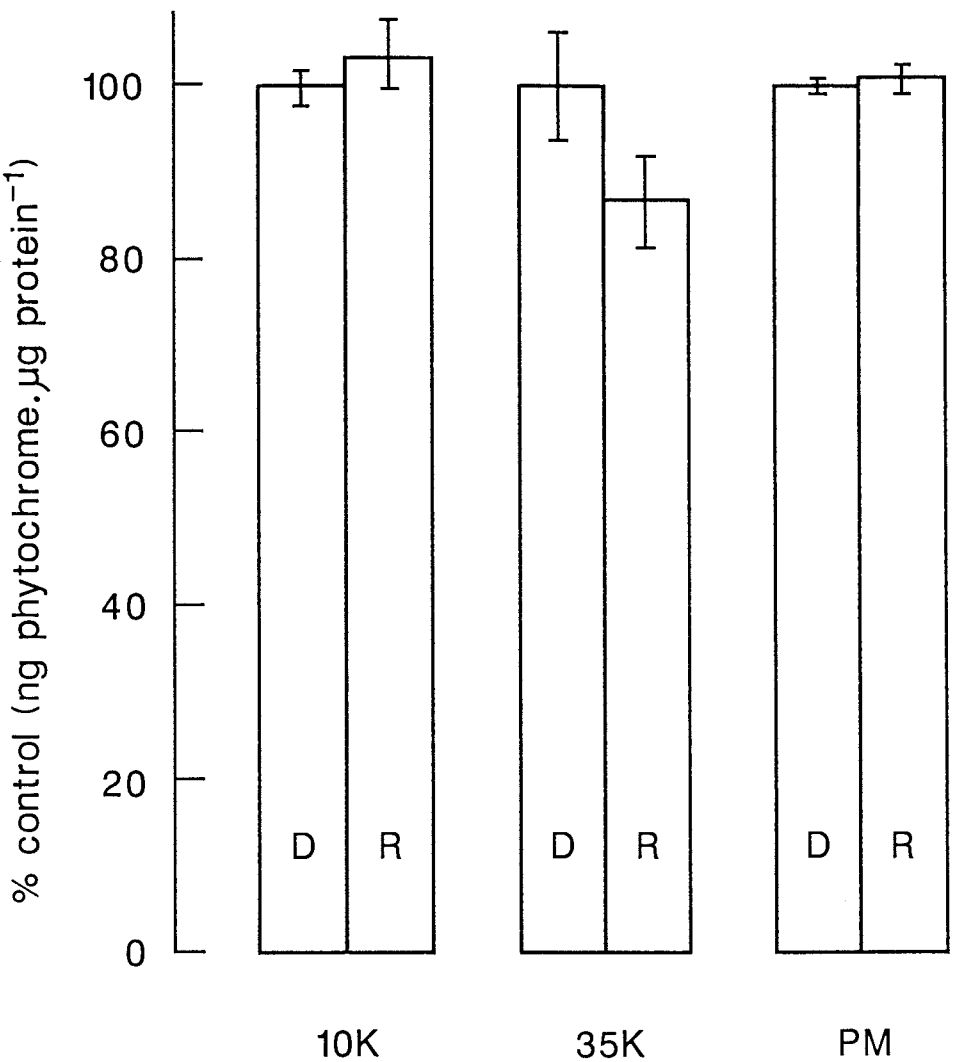


Figure 6.5: The effect of different *in vivo* light treatments on the binding of phytochrome to plasma membranes (PM), microsomal membranes (35K) and a 10,000g membrane pellet (10K) after homogenization at pH 8.2 in the absence of $Mg^{2+}$ ions. Results are from a single experiment (except for plasma membrane-bound phytochrome, see Figure 6.4) and are expressed as mean % of control (D) ± standard error ($n \geq 6$).

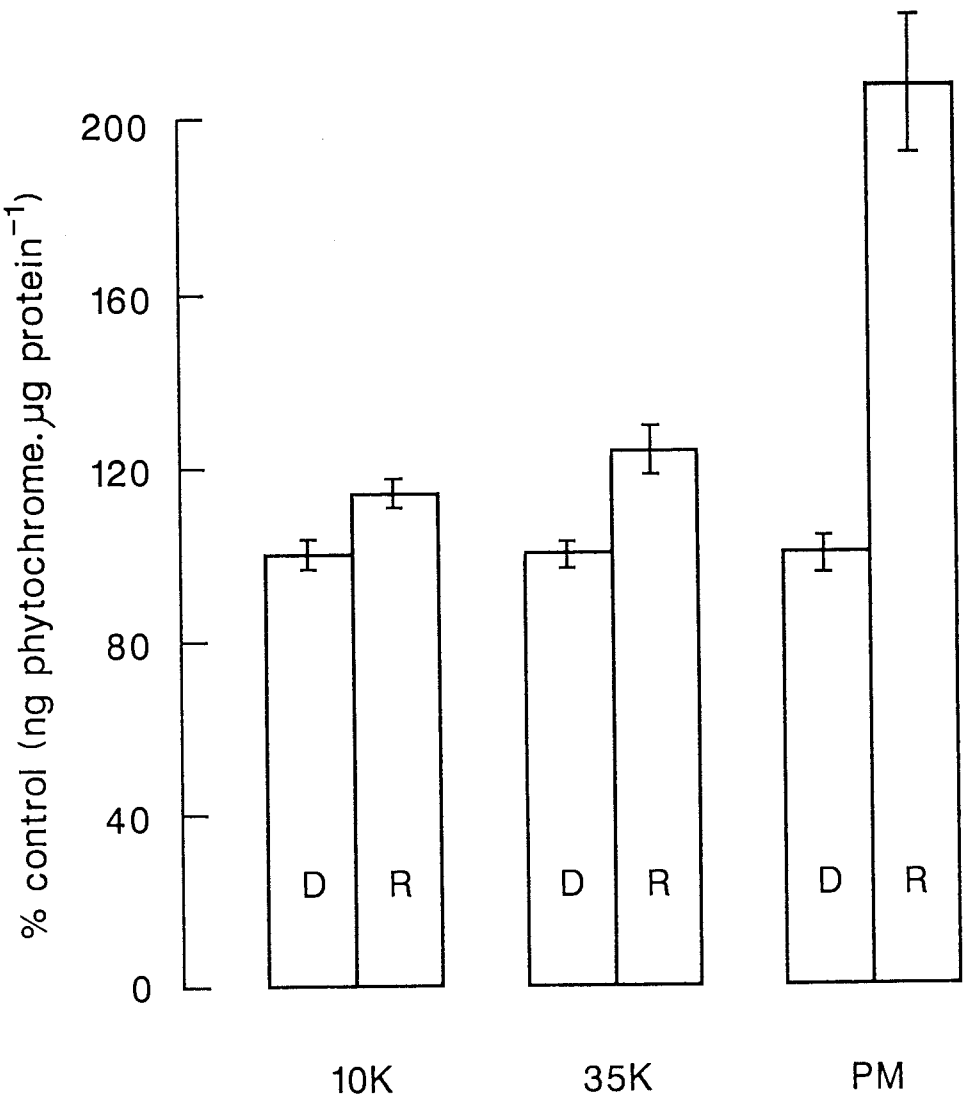


Figure 6.6: The effect of different *in vivo* light treatments on the binding of phytochrome to plasma membranes (PM), microsomal membranes (35K) and a 10,000g membrane pellet (10K) after homogenization at pH 7.0 in the presence of 10 mol $m^{-3}$ $Mg^{2+}$ ions. Results are from a single experiment and are expressed as mean % of control (D) ± standard error (n=6).

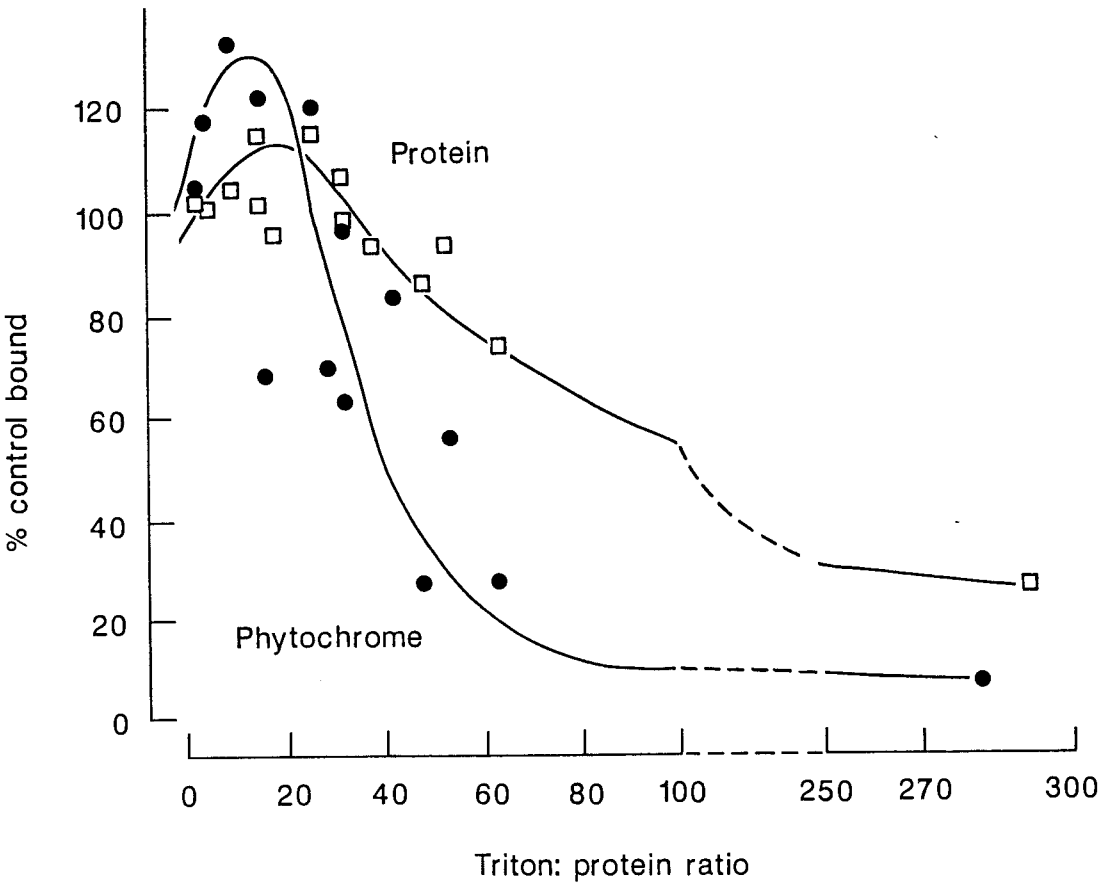


Figure 6.7: The effect of Triton X-100 concentration on the binding of phytochrome and total protein to plasma membranes after 5 min R *in vivo*. Results are from three experiments and are expressed as mean % of control (amount bound in the absence of Triton).

phytochrome after a 5 min R *in vivo* treatment was investigated. Figure 6.7 shows the amount of phytochrome and total protein bound as a function of the Triton:protein (t:p) ratio, a more accurate measure of Triton activity than detergent concentration. The bound phytochrome was released from the membrane at t:p ratios of between 20 and 50 with total protein being released more gradually at t:p ratios above 40. The phytochrome and protein removed from the membrane does not represent the release of trapped molecules from within the membrane vesicles because other membrane-affecting agents (Table 6.3) which are able to remove phytochrome are added in the presence of only 0.001% (w/w) Triton which corresponds to a t:p ratio of ~2.5; *i.e.* at this concentration the detergent can form holes in the plasma membrane vesicles that are large enough to allow phytochrome to pass through.

Figure 6.8 shows the effect of KI on both bound phytochrome and total protein. Phytochrome binding was more sensitive to KI than total protein with $1x10^3$ mol $m^{-3}$ being sufficient to remove all the bound phytochrome but only 38% of the total protein. The effectiveness of the KI treatment was expected as KI can disrupt both hydrophobic and hydrophilic (electrostatic) interactions (Maddy and Dunn 1976). Other membrane-affecting agents were also investigated and these results are shown in Table 6.3. Although no treatment appeared to have an effect on the total plasma membrane-associated protein, most of the treatements used reduced the amount of plasma-membrane bound phytochrome. KCl, which would be expected to disrupt electrostatic interactions, was only effective at a concentration of $1x10^3$ mol $m^{-3}$ at which point ~37% of the phytochrome was removed. Low concentrations (10 mol $m^{-3}$) of both EDTA and $Ca^{2+}$ ions also removed bound phytochrome, probably by breaking salt bridges and reorganising lipid head groups respectively. Phytochrome binding was also reduced (29%) by 10 mol $m^{-3}$ $MgCl_2$. There is no obvious explanation for this result particularly as $Mg^{2+}$ ions have been used widely to make phytochrome 'stick' to membranes (see 6.1).

The results detailed above used plasma membranes that had been given a 5 min R *in vivo* treatment before isolation (*i.e.* phytochrome was in the $P_{fr}$ form). Although this treatment had no effect on the amount of phytochrome bound to the plasma membranes it was possible that the nature of the phytochrome-membrane interaction may have been altered. Table 6.4 shows a summary of similar results obtained using plasma membranes from tissue which had received no *in vivo* light treatment (*i.e.* phytochrome was in the $P_r$ form). There was very little detectable difference between the interactions of $P_r$ and $P_{fr}$. The results with all Triton and KI concentrations examined and EDTA were essentially the same. However, both $1x10^3$ mol $m^{-3}$ KCl and 10 mol $m^{-3}$ $MgCl_2$ were

| Treatment | % control bound | |
|---|---|---|
| | phytochrome | total protein |
| control | 100.00 ± 1.81 | 100.00 ± 1.06 |
| KCl (100 mol $m^{-3}$) | 107.89 ± 3.17 | 104.45 ± 6.15 |
| KCl ($1x10^3$ mol $m^{-3}$) | 63.26 ± 1.52 | 107.72 ± 2.87 |
| EDTA (10 mol $m^{-3}$) | 81.20 ± 2.20 | 112.90 ± 2.18 |
| $MgCl_2$ (10 mol $m^{-3}$) | 71.04 ± 5.48 | 109.93 ± 0.61 |
| $CaCl_2$ (1 mol $m^{-3}$) | 91.94 ± 3.16 | 102.92 ± 2.86 |
| $CaCl_2$ (10 mol $m^{-3}$) | 57.74 ± 1.03 | 105.31 ± 2.63 |

Table 6.3: The effect of various treatments on the binding of both phytochrome and total protein to plasma membranes purified after an *in vivo* 5 min R treatment. Treatments were added in the presence of 0.001% (w/w) Triton X-100. Results are from three experiments and values are expressed as mean % of control (amount bound with Triton alone) ± standard error ($n \geq 6$).

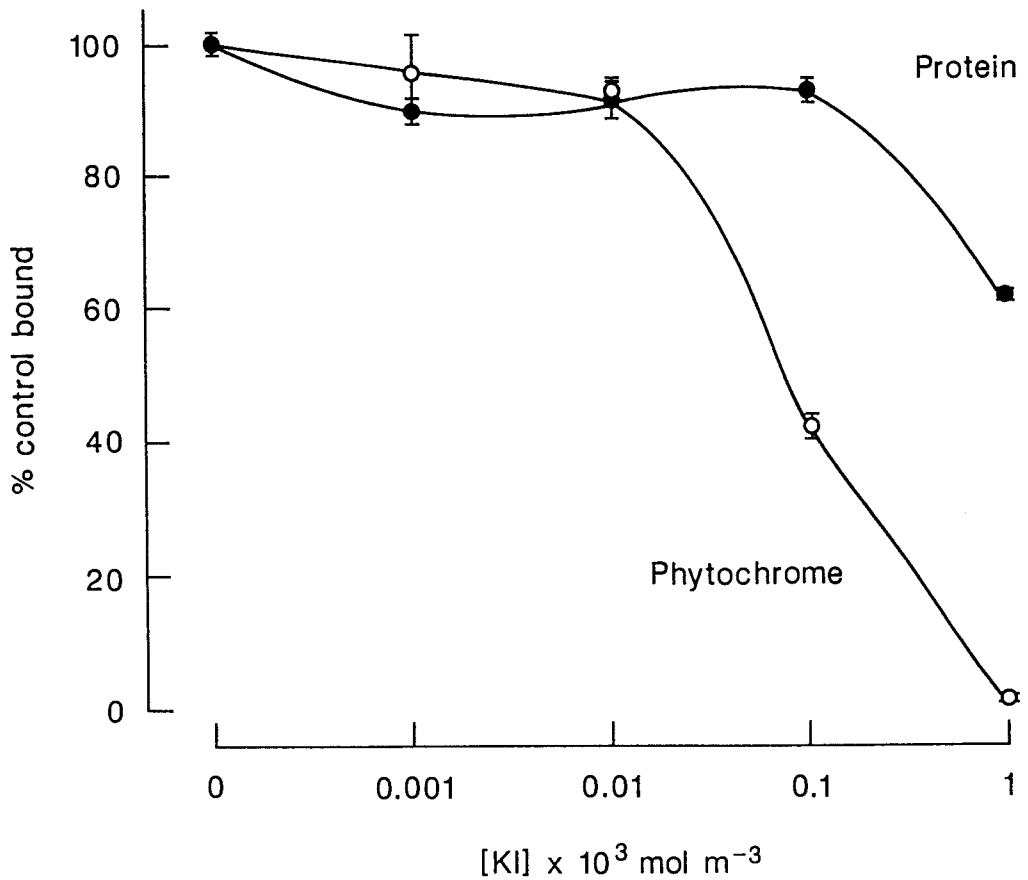


Figure 6.8: The effect of KI concentration on the binding of phytochrome and total protein to plasma membranes after 5 min R *in vivo*. KI was added in the presence of 0.001% (w/w) Triton X-100. Results are from three experiments and are expressed as mean % of control (amount bound with Triton alone) ± standard error ($n \geq 6$).

| Treatment | % control bound | |
|---|---|---|
| | phytochrome | total protein |
| control | 100.00 ± 4.57 | 100.00 ± 4.80 |
| Triton (0.005%, t:p 28.15) | 103.16 ± 3.22 | 98.27 ± 5.31 |
| Triton (0.01%, t:p 56.29) | 13.26 ± 1.49 | 43.62 ± 2.97 |
| Triton (0.05%, t:p 281.46) | 6.91 ± 2.33 | 41.16 ± 1.46 |
| Triton (0.1%, t:p 562.92) | 5.65 ± 2.05 | 31.38 ± 1.31 |
| control | 100.00 ± 4.15 | 100.00 ± 7.31 |
| KI (10 mol $m^{-3}$) | 90.80 ±13.22 | 113.79 ± 4.67 |
| KI (100 mol $m^{-3}$) | 38.95 ± 3.25 | 99.39 ± 4.28 |
| KI ($1x10^3$ mol $m^{-3}$) | 1.09 ± 0.51 | 33.48 ± 0.85 |
| KCl (100 mol $m^{-3}$) | 119.82 ±11.56 | 104.21 ± 6.57 |
| KCl ($1x10^3$ mol $m^{-3}$) | 40.23 ± 5.55 | 93.31 ± 2.76 |
| EDTA (10 mol $m^{-3}$) | 75.06 ±12.03 | 100.17 ± 0.63 |
| $MgCl_2$ (10 mol $m^{-3}$) | 47.42 ± 5.22 | 90.75 ± 3.13 |

Table 6.4: The effect of various treatments on the binding of both phytochrome and total protein to plasma membranes purified in the absence of an *in vivo* R treatment. The concentration of Triton X-100 added is expressed as % (w/w) with the Triton:protein ratio also given. The other treatments were added in the presence of 0.001% (w/w) Triton and the control value for these treatments is the amount bound with Triton alone. Results are from three experiments and values are expressed as mean % of control ± standard error ($n \geq 3$).

slightly more effective on $P_r$ than $P_{fr}$.

### 6.3.5. Does membrane-bound $P_{fr}$ exhibit different destruction kinetics to the total type I pool?

#### 6.3.5.1. Destruction in continuous R.

The destruction kinetics of $P_{fr}$ in continuous R were followed for both plasma membrane-bound phytochrome and total type I phytochrome (Figure 6.9). These results were calculated by regression analysis, a variation of analysis of variance which can be used when the treatment blocks are incomplete. The means and standard errors which were derived from pooled variance are shown in Table 6.5. The patterns of $P_{fr}$ destruction were quite different. After 1h continuous R there was no decrease in membrane-bound phytochrome (the small increase detected was not significant at the 5% level) whereas the total type I pool was reduced to 60% of the control level. Although there is rapid destruction of membrane-bound phytochrome between 1h and 3h continuous R the level appears to stabilize at about 15% of the dark control. The total pool continues to decrease and after 4h is reduced to 2.18 ± 3.05% of the control level. Pre-immune rat IgG controls for this assay are 1.67 ± 0.15% of the control value so after 4h R there is effectively no type I phytochrome remaining.

Figure 6.10 shows the destruction of phytochrome in continuous R followed spectrophotometrically. The homogenate was centrifuged for 10 min at 10,000g to remove large particles which caused light scattering and interfered with the assay. The pattern of phytochrome destruction was similar to that determined by ELISA but was not as rapid, with some phytochrome still detectable after 4h. This is probably because the more stable type II phytochrome is also being measured. The linearity of the logarithmic plot of this data indicates that total phytochrome destruction is first order with a half-life ($t_{0.5}$) ≃100 min.

The destruction of 124 kDa phytochrome can also be followed visually by western blotting. Figure 6.11 shows the destruction in continuous R of both membrane-bound and total type I phytochrome. For the plasma membrane-bound phytochrome particularly, not all the phytochrome has gone into the gel and this is seen in the higher molecular weight band at ⩾180 kDa (Figure 6.11a). This was probably due the presence of phytochrome dimers or aggregates and was most apparent in the dark treated membranes, when the phytochrome was in the $P_r$ form. $P_{fr}$ is the phytochrome species which normally forms aggregates, for example in the pelletability response (6.1.1.), so this result is unexpected. By scanning across the western blot transversely using a densitometer, a quantitative

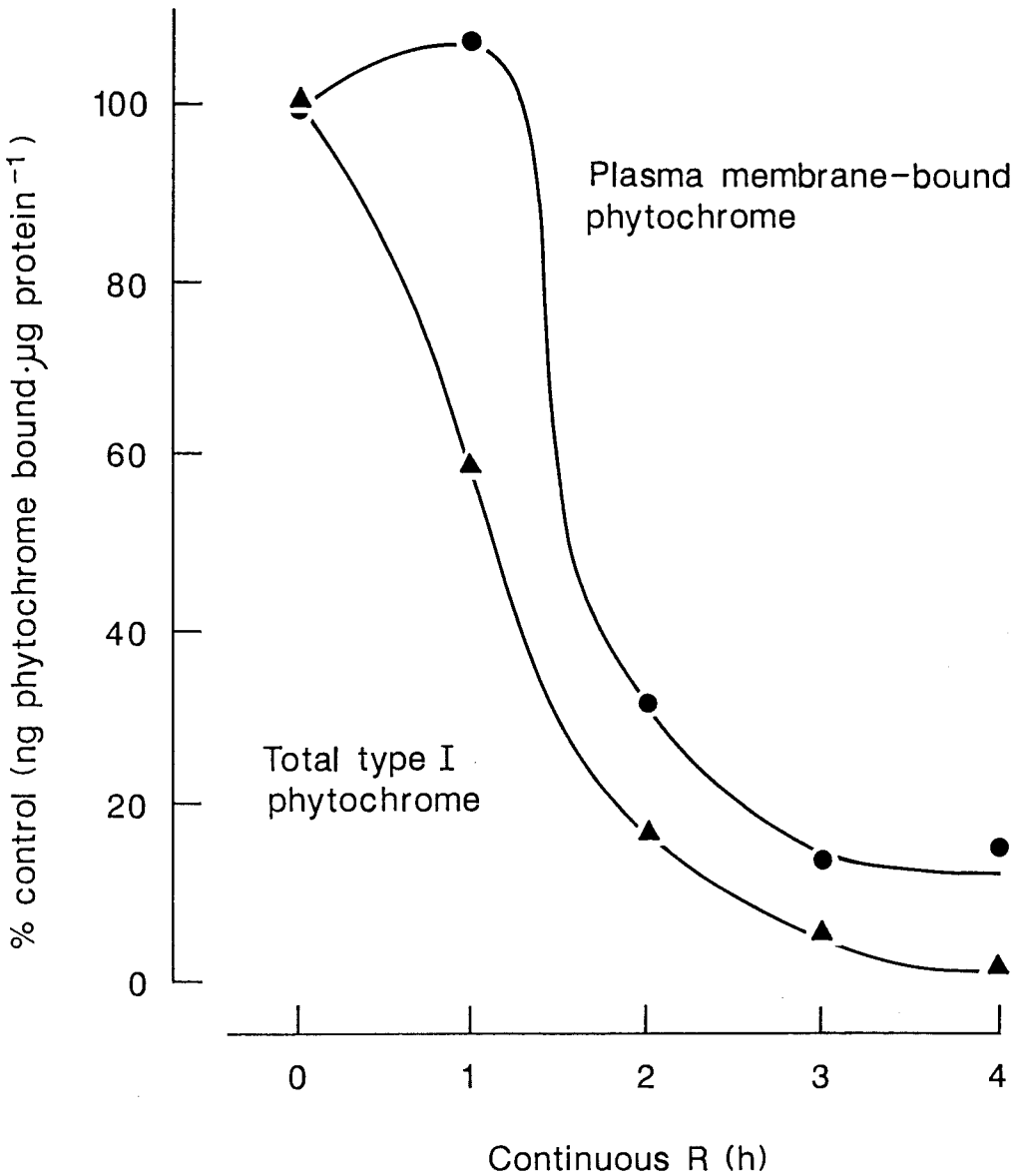


Figure 6.9: The effect of continuous R *in vivo* on plasma membrane-bound and total type I phytochrome. Results are from four experiments and points are expressed as mean % of control (D). The data were calculated by regression analysis and errors are shown in Table 6.5.

| Treatment | % control | |
|---|---|---|
| | ELISA | western blot |
| plasma membrane-bound phytochrome | | |
| 0h R | 98.52 ± 3.08 | 100.00 |
| 1h R | 106.74 ± 3.08 | 110.35 |
| 2h R | 32.01 ± 3.64 | 74.72 |
| 3h R | 13.49 ± 5.75 | |
| 4h R | 15.14 ± 3.65 | 11.60 |
| total type I phytochrome | | |
| 0h R | 100.04 ± 2.69 | 100.00 |
| 1h R | 59.35 ± 2.69 | 54.78 |
| 2h R | 17.80 ± 2.69 | 6.08 |
| 3h R | 5.53 ± 5.71 | |
| 4h R | 2.18 ± 3.05 | 1.34 |

Table 6.5: The destruction of phytochrome in continuous red light (R) measured by ELISA and western blotting. The ELISA data are from four experiments and values are expressed as the mean % of control (Oh R) ± standard error. The standard error is derived from a pooled variance, calculated using regression analysis (see Figure 6.9). The western blotting data are from a single experiment and were determined by densitometry (see Figures 6.11 and 6.12).

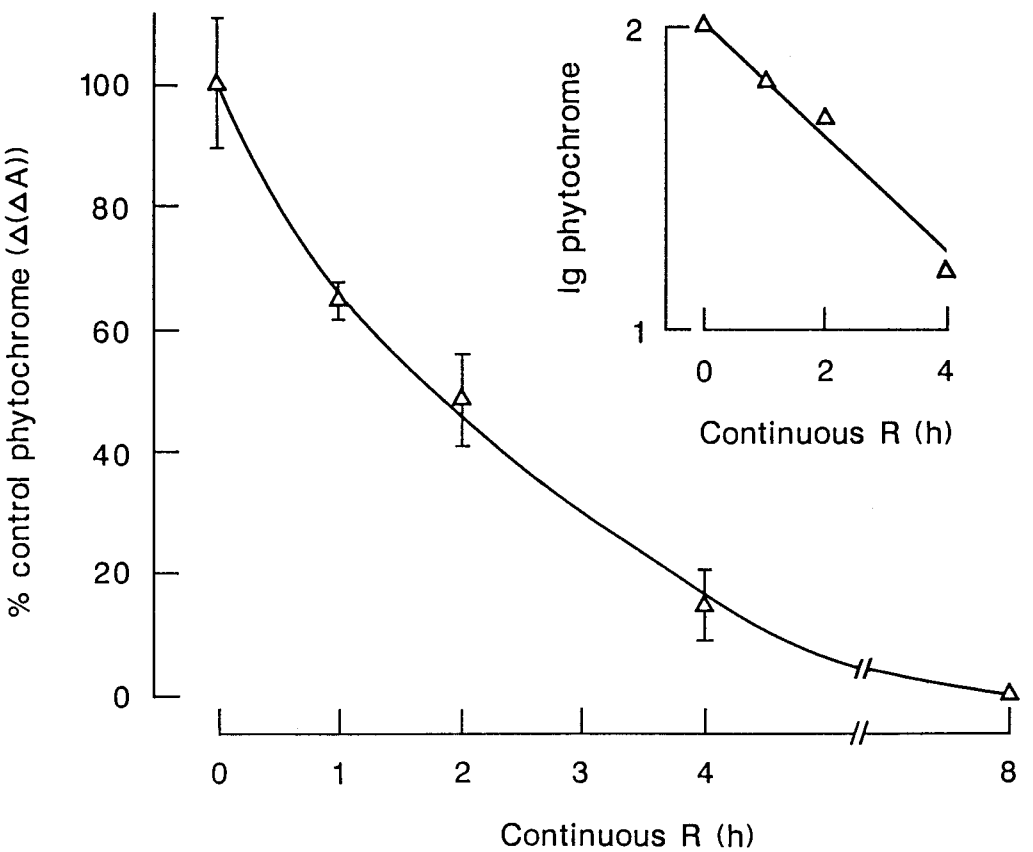


Figure 6.10: The effect of continuous R *in vivo* on total cell phytochrome determined spectrophotometrically. Insert shows a logarithmic plot of the same data. Results are from a single experiment and points are expressed as mean % of control (0h R) ± standard error (n≥2).

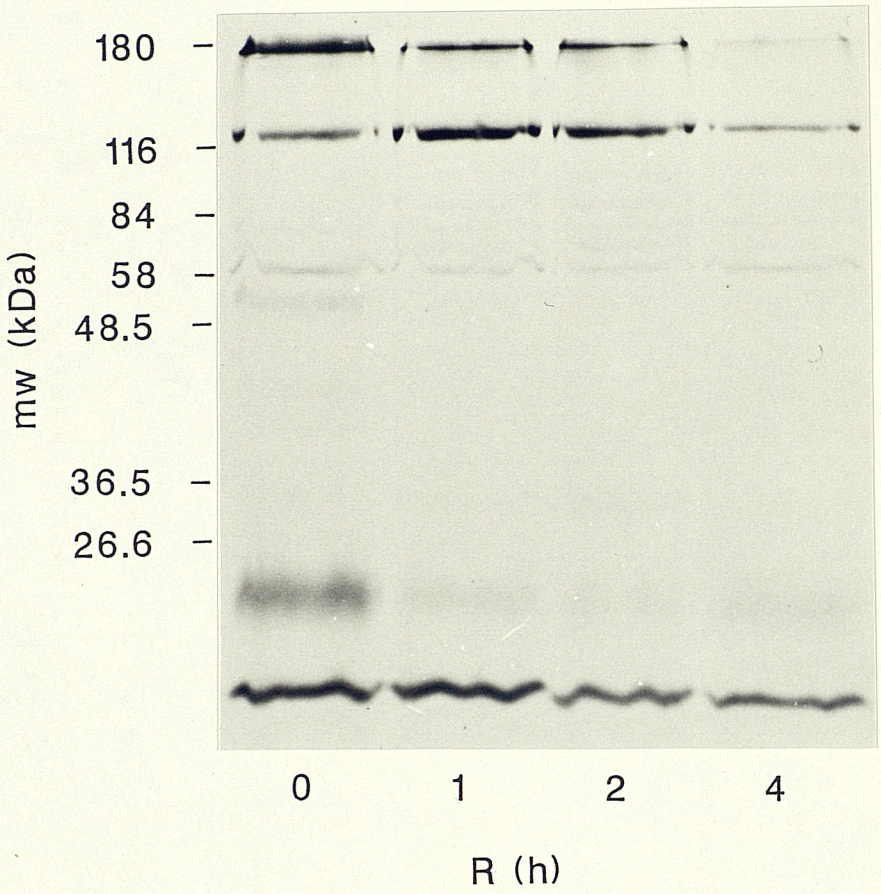


Figure 6.11a: Western blot of the destruction in continuous R *in vivo* of plasma membrane–bound phytochrome. Blots were stained using anti–phytochrome monoclonal antibody supernatants MAC 197, MAC 199 and MAC 200 (~2 $\mu g/cm^3$ each) with a biotin–streptavidin amplification step. Each lane was loaded equally with ~25 $\mu g$ protein.

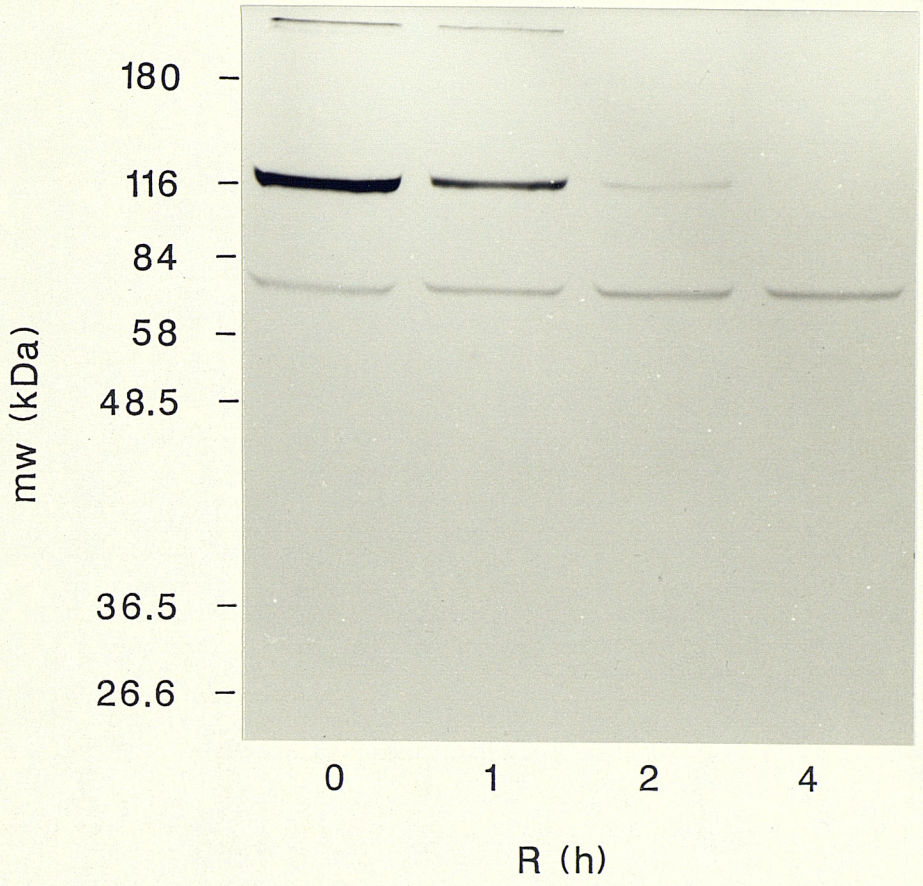


Figure 6.11b: Western blot of the destruction in continuous R *in vivo* of total type I phytochrome. Blots were stained using anti-phytochrome monoclonal antibodies MAC 52 and MAC 199 (3 $\mu g/cm^3$ each) and monoclonal antibody supernatant MAC 197 (~2 $\mu g/cm^3$) with a biotin-streptavidin amplification step. Each lane was loaded equally with ~15 $\mu g$ protein.

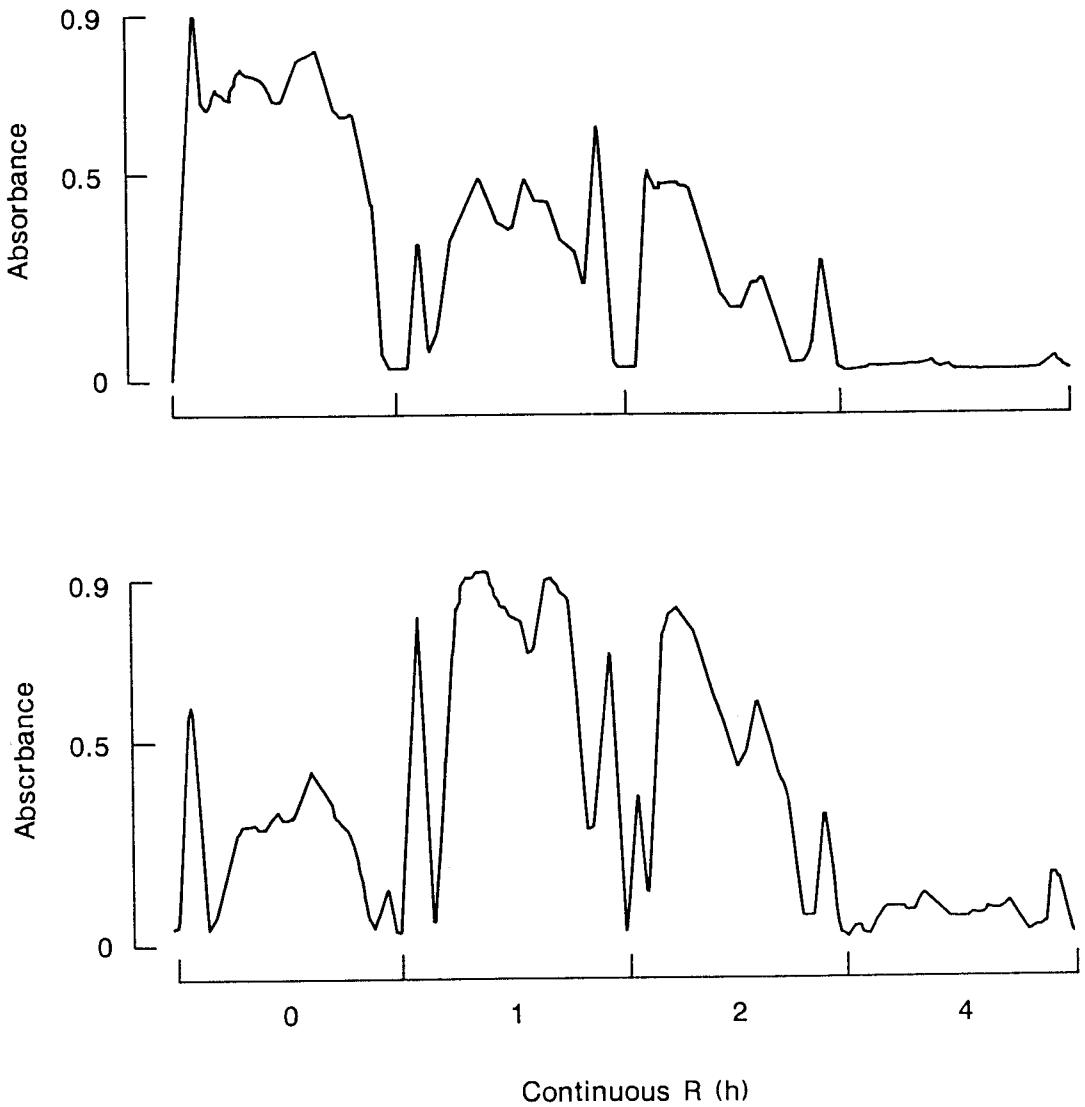


Figure 6.12: Transverse densitometry scans of bands stained for phytochrome at ≥180 kDa (above) and 124 kDa (below) from Figure 6.11a. The intensity of each band is proportional to the area under each curve and is given numerically in Table 6.5.

measure of the intensity of each band can be determined. Figure 6.12 shows densitometry scans for the destruction of plasma membrane-bound phytochrome. By adding the absorbance from both phytochrome bands (124 kDa and ≥180 kDa) the amount of phytochrome at each stage of the light treatment can be calculated. These values are given in Table 6.5 and approximate to those determined more rigorously using ELISA. Values from similar densitometry scans of Figure 6.11b are also given.

The origin of some of the lower bands in Figure 6.11a is not clear. The bands at 62 kDa, 52 kDa and 25 kDa are probably breakdown products of phytochrome. The unusual band at 19 kDa and the 79 kDa band in Figure 6.11b may be related to the use of the biotin-streptavidin amplification system which would also detect any protein in the sample which had a biotin group attached to it. In a later experiment, where the western blot was divided to allow different treatments to each lane, controls were included but the 19 kDa band was not detected again under any conditions. This result can be seen in Figure 6.13. The experiment was designed principally to find out whether the population of plasma membrane-bound phytochrome remaining after 4h continuous R was antigenically different to the population of membrane-bound phytochrome in dark tissue. There was little detectable difference, although MAC 200 was comparatively more effective at detecting membrane-bound phytochrome from dark tissue. MAC 197 and MAC 199 were also effective but, although very faint bands can be seen on Figure 6.13a, no other anti-type I oat phytochrome monoclonal antibody was able to detect plasma membrane-bound phytochrome from wheat leaves on a western blot. Figure 6.13 also demonstrates clearly the advantage of using the biotin-streptavidin system. In the absence of this amplification step no band was detected using MAC 200 by the usual procedure.

### 6.3.5.2. Destruction in continuous FR.

The destruction kinetics of phytochrome in continuous FR were followed as for continuous R (Figure 6.14). There was little destruction of either plasma membrane-bound or total type I phytochrome although, interestingly, this rate of destruction was greater for plasma membrane-bound phytochrome than the total type I phytochrome. After 1h FR the amount of membrane-bound phytochrome started to increase. This effect was also seen in the total type I pool, but the increase was much slower.

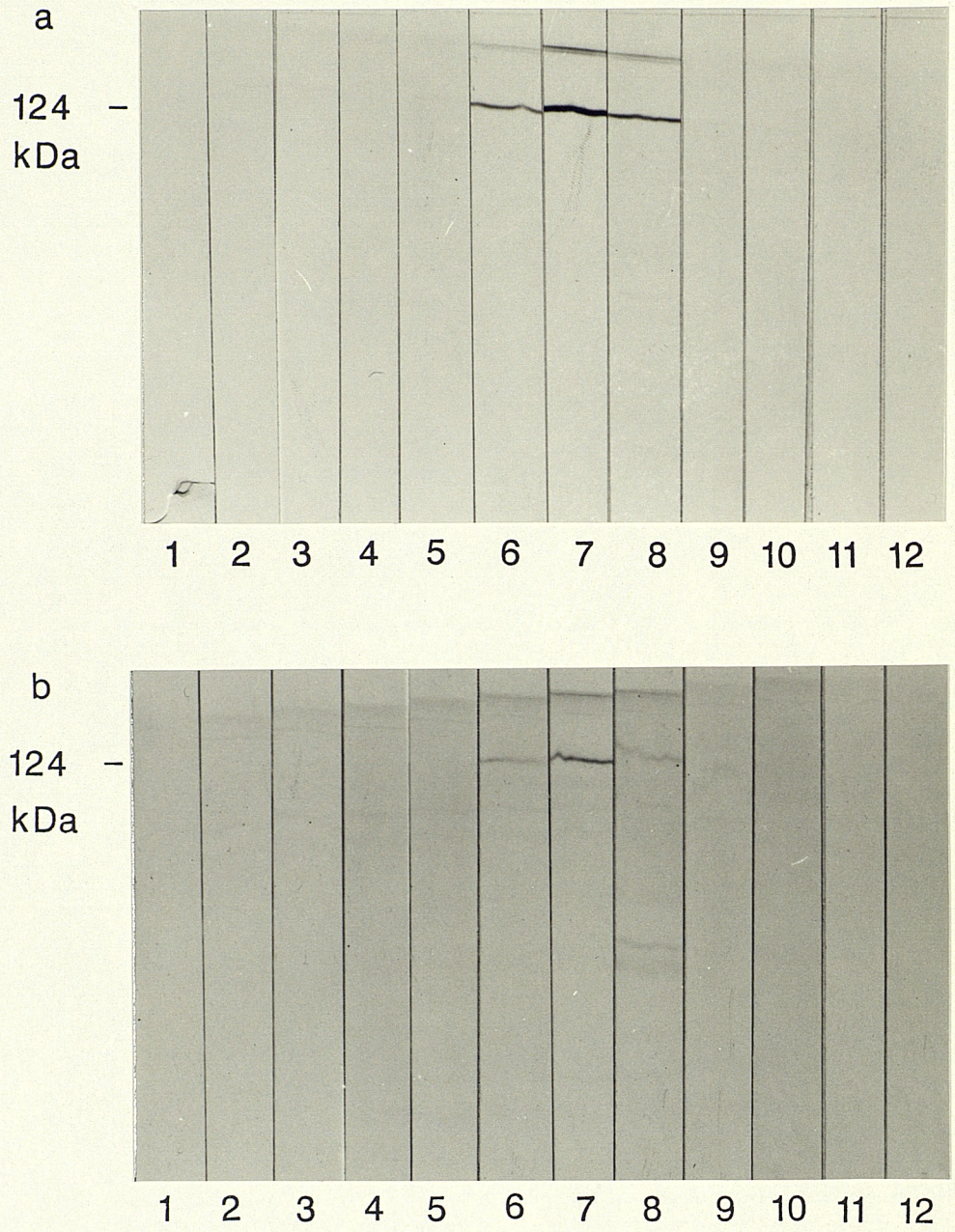


Figure 6.13: Western blots of plasma membrane-bound phytochrome (124 kDa) after no *in vivo* light treatment (a) or 4h continuous R *in vivo* (b). Blots were stained using a biotin-streptavidin amplification step (except where stated). Lane 1: pre-immune rat IgG (no amplification step); lane 2: MAC 49 (2$\mu$g/cm$^3$); lane 3: MAC 52 (2$\mu$g/cm$^3$); lane 4: MAC 111 supernatant (~2$\mu$g/cm$^3$); lane 5: MAC 112 supernatant (~2$\mu$g/cm$^3$); lane 6: MAC 197 supernatant (~2$\mu$g/cm$^3$); lane 7: MAC 199 supernatant (~2$\mu$g/cm$^3$); lane 8: MAC 200 supernatant (~2$\mu$g/cm$^3$); lane 9: MAC 56 (2$\mu$g/cm$^3$); lane 10: MAC 50 (2$\mu$g/cm$^3$); lane 11: pre-immune rat IgG; lane 12: MAC 200 supernatant (~2$\mu$g/cm$^3$, no amplification step). Each lane was loaded identically with ~13 $\mu$g protein.

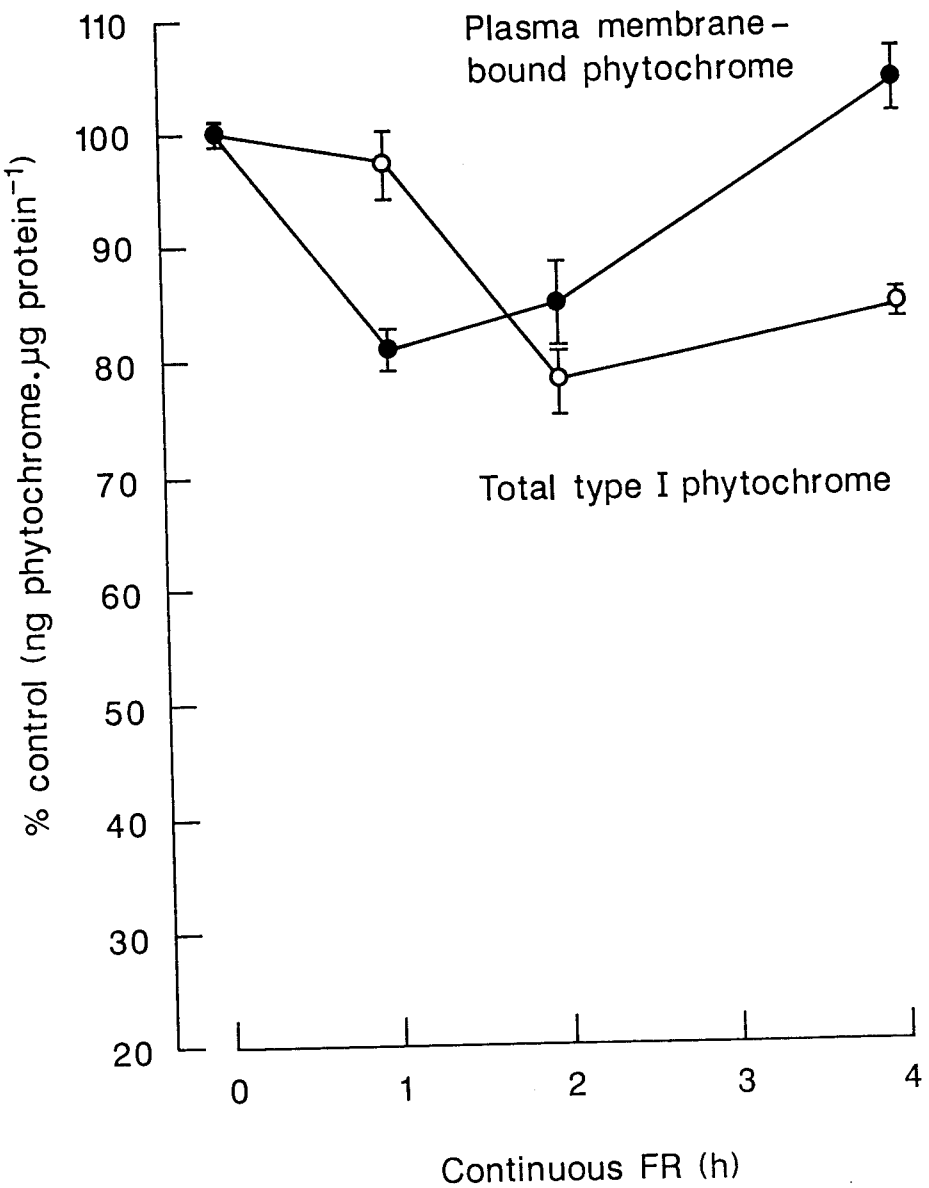


Figure 6.14: The effect of continuous FR *in vivo* on plasma membrane-bound and total type I phytochrome. Results are from two experiments and points are expressed as mean % of control (0h FR) ± standard error (n=24).

### 6.3.6. Does membrane-bound $P_r$ exhibit different synthesis kinetics to the total type I pool?

The possibility that the plasma membrane-bound phytochrome represented a different population from the total type I pool was also investigated by manipulating the level of $P_r$ in the plant. This was achieved by blocking phytochrome synthesis with light treatments given at 12h intervals for a various number of days before harvesting. Initially the effects of 5 min R treatments were examined. Treatments for one day were sufficient to completely block type I phytochrome synthesis (data not shown) and R treatments were therefore unsuitable for investigating differences in the kinetics of phytochrome synthesis between the two pools. Alternative treatments were tried and Figures 6.15 and 6.16 show the effect of 5 min and 10 min FR treatments respectively. In both experiments the blocking of $P_r$ synthesis reduced the level of total type I phytochrome more than that of the membrane-bound phytochrome.

The relationship between plasma membrane-bound phytochrome and the total type I pool can be shown graphically by plotting one against the other for each treatment in which phytochrome levels were manipulated (Figure 6.17). The graph shows that only after the total type I phytochrome has been reduced to ~60% of the control level is there any reduction in plasma membrane-bound phytochrome. The only exception appears to be after 1h continuous FR which was able to reduce the level of membrane-bound phytochrome more than the total type I pool. The average control values for all experiments in this chapter were 1.89 ± 0.18 ng phytochrome/$\mu$g protein for the plasma membrane-bound phytochrome and 10.55 ± 0.95 ng phytochrome/$\mu$g protein for the total type I pool.

## 6.3. DISCUSSION.

The results in this chapter demonstrate clearly that a small proportion of the total type I phytochrome ($\ll$0.7%) is associated with plasma membranes purified from wheat leaves by phase partition. This plasma membrane-bound phytochrome has an apparent molecular mass of 124 kDa. Although phytochrome has been shown previously to separate with putative plasma membrane markers on sucrose density gradients (Marme *et al* 1976, Gallagher *et al* 1988) and also to be associated with oat plasma membranes purified by phase partition (Widell and Sundqvist 1984), this is the first detailed quantitative and qualitative study of phytochrome binding to the plasma membrane.

Perhaps the most striking feature of this phytochrome-membrane interaction is that, in contrast to almost all previous studies, there is no increase

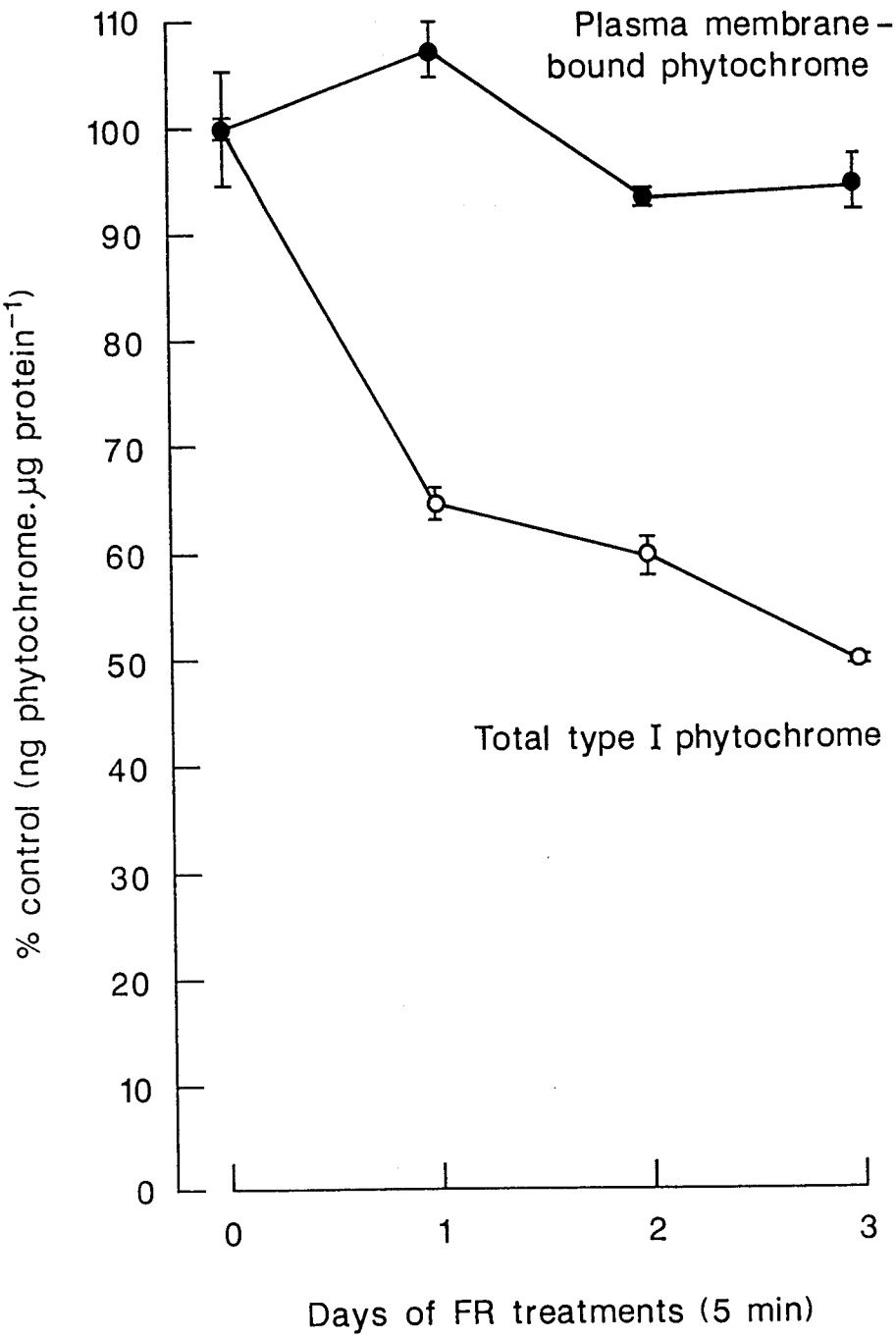


Figure 6.15: The effect of two 5 min FR *in vivo* light treatments given 12h apart on successive days before harvesting on plasma membrane-bound and total type I phytochrome. Results are from a single experiment and points are expressed as mean % of control (D) ± standard error (n=6).

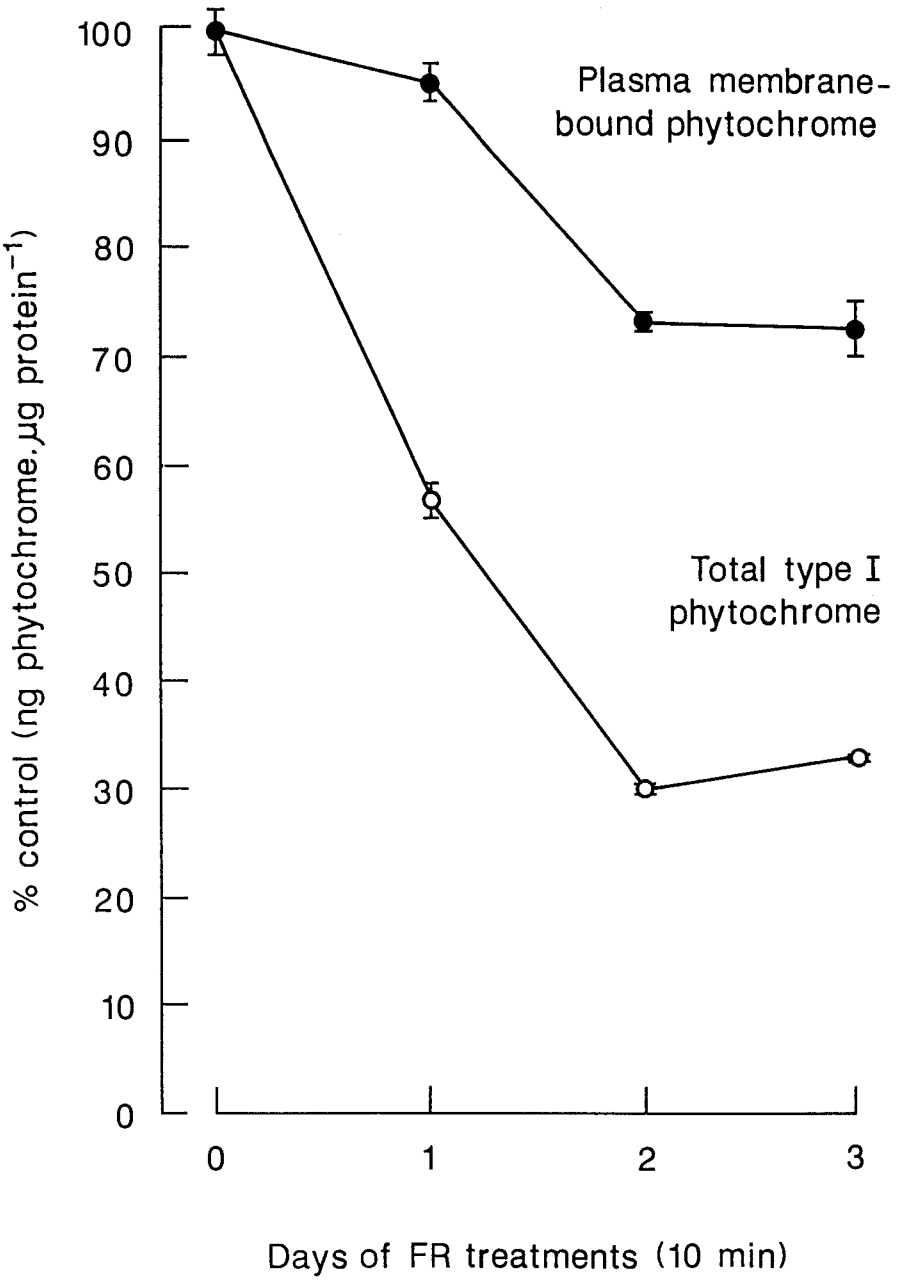


Figure 6.16: The effect of two 10 min FR *in vivo* light treatments given 12h apart on successive days before harvesting on plasma membrane-bound and total type I phytochrome. Results are from two experiments and points are expressed as mean % of control (D) ± standard error (n≥6).

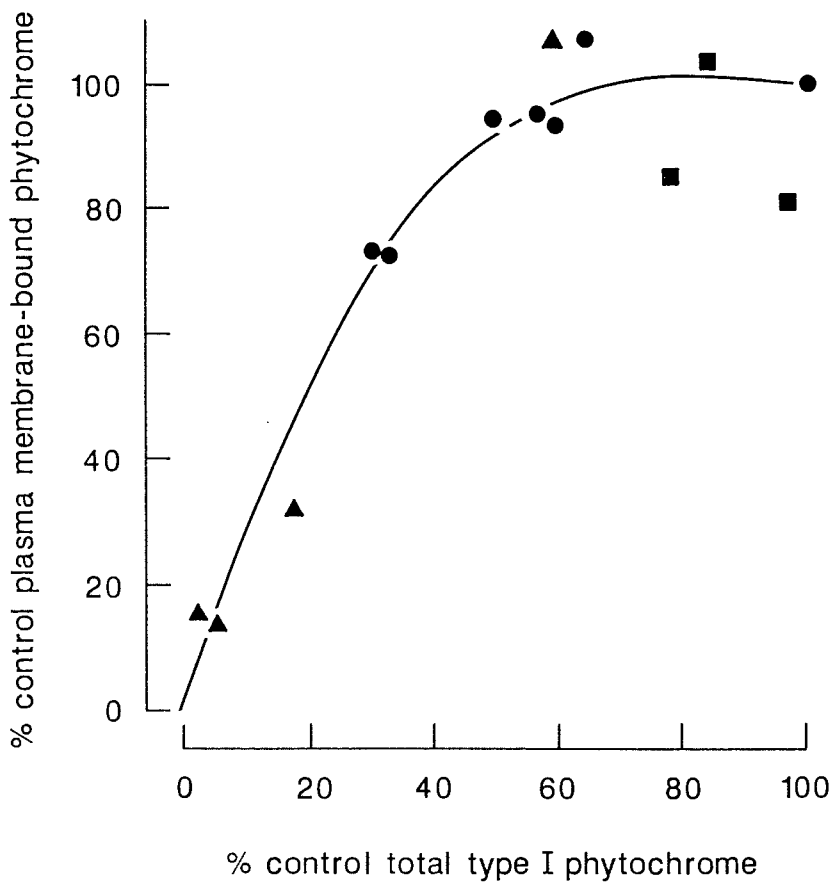


Figure 6.17: Plot of % of control plasma membrane-bound phytochrome against total type I phytochrome for each data point from the experiments shown in Figures 6.9, 6.14, 6.15 and 6.16. Points represent destruction in continuous R ( ▲ ), destruction in continuous FR ( ■ ) and inhibition of synthesis ( ● ).

in membrane-bound phytochrome after an *in vivo* red light (R) treatment (Figure 6.4). Both Watson and Smith (1982b) and Napier and Smith (1987a) reported an increase of hydrophobically-bound phytochrome in heterogeneous membrane preparations from oat seedlings after R *in vivo*. An obvious explanation of the difference in the amount of R-induced binding observed is that phytochrome associated with the microsomal membrane fraction is binding to a membrane type(s) other than the plasma membrane. However, there was also no increase in the amount of phytochrome associated with wheat leaf microsomal membranes (Figure 6.5), so the difference may be due to a difference in the species used. This explanation is supported by Marme *et al* (1976) who detected, under homogenization conditions of pH 8.0 and no $Mg^{2+}$ ions, a significant increase in membrane-bound phytochrome in *Zucchini* after an *in vivo* R treatment, but were unable to detect a similar increase in corn. Similarly with plasma membrane preparations, Widell and Sundqvist (1984) were able to demonstrate an R-induced increase in hydrophobically-bound phytochrome in oat seedlings. The difference between this result and the one reported in this chapter may also represent a difference between species, although the plasma membrane preparation used by Widell and Sundqvist (1984) was not very pure, as judged by the decrease in specific activity of the plasma membrane markers glucan synthetase II and LIAC compared with a microsomal preparation, and the increase in phytochrome binding could be the result of an association with a contaminating membrane(s).

Another possible explanation for the differences between the binding data described in this chapter and that in the literature is that two different phytochrome assays have been used and so the same phytochrome populations are not being measured. The ELISA assay used in this study measures only type I phytochrome, whereas the spectrophotometric assay normally used measures both type I and type II phytochrome. If type II phytochrome only was associating with membranes after R this would only be detected spectrophotometrically. Jordan *et al* (1984) were unable to immunologically detect an R-induced increase in phytochrome bound to oat microsomal membranes in the absence of $Mg^{2+}$ ions, but an increase could be detected spectrophotometrically. It is therefore possible that the observed increase in hydrophobically-bound phytochrome after an *in vivo* treatment is through the association of type II phytochrome.

The nature of the phytochrome-membrane association was investigated using a range of reagents which modify membrane properties. The mechanisms by which an extrinsic protein could bind to a membrane can be separated into two general categories. There could be a hydrophobic interaction between the protein and the lipids and/or integral proteins of the membrane. Alternatively, the interaction could be a hydrophilic or electrostatic one. This electrostatic interaction

may be a direct or an indirect interaction where, for example, $Mg^{2+}$ ions bridge between the protein and the membrane. These are termed salt bridges. The chaotropic agent, KI, is principally considered to break hydrophobic bonds (Maddy and Dunn 1976) and the complete removal of plasma membrane-bound phytochrome (Figure 6.8) indicates that this association is hydrophobic. Similar results were reported by Watson and Smith (1982b) and Widell and Sundqvist (1984). However, KI should also be able to break hydrophilic interactions as it can dissociate to give free $K^+$ and $I^-$ ions and a purely hydrophobic association cannot therefore be assumed.

Napier and Smith (1987a) demonstrated that 1% (v/v) Triton did not release phytochrome pelleted at low pH but completely dissociated phytochrome bound at pH 7.85. This suggests that Triton will only remove hydrophobically-bound phytochrome and therefore the results in Figure 6.7 confirm that the phytochrome association with plasma membranes from wheat leaves is largely hydrophobic. Similar results were found by Watson and Smith (1982b) but not by Widell and Sundqvist (1984) where phytochrome associated with both plasma membranes and microsomal membranes was unaffected by 1% (w/v) Triton.

There was a small increase in both phytochrome and protein binding at low Triton concentrations. The reason for this is not clear but Triton is known to bind soluble proteins *e.g.* BSA (Sukow *et al* 1980) and also to have a binding site on phytochrome (Kim *et al* 1983). It is therefore possible that Triton is binding soluble proteins to the plasma membranes. The structure of Triton X-100 is shown in Figure 6.18. The Triton molecule consists of a hydrophilic tail which could bind to soluble proteins and a hydrophobic head that could bind covalently to the membrane. Because any soluble protein trapped in sealed vesicles would be pelleted in the absence of Triton the additional bound protein must arise from soluble proteins that have partitioned into the upper phase. Phytochrome is known to have a partition ratio similar to that of plasma membranes (Tokutomi *et al* 1981) and it can be assumed that this will be the case for some other soluble proteins.

There also appeared to be a small phytochrome pool that was not removed from the membrane even with high concentrations of Triton. The critical micelle concentration (cmc) for Triton X-100 ranges from 0.39 mol $m^{-3}$ (Sukow *et al* 1980) to 0.25 mol $m^{-3}$ (Egan *et al* 1976). This is equivalent to a concentration of between 0.016% and 0.024% (w/w) and approximates to a t:p ratio of 100 in these experiments. Once the Triton concentration reaches the cmc it will form micelles. The residual levels of phytochrome and protein that are seen at these higher concentrations of Triton are probably attributable to entrapment and pelleting in these detergent vesicles.

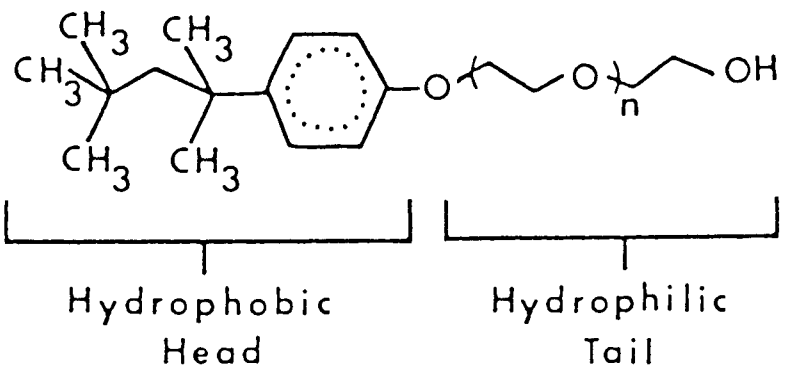


Figure 6.18: The chemical structure of Triton X where n=9 for Triton X–100. From Sukow *et al* (1980).

The association between phytochrome and plasma membranes does not appear to be entirely hydrophobic as 1 mol $m^{-3}$ KCl can remove ~37% of the bound phytochrome (Table 6.3). In previous studies this treatment was not shown to remove any membrane-bound phytochrome (Watson and Smith 1982b, Widell and Sundqvist 1984, Gallagher *et al* 1988). Relatively low concentrations (10 mol $m^{-3}$) of EDTA, $Mg^{2+}$ ions and $Ca^{2+}$ ions were also able to remove about 20, 30 and 40% of the bound phytochrome respectively (Table 6.3). $Ca^{2+}$ ions, which stabilise membranes by reorganising lipid head groups, have not previously been shown to remove membrane-bound phytochrome (Watson and Smith 1982b, Widell and Sundqvist 1984). However, the effect of $Ca^{2+}$ ions would appear to indicate a strong interaction with the lipid bilayer although some of this perceived effect may be attributable to the same mechanism which results in the loss of ~29% of the bound phytochrome with 10 mol $m^{-3}$ $Mg^{2+}$. EDTA presumable acts by breaking salt bridges. No effect of EDTA was detected by Widell and Sundqvist (1984) or Gallagher *et al* (1988), but a similar reduction in bound phytochrome was found by Watson and Smith (1982b).

These results taken together appear to demonstrate that the binding of phytochrome to plasma membranes is predominantly hydrophobic although some electrostatic interactions are also present. Whether this represents one phytochrome population bound by a combination of interactions or two separate populations bound by different mechanisms is not known. However, it would be expected that phytochrome bound hydrophobically might also form electrostatic interactions which could stabilise the overall binding. In addition to the evidence described above, there are further indications that phytochrome can interact hydrophobically with membranes. For example, Jordan (1982) showed that phospholipase $A_2$ could alter the spectral properties of phytochrome bound to microsomal membranes from mung bean hypocotyl hooks. This result suggests a close association between phytochrome and the lipid bilayer although the change in spectral properties may be mediated by a membrane-bound reaction partner. A hydrophobic association has also been indicated by Roth-Bejerano and Kendrick (1979), who looked at filipin-sensitive binding to sterol-containing membranes, and both Kim and Song (1981) and Krieg *et al* (1988) who investigated phytochrome binding to preformed liposomes. An examination of the four available phytochrome amino acid sequences shows quite clearly that phytochrome cannot be an intrinsic membrane protein. However, a region near to the C-terminus has recently been identified which shows similar properties of hydrophobicity to some known membrane-associated peptides (M.D. Partis pers. comm.).

The crucial question concerning the plasma membrane-bound phytochrome is whether this binding has any physiological significance. An

important step towards answering this question would be to demonstrate that the phytochrome is bound *in vivo*. Unfortunately, this information is not currently available. Previous attempts at determining intracellular phytochrome localization by using immunocytochemical techniques have been unable to demonstrate a plasma membrane association (Thomas *et al* 1986 for review), although such a small proportion of the total phytochrome might only appear as background noise compared with the staining of the phytochrome located in the cytoplasm. Another problem associated with immunocytochemical detection is that there may be a loss of antigenicity during the rigorous procedures required for tissue fixation (Roux 1987). One way of overcoming these problems could be to examine the isolated plasma membrane vesicles themselves.

Another approach to answering this important question is to investigate whether the pool of plasma membrane-bound phytochrome exhibits different kinetics to the total phytochrome pool. There may always be a relatively constant proportion of the total phytochrome which is associated with (or associates with on homogenization) numerous cellular structures, including the plasma membrane. If this were the case then the amount of phytochrome bound would change proportionally as the level of the total cellular phytochrome was altered. This type of association would probably be an artifactual one. If, however, the amount of bound phytochrome was independent of the amount in the total phytochrome pool it would suggest that the binding might be significant.

The results described in this chapter demonstrate that the plasma membrane-bound and total type I phytochrome pools behave differently under conditions where both the levels of $P_r$ and $P_{fr}$ are altered. The most detailed analysis was performed on the destruction of $P_{fr}$ in continuous R (Figures 6.9 and 6.11, Table 6.5). The destruction of the total type I phytochrome was first order and followed the pattern observed for the total cellular phytochrome. In contrast, the plasma membrane-bound phytochrome had a lag period of 1h before destruction started and also appeared to stabilise at ~15% of the dark level. The destruction between 1h and 3h was also first order ($t_{0.5}$ ≃50 min). Western blots of the destruction of both phytochrome pools demonstrated that the remaining phytochrome was full size. An exact interpretation of this result is difficult. It is possible that the different destruction kinetics are attributable to logistical difficulties that the cell may have in destroying phytochrome that is associated with a membrane structure. The first event in phytochrome destruction is probably an aggregation (sequestering) of $P_{fr}$ (McCurdy and Pratt 1986) and this aggregation may be prevented by membrane binding. Alternatively, if the phytochrome is destroyed while still on the membrane, there could be problems of accessability (steric hindrance) for components of the destruction machinery such as proteases

or ubiquitin, which is conjugated to phytochrome after photoconversion to $P_{fr}$ (Shanklin *et al* 1987) and is involved in the destruction of plant proteins (Vierstra 1987). It may be that some of these factors are used 'deliberately' to produce an independent phytochrome pool, although an easier mechanism by which the cell could differentially regulate two phytochrome pools would be to have a second population of 'different' phytochrome that was destroyed by a fundamentally different mechanism.

Experiments were also undertaken to examine the two phytochrome pools under conditions where the amount of $P_r$ synthesized had been manipulated (Figures 6.15 and 6.16). These results show that for a given light treatment plasma membrane-bound phytochrome is apparently synthesized preferentially to the total type I pool compared with the dark control. Again this result could be interpreted in two ways. There could be two populations that have different mechanisms by which synthesis is controlled or, alternatively, phytochrome binding sites on the plasma membrane could be filled by soluble phytochrome as a function of the concentration of this soluble pool. Figure 6.18 indicates that only when the amount of total type I phytochrome falls below ~60% of the dark control will any be lost from the plasma membrane.

The data from the experiments using a continuous far-red light (FR) treatment are not so straightforward (Figure 6.14). There does appear to be some difference between the two pools but it is not as clear as that seen during previous light treatments. The data are from two experiments and more are required if such apparently subtle differences are to be interpreted correctly. The time course of the experiment could also be lengthened to include data after 6h and 8h continuous FR as in light-grown wheat, continuous FR leads to an increase in total type I phytochrome levels (H. Carr-Smith and B. Thomas pers. comm.). Under conditions where there is a greater change in phytochrome levels, changes between the pools might be more apparent. The faster destruction of plasma membrane-bound phytochrome does, however, support the idea that this phytochrome pool is destroyed by a different mechanism to the total type I pool.

Differences in the kinetics of membrane-bound and soluble phytochrome pools have been noted before. Jordan *et al* (1984) reported different immunological responses for the two phytochrome pools. In addition, both Watson and Smith (1982c) and Napier and Smith (1987a) showed differences in the pattern of destruction for the two pools (see 6.1.2.1.) although Furuya and Manabe (1976) reported similar destruction rates for both total and microsomal membrane-bound phytochrome. The concept of two different phytochrome pools was first proposed by Hillman (1966) and Briggs and Chon (1966) to explain paradoxes where $P_{fr}$ measured did not correlate with physiological responses. However, in all reports

where two pools of phytochrome have been demonstrated (*e.g.* Brockmann and Schafer 1982), these refer to type I and type II phytochromes. Multiple gene loci for phytochrome have been identified in some species (*e.g.* oat, Hershey *et al* 1985) and the possibility that there is a separately transcribed population of type I-like phytochrome cannot be excluded. Both Hershey *et al* (1985) and Grimm *et al* (1987) have demonstrated the existence of multiple isoforms of oat phytochrome. Evidence against a distinct population of membrane-bound phytochrome comes from peptide mapping experiments where it has been demonstrated that soluble and membrane-bound phytochrome give identical patterns after proteolytic digestion in both pea (Manabe *et al* 1984) and oat (M.D. Partis pers. comm.).

The differences between the two phytochrome pools could also be explained by postulating that the phytochrome is binding to a reaction partner on the plasma membrane. If this were the case then the observed changes in bound phytochrome would reflect the regulation of the abundance of the reaction partner with the bound phytochrome being identical to and in equilibrium with the total soluble pool. The possible existence of a reaction partner (receptor) for phytochrome could be investigated using cross-linking studies. It has been demonstrated previously that phytochrome exists near a 90 kDa protein in a membrane fraction from pea (Manabe 1987) and also that phytochrome interacts with two etioplast membrane components of 18 and 24 kDa (M.D. Partis pers. comm.). The identification of a receptor would be the crucial step in the elucidation of the mechanism of action of phytochrome.

# CHAPTER SEVEN

# GENERAL CONCLUSIONS

In most respects the initial aims of the project have been achieved. The conclusions that can be drawn from the results presented in the earlier chapters are outlined here and fall conveniently into two categories. The conclusions from the characterization of wheat leaf ATPase activity are quite straightforward and are therefore dealt with only briefly. The conclusions that can be made about the action of phytochrome at the plasma membrane are not so obvious and therefore, to put these into perspective, the possible mechanism of phytochrome action at the plasma membrane is discussed in some detail.

## 7.1 Wheat leaf ATPase activity.

By purifying plasma membranes and characterizing the associated ATPase activity it has been demonstrated, for the first time, that wheat leaves possess a plasma membrane ATPase activity that is similar to that associated with the plasma membrane in most plant species. The characteristics of this ATPase are a dependence on $Mg^{2+}$ ions, stimulation by $K^+$ ions and sensitivity to vanadate. The similarity of these properties and others, such as pH optimum and substrate specificity, with those of the plasma membrane $H^+$–ATPase of higher plant cells (Sze 1985, Serrano 1989) suggests that this wheat leaf plasma membrane ATPase is probably a plasma membrane $H^+$–ATPase.

The nature of the predominant ATPase activity in wheat leaf microsomal membrane preparations (termed non–specific ATPase activity) was also characterized. Unfortunately this investigation has not shed any light on the possible origin of this activity and some important questions remain to be answered. Is this non–specific ATPase activity confined to certain plant species (*e.g.* wheat and barley) or do these species just have a relative abundance of this enzyme? If the latter is the case, why is this non–specific ATPase considerably more active in these species? What is the function of this enzyme? Perhaps the only real progress that has been made in understanding the origin of this ATPase activity is the conclusion that it is unlikely to be a transport protein based upon the limited evidence from the proton pumping experiments described in Chapter 3. Subsequent investigators of the non–specific ATPase activity present in wheat will probably be more concerned with plant metabolism than transport processes.

## 7.2. Mechanism of phytochrome action.

The high degree of purity of these plasma membranes has enabled a detailed examination of the possible photoregulation of this plasma membrane ATPase activity and of plasma membrane-bound phytochrome. The experiments detailed in Chapter 5 demonstrated that there was no effect of R on plasma membrane ATPase activity measured *in vitro*, whether the light treatment was given *in vivo* or *in vitro*. The lack of an *in vitro* effect may represent the lability of a membrane-associated transduction chain or the requirement of components of this chain which are absent in an *in vitro* system. Work on purified organelles such as mitochondria (Serlin *et al* 1984) and nuclei (Chen and Roux 1986) has indicated that the light-regulation of an ATPase and NTPase respectively, which are under phytochrome control, might be mediated *via* $Ca^{2+}$ ion fluxes. This type of regulatory mechanism would not be detected using unsealed membrane vesicles as in the ATPase assay used, but might be seen (as a fall in quinacrine fluorescence quenching on the addition of $Ca^{2+}$ ions) using sealed, right-side out, plasma membrane vesicles that had been loaded during homogenization with ATP and $Mg^{2+}$ ions in the presence of EGTA.

As discussed earlier (1.3.4.2.) $Ca^{2+}$ ions appear to play an important role in mediating many phytochrome responses and have been implicated in plasma membrane-localized phytochrome responses. Das and Sopory (1985) demonstrated an enhanced, transient uptake of $^{45}Ca^{2+}$ after R and both Bossen *et al* (1988) and Tretyn *et al* (1990) have reported considerable circumstantial evidence for $Ca^{2+}$ influx at the plasma membrane mediating the protoplast swelling response. However, light-regulated $Ca^{2+}$ influxes into isolated leaf protoplasts have not always been detected (Akerman *et al* 1983). The existence of $Ca^{2+}$ channels at the plasma membrane has been demonstrated using [$^3$H]nitrendipine (Hetherington and Trewavas 1984) and [$^3$H]verapamil (Andrejauskas *et al* 1985). Assuming that the primary action of phytochrome is to open these $Ca^{2+}$ channels at the plasma membrane (or other internal stores such as mitochondria or endoplasmic reticulum, see 1.3.4.2.), a simple control mechanism is suggested whereby changes in intracellular $Ca^{2+}$ regulate the activities of protein kinases or phosphatases which in turn control the activity of various cell components.

The regulatory role of phosphorylation/dephosphorylation cycles in plants is now well established (Ranjeva and Boudet 1987, Budde and Chollet 1988), and there have been recent reports of both $Ca^{2+}$-dependent (Klucis and Polya 1988, Ladror and Zielinski 1989) and $Ca^{2+}$-calmodulin regulated (Blowers and Trewavas 1989) protein kinases on the plant plasma membrane. Interestingly, calmodulin has been reported to be associated in small quantities with pea plasma membranes

(Collinge and Trewavas 1989). This association has parallels with the results described here for phytochrome binding as calmodulin is normally found exclusively in the cytoplasm. Protein phosphatase activity has also been detected in plasma membrane preparations (Ladror and Zielinski 1989). A report that phytochrome is a protein kinase (Wong *et al* 1986) may be premature (Grimm *et al* 1989, Kim *et al* 1989), but phytochrome undoubtedly has a very close, physical association with a polycation–stimulated protein kinase and this may also play a role in signal transduction (Wong *et al* 1989).

The targets for the protein kinases described above might include the plasma membrane ATPase (see 5.4.), other ion channels (as has been found in animal systems, Levitan 1985) such as the $Ca^{2+}$ channel of *Nitellopsis* (Shiina *et al* 1988), numerous soluble proteins (Ranjeva and Boudet 1987) or even phytochrome itself (Wong *et al* 1986). There are also a number of reports of phytochrome–mediated changes in the level of protein phosphorylation. Datta *et al* (1985) reported a phytochrome–mediated, *in vitro* stimulation of protein phosphorylation in pea nuclei and Park and Chae (1989) reported that R stimulated the $Ca^{2+}$–dependent phosphorylation of 15 different proteins in oat protoplasts. They were also able to show that the phosphorylation of at least two of these proteins was under phytochrome control *in vivo*. Recently, Otto and Schafer (1988) demonstrated *in vivo* phytochrome–mediated changes in the phosphorylation of three proteins from oat coleoptile tips. Phosphorylation was seen to decrease in two of these proteins and increased in the third. The half–life of 2s at 0°C for this response (the most rapid $P_{fr}$–mediated response yet recorded) suggests that it is a very early event of phytochrome action, possibly the first.

The simple transduction scheme outlined above would require a direct interaction of phytochrome with the $Ca^{2+}$ channels involved. This scenario cannot be ruled out but the lag periods of between 4.5 and 90s that have variously been reported for phytochrome–mediated changes in membrane potential (Quail 1983) are consistent with the presence of an intermediate in this reaction as the rate–limiting step is not the rate of photoconversion, the movement of phytochrome to the site of action, nor the interaction itself (Quail 1983 for discussion).

In animal systems, complex signal transduction chains have been identified. These can be categorised into two major pathways. One involves the regulation of cAMP–dependent protein kinases through the formation of the secondary messenger cAMP by adenylate cyclase. The other involves the release of intracellular $Ca^{2+}$ and the activation of protein kinase C, both of which are regulated by the breakdown of inositol phospholipids (Berridge 1987). Evidence for the role of cAMP in plants is limited (Brown and Newton 1981), but recently

many of the components required for a inositol phospholipid–mediated signal transduction pathway have been identified in plant cells (Morse *et al* 1989). Some of these components have been localized to the plasma membrane, for example, phosphatidylinositol kinase, phosphatidylinositol phosphate kinase (Sommarin and Sandelius 1988) and phospholipase C activity (Einspahr *et al* 1989). In addition, inositol phospholipid turnover has been shown to be regulated by light (Morse *et al* 1987, 1989) and protein kinase C has been shown to be involved in the regulation of $Ca^{2+}$ channels in the plasma membrane of *Nitella syncarpa* (Zherelova 1989).

These components of an, as yet unidentified, signal transduction chain may well constitute the intermediate step between $P_{fr}$ formation and the regulation of, for example, a $Ca^{2+}$ channel. Another interesting category of molecules that may also be involved in the mediation of the phytochrome signal are the G–proteins. In animal systems these proteins transfer information within the plane of the membrane between receptors and effectors such as adenylate cyclase and phospholipase C (Gilman 1987). They have recently been identified in plasma membranes of several higher plants (Blum *et al* 1988) and have also been shown to mediate $Ca^{2+}$ release from a plant microsomal fraction (Allan *et al* 1989). Recent work on the wheat leaf protoplast swelling response has shown that R–induced swelling can be prevented by the G–protein inhibitor GDP–$\beta$–S and is stimulated by the activator GTP–$\gamma$–S. Swelling was also prevented by the phospholipase C inhibitors, neomycin and $Li^{+}$, and by the protein kinase C inhibitor, $H_7$ (Bossen 1990).

It is therefore beginning to appear that the plasma membrane is an important signal transducing membrane in plant cells as it is in animal cells. By analogy with animal systems an obvious requirement for phytochrome action at the plasma membrane would be the presence of a reaction partner or receptor. Progress has already been made in the molecular characterization of plasma membrane receptors for both the fungal phytotoxin, fusicoccin (DeMichelis *et al* 1989, DeBoer *et al* 1989, Feyerabend and Weiler 1989) and the plant growth regulator, auxin (Barbier–Brygoo *et al* 1989). Identification and charaterization of a plasma membrane–bound phytochrome receptor would be the most important step forward for understanding the mechanism of phytochrome action at this membrane site. Identification could be achieved initially by using cross–linking agents or by photoaffinity labeling. Phytochrome might be expected to have numerous interactions in a crude membrane preparation, but by using a purified plasma membrane preparation, such as the one described here, detection of this receptor, should it exist, would be simplified.

What can the results obtained in this investigation say about the

mechanism of action of phytochrome at the plasma membrane? Undoubtedly the demonstration that a differently regulated phytochrome pool exists on the plasma membrane supports the view that phytochrome acts directly at the plasma membrane and not by an indirect route such as the release of gibberellic acid from etioplasts (Cooke and Kendrick 1976). Further strong but circumstantial evidence for this proposal could be obtained by demonstrating the presence of phytochrome on the plasma membrane *in vivo*. The proposal that the wheat leaf protoplast swelling response is the result of a change in plasma membrane $H^+$–ATPase activity (Blakeley *et al* 1983) cannot be discounted from these results. There is, however, no direct interaction between phytochrome and the ATPase as, in the experiments where phytochrome (and an associated protein kinase?) was added to the membrane vesicles, a change in ATPase activity was not seen. Furthermore, the absence of a change in ATPase activity after an *in vivo* light treatment argues against a (semi)permanent alteration of the enzyme as might be expected with, for example, a change in phosphorylation state.

In view of the evidence for the role of $Ca^{2+}$ ions in phytochrome–mediated responses, it is not surprising that phytochrome has no direct effect on the plasma membrane $H^+$–ATPase. Previous reports on the light regulation of ATPases in plant microsomal and plasma membrane preparations (see 5.1) have not provided any clear evidence to suggest otherwise. However, the effect of $Ca^{2+}$ ions on wheat leaf plasma membrane ATPase activity reported in this study (Figure 4.11) demonstrates that a change in intracellular $Ca^{2+}$ ion concentration would probably effect $H^+$–ATPase activity *in vivo*, albeit transiently. It remains to be seen during this decade whether phytochrome action at the plasma membrane is mediated by a signal transduction route similar to those so ubiquitous in animal cells. The recent demonstration that inositol phospholipids activate the plasma membrane ATPase of both sunflower hypocotyls and carrot cells (Memon *et al* 1989) suggests that the signal transduction pathway of phytochrome and the regulatory pathway of the plasma membrane ATPase may yet prove to be inseparably intertwined.

## REFERENCES


AKERMAN, K.E.O., M.O. PROUDLOVE and A.L. MOORE (1983) Evidence for a $Ca^{2+}$ gradient across the plasma membrane of wheat protoplasts. *Biochem. Biophys. Res. Comm.* 113, 171–177.

ALBERTSSON, P–A. (1971) Partition of Cell Particles and Macromolecules. Wiley, New York.

ALBERTSSON, P–A., B. ANDERSSON, C. LARSSON and H–E. AKERLUND (1982) Phase partition – a method for purification and analysis of cell organelles and membrane vesicles. In: GLICK, D. (ed) Methods of Biochemical Analysis vol. 28, *pp.* 115–150. Wiley, New York.

ALESHINA, N.V., K.K. BORUAKH, N.V. ASTAKHOVA and T.I. TRUNOVA (1988) Isolation and properties of the plasma membrane from etiolated winter wheat seedlings. *Sov. Plant Physiol.* 35, 797–804.

ALLAN, E., A. DAWSON, B. DROBAK and K. ROBERTS (1989) GTP causes calcium release from a plant microsomal fraction. *Cell. Signal.* 1, 23–29.

ANDERSEN, J.P., J.V. MOLLER and P.L. JORGENSEN (1982) The functional unit of sarcoplasmic reticulum $Ca^{2+}$–ATPase. *J. Biol. Chem.* 257, 8300–8307.

ANDREJAUSKAS, E., R. HERTEL and D. MARME (1985) Specific binding of the calcium antagonist [$^3$H]verapamil to membrane fractions from plants. *J. Biol. Chem.* 260, 5411–5414.

ANTHON, G.E. and R.M. SPANSWICK (1986) Purification and properties of the $H^+$–translocating ATPase from the plasma membrane of tomato roots. *Plant Physiol.* 81, 1080–1085.

ARNON, D.I. (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24, 1–15.

ASSMANN, S.M., L. SIMONCINI and J.I. SCHROEDER (1985) Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba*. *Nature* 318, 285–287.

BALKE, N.E. and T.K. HODGES (1975) Plasma membrane adenosine triphosphatase of oat roots. *Plant Physiol.* 55, 83–86.

BALKE, N.E., H. SZE, R.T. LEONARD and T.K. HODGES (1974) Cation sensitivity of the plasma membrane ATPase of oat roots. In: DAINTY, J. and U. ZIMMERMAN (eds) Membrane Transport in Plants and Plant Organelles. *pp.* 301–306. Springer–Verlag, Berlin.

BALL, J.H. (1988) Properties of membrane associated ATPases and their relation to cell growth in the hypocotyl of *Cucumis sativus* L. PhD Thesis, University of Southampton.

BALL, J.H., L. WILLIAMS and J.L. HALL (1987) Effect of SW26 and erythrosin

B on ATPase activity and related processes in *Ricinus* cotyledons and cucumber hypocotyls. *Plant Sci.* 52, 1–5.

BARBIER-BRYGOO, H., G. EPHRITIKHINE, D. KLAMBT, M. GHISLAIN and J. GUERN (1989) Functional evidence for an auxin receptor at the plasmalemma of tabacco mesophyll protoplasts. *Proc. Natl. Acad. Sci. USA* 86, 891–895.

BENNETT, A.B., S.A. O'NEILL, M. EILMANN and R.M. SPANSWICK (1985) Proton–ATPase activity from storage tissue of *Beta vulgaris* : 3. Modulation of ATPase activity by reaction substrates and products. *Plant Physiol.* 78, 495–499.

BENNETT, A.B. and R.M. SPANSWICK (1983) Optical measurements of ΔpH and $\Delta\psi$ in corn root membrane vesicles: kinetic analysis of $Cl^-$ effects on a proton–translocating ATPase. *J. Memb. Biol.* 71, 95–107.

BERCZI, A. (1988) Influence of EDTA on the ATPase activities of plasma membranes from winter wheat roots. *Acta Biochim. Biophys. Hung.* 23, 175–179.

BERCZI, A. and I.M. MOLLER (1986) Comparison of the properties of plasmalemma vesicles purified from wheat roots by phase partitioning and by discontinuous sucrose gradient centrifugation. *Physiol. Plant.* 68, 59–66.

BERCZI, A. and I.M. MOLLER (1987) $Mg^{2+}$–ATPase activity in wheat root plasma membrane vesicles: time–dependence and effect of sucrose and detergents. *Physiol. Plant.* 70, 583–589.

BERRIDGE, M.J. (1987) Inositol triphosphate and diacylglycerol: two interacting second messengers. *Ann. Rev. Biochem.* 56, 159–193.

BIDWAI, A.P. and J.Y. TAKEMOTO (1987) Bacterial phytotoxin, syringomycin, induces a protein kinase–mediated phosphorylation of red beet plasma–membrane polypeptides. *Proc. Natl. Acad. Sci. USA* 84, 6755–6759.

BLAKELEY, S.D. (1983) The regulation of protoplast swelling by light. D. Phil. Thesis, University of Sussex.

BLAKELEY, S.D., B. THOMAS and J.L. HALL (1987) The role of microsomal ATPase activity in light–iduced protoplast swelling in wheat. *J. Plant Physiol.* 127, 187–191.

BLAKELEY, S.D., B. THOMAS, J.L. HALL and D. VINCE–PRUE (1983) Regulation of swelling of etiolated–wheat–leaf protoplasts by phytochrome and gibberellic acid. *Planta* 158, 416–421.

BLEIN, J–P., X. De CHERADE, M. BERGON, J–P. CALMON and R. SCALLA (1986) Inhibition of adenosine triphosphatase activity from a plasma membrane fraction from *Acer pseudoplatanus* cells by 2,2,2–trichloroethyl 3,4–dichlorocarbanilate. *Plant Physiol.* 80, 782–785.

BLEIN, J-P., J. MARTINEZ, M. BERGON, J-P. CALMON and R. SCALLA (1987) Inhibition of adenosine triphosphatase activity from a plasma membrane fraction from *Acer pseudoplatanus* cells by carbanilate derivatives. *Plant Physiol.* 83, 469–471.

BLOWERS, D.P. and A.J. TREWAVAS (1989) Rapid cycling of autophosphorylation of a $Ca^{2+}$–calmodulin regulated plasma membrane located protein kinase from pea. *Plant Physiol.* 90, 1279–1285.

BLUM, W., K-D. HINSCH, G. SCHULTZ and E.W. WEILER (1988) Identification of GTP-binding proteins in the plasma membrane of higher plants. *Biochem. Biophys. Res. Comm.* 156, 954–959.

BORST-PAUWELS, G.W.F.H. and P.H.J. PETERS (1981) Factors affecting the inhibition of yeast plasma membrane ATPase by vanadate. *Biochim. Biophys. Acta* 642, 173–181.

BOSS, W.F. and A.W. RUESINK (1979) Isolation and characterization of concanavalin A-labeled plasma membranes of carrot protoplasts. *Plant Physiol.* 64, 1005–1011.

BOSSEN, M.E. (1990) Plant protoplasts as a model system to study phytochrome-regulated changes in the plasma membrane. PhD Thesis, Agricultural University, Wageningen, The Netherlands.

BOSSEN, M.E., H.H.A. DASSEN, R.E. KENDRICK and W.J. VREDENBERG (1988) The role of calcium ions in phytochrome-controlled swelling of etiolated wheat (*Triticum aestivum* L.) protoplasts. *Planta* 174, 94–100.

BOWMAN, B.J., C.J. BERENSKI and C.Y. JUNG (1985) Size of the plasma membrane $H^+$–ATPase from *Neurospora crassa* determined by radiation inactivation and comparison with the sarcoplasmic reticulum $Ca^{2+}$–ATPase from skeletal muscle. *J. Biol. Chem.* 260, 8726–8730.

BOWMAN, B.J. and E.J. BOWMAN (1986) $H^+$–ATPases from mitochondria, plasma membranes, and vacuoles of fungal cells. *J. Memb. Biol.* 94, 83–97.

BOWMAN, B.J. and C.W. SLAYMAN (1979) The effects of vanadate on the plasma membrane ATPase of *Neurospora crassa*. *J. Biol. Chem.* 254, 2928–2934.

BOWMAN, E.J., S. MANDALA, L. TAIZ and B.J. BOWMAN (1986) Structural studies of the vacuolar membrane ATPase from *Neurospora crassa* and comparison with the tonoplast membrane ATPase from *Zea mays*. *Proc. Natl. Acad. Sci. USA* 83, 48–52.

BRADFORD, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.

BRIARS, S.A. and D.E. EVANS (1989) The calmodulin-stimulated ATPase of

maize coleoptiles forms a phosphorylated intermediate. *Biochem. Biophys. Res. Comm.* 159, 185–191.

BRIARS, S.A., F. KESSLER and D.E. EVANS (1988) The calmodulin–stimulated ATPase of maize coleoptiles is a 140 000–$M_r$ polypeptide. *Planta* 176, 283–285.

BRIGGS W.R. and H.P. CHON (1966) The physiological versus the spectrophotometric status of phytochrome in corn coleoptiles. *Plant Physiol.* 41, 1159–1166.

BRISKIN, D.P. (1986) Plasma membrane $H^+$–translocating ATPase: role in potassium ion transport? *Physiol. Plant.* 68, 159–163.

BRISKIN, D.P. and R.T. LEONARD (1982) Partial characterization of a phosphorylated intermediate associated with the plasma membrane ATPase of corn root. *Proc. Natl. Acad. Sci. USA* 79, 6922–6926.

BRISKIN, D.P. and R.J. POOLE (1983) Plasma membrane ATPase of red beet forms a phosphorylated intermediate. *Plant Physiol.* 71, 507–512.

BRISKIN, D.P. and I. REYNOLDS–NIESMAN (1989) Change in target molecular size of the red beet plasma membrane ATPase during solubilization and reconstitution. *Plant Physiol.* 90, 394–397.

BRISKIN, D.P. and W.R. THORNLEY (1985) Plasma membrane ATPase from sugarbeet. *Phytochem.* 24, 2797–2802.

BRISKIN, D.P., W.R. THORNLEY and J.L. ROTI–ROTI (1985) Target molecular size of the red beet plasma membrane ATPase. *Plant Physiol.* 78, 642–644.

BROCKMANN, J. and E. SCHAFER (1982) Analysis of $P_{fr}$ destruction in *Amaranthus caudatus* L.–evidence for two pools of phytochrome. *Photochem. Photobiol.* 35, 555–558.

BROWN, E.G. and R.P. NEWTON (1981) Cyclic AMP and higher plants. *Phytochem.* 20, 2453–2463.

BROWNLEE, C. and R.E. KENDRICK (1979) Ion fluxes and phytochrome protons in mung bean hypocotyl segments. *Plant Physiol.* 64, 211–213.

BUDDE, R.J.A. and R. CHOLLET (1988) Regulation of enzyme activity in plants by reversible phosphorylation. *Physiol. Plant.* 72, 435–439.

BUTCHER, R.D. and D.E. EVANS (1987a) Calcium transport by pea root membranes. I. Purification of membranes and characteristics of uptake. *Planta* 172, 265–272.

BUTCHER, R.D. and D.E. EVANS (1987b) Calcium transport by pea root membranes. II. Effects of calmodulin and inhibitors. *Planta* 172, 273–279.

BUTLER, W.L. and H.C. LANE (1965) Dark transformations of phytochrome *in vivo* II. *Plant Physiol.* 40, 13–17.

BUTLER, W.L., K.H. NORRIS, H.W. SIEGELMAN and S.B. HENDRICKS (1959)

Detection, assay, and preliminary purification of the pigment controlling photoresponsive development of plants. *Proc. Natl. Acad. Sci. USA* 45, 1703–1708.

CALDWELL, C.R. and A. HAUG (1980) Kinetic characterization of barley root plasma membrane–bound $Ca^{2+}$– and $Mg^{2+}$–dependent adenosine triphosphatase activities. *Physiol. Plant.* 50, 183–193.

CALDWELL, C.R. and A. HAUG (1982) Divalent cation inhibition of barley root plasma membrane–bound $Ca^{2+}$–ATPase activity and its reversal by monovalent cations. *Physiol. Plant.* 54, 112–118.

CANTLEY, L.C. (1981) Structure and mechanism of the (Na,K)–ATPase. *Curr. Top. Bioenerg.* 11, 201–237.

CANTLEY, L.C., L.G. CANTLEY and L. JOSEPHSON (1978) A characterization of vanadate interactions with the (Na,K)–ATPase. *J. Biol. Chem.* 253, 7361–7368.

CANUT, H., A. BRIGHTMAN, A.M. BOUDET and D.J. MORRE (1988) Plasma membranes of opposite sidedness from soybean hypocotyls by preparative free–flow electrophoresis. *Plant Physiol.* 86, 631–637.

CARTER, S.G. and C.L. TIPTON (1987) A high–affinity ($Ca^{2+}$+$Mg^{2+}$)–ATPase from *Zea mays* microsomes. *Phytochem.* 26, 2191–2196.

CEDEL, T.E. and S.J. ROUX (1980) Further characterization of the *in vitro* binding of phytochrome to a membrane fraction enriched for mitochondria. *Plant Physiol.* 66, 696–703.

CHADWICK, C.C., E. GOORMAGHTIGH and G.A. SCARBOROUGH (1987) A hexameric form of the *Neurospora crassa* plasma membrane $H^+$–ATPase. *Arch. Biochem. Biophys.* 252, 348–356.

CHANSON, A., E. McNAUGHTON and L. TAIZ (1984) Evidence for a KCl–stimulated, $Mg^{2+}$–ATPase on the Golgi of corn coleoptiles. *Plant Physiol.* 76, 498–507.

CHEN, Y–R. and S.J. ROUX (1986) Characterization of nucleoside triphosphatase activity in isolated pea nuclei and its photoreversible regulation by light. *Plant Physiol.* 81, 609–613.

CHUNG, C.H., S–U. LIM, P–S. SONG, J.D. BERLIN and J.R. GOODIN (1988) Swelling of etiolated oat protoplasts induced by phytochrome, cAMP and gibberellic acid: a kinetic study. *Plant Cell Physiol.* 29, 855–860.

COCUCCI, M.C. (1986) Inhibition of plasma membrane and tonoplast ATPase by erythrosin B. *Plant Sci.* 47, 21–27.

COCUCCI, M.C., A. BALLARIN–DENTI and M.T. MARRE (1980) Effects of orthovanadate on $H^+$ secretion, $K^+$ uptake, electric potential difference and membrane ATPase activities of higher plant tissues. *Plant Sci. Lett.* 17,

391-400.

COCUCCI, M.C. and E. MARRE (1984) Lysophosphatidylcholine-activated, vanadate-inhibited $Mg^{2+}$-ATPase from radish microsomes. *Biochim. Biophys. Acta* 771, 42-52.

COLBERT, J.T. (1988) Molecular biology of phytochrome. *Plant Cell Environ.* 11, 305-318.

COLBERT, J.T., H.P. HERSHEY and P.H. QUAIL (1985) Phytochrome regulation of phytochrome mRNA abundance. *Plant Mol. Biol.* 5, 91-101.

COLLINGE, M. and A.J. TREWAVAS (1989) The location of calmodulin in the pea plasma membrane. *J. Biol. Chem.* 264, 8865-8872.

COOKE R.J. and R.E. KENDRICK (1976) Phytochrome controlled gibberellin metabolism in etioplast envelopes. *Planta* 131, 303-307.

CORDONNIER, M.M. (1989) Monoclonal antibodies: Molecular probes for the study of phytochrome. *Photochem. Photobiol.* 49, 821-831.

CORDONNIER, M.M., H. GREPPIN and L.H. PRATT (1985) Monoclonal antibodies with differing affinities to the red-absorbing and far-red-absorbing forms of phytochrome. *Biochem.* 24, 3246-3253.

CORDONNIER, M.M., H. GREPPIN and L.H. PRATT (1986) Phytochrome from green *Avena* shoots characterized with a monoclonal antibody to phytochrome from etiolated *Pisum* shoots. *Biochem.* 25, 7657-7666.

DAS, R. and S.K. SOPORY (1985) Evidence of regulation of calcium uptake by phytochrome in maize protoplasts. *Biochem. Biophys. Res. Comm.* 128, 1455-1460.

DATTA, N., Y-R. CHEN and S.J. ROUX (1985) Phytochrome and calcium stimulation of protein phosphorylation in isolated pea nuclei. *Biochem Biophys. Res. Comm.* 128, 1403-1408.

De BOER, A.H., B.A. WATSON and R.E. CLELAND (1989) Purification and identification of the fusicoccin binding protein from oat root plasma membrane. *Plant Physiol.* 89, 250-259.

De GREEF, J.A., R. CAUBERGS, J.P. VERBELEN and E. MOEREELS (1976) Phytochrome-mediated inter-organ dependence and rapid transmission of the light stimulus. In: SMITH, H. (ed) Light and Plant Development. *pp.* 295-316. Butterworths, London.

De MICHELIS, M.I., M.C. PUGLIARELLO and F. RASI-CALDOGNO (1989) Fusicoccin binding to its plasma membrane receptor and the activation of the plasma membrane $H^+$-ATPase. *Plant Physiol.* 90, 133-139.

De MICHELIS, M.I. and R.M. SPANSWICK (1986) $H^+$-pumping driven by the vanadate-sensitive ATPase in membrane vesicles from corn roots. (1986) *Plant Physiol.* 81, 542-547.

De VRIES, S.C., J. SPRINGER and J.G.H. WESSELS (1982) Diversity of abundant mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. *Planta* 156, 129–135.

DIAZ De LEON, J.L. and R.G. WYN JONES (1985) $Ca^{2+}$–ATPases and their occurrence in vacuoles of higher plants. In: MARIN, B.P. (ed) Biochemistry and Function of Vacuolar ATPase in Fungi and Plants. *pp.* 57–66. Springer–Verlag, Berlin.

DOOSKIN, R.H. and A.L. MANCINELLI (1968) Phytochrome decay and coleoptile elongation in *Avena* following various light treatments. *Bull. Torrey Bot. Club.* 95, 474–487.

DUFOUR, J–P., M. BOUTRY and A. GOFFEAU (1980) Plasma membrane ATPase of yeast. *J. Biol. Chem.* 255, 5735–5741.

DUFOUR, J–P. and A. GOFFEAU (1980) Molecular and kinetic properties of the purified plasma membrane ATPase of the yeast *Schizosaccharomyces pombe*. *Eur. J. Biochem.* 105, 145–154.

DUPONT, F.M., D.L. GIORGI and R.M. SPANSWICK (1982) Characterization of a proton–translocating ATPase in microsomal vesicles from corn roots. *Plant Physiol.* 70, 1694–1699.

DUPONT, F.M. and W.J. HURKMAN (1985) Separation of the $Mg^{2+}$–ATPases from the $Ca^{2+}$–phosphatase activity of microsomal membranes prepared from barley roots. *Plant Physiol.* 77, 857–862.

EGAN, R.W., M.A. JONES and A.L. LEHNINGER (1976) Hydrophile–lipophile balance and critical micelle concentration as key factors influencing surfactant disruption of mitochondrial membranes. *J. Biol. Chem.* 251, 4442–4447.

EINSPAHR, K.J., T.C. PEELER and G.A. THOMPSON Jr. (1989) Phosphatidylinositol 4,5–bisphosphate phospholipase C and phosphomonoesterase in *Dunaliella salina* membranes. *Plant Physiol.* 90, 1115–1120.

ELICH, T.D. and J.C. LAGARIAS (1987) Phytochrome chromophore biosynthesis. *Plant Physiol.* 84, 304–310.

ENZYME NOMENCLATURE COMMITTEE, UNION INTERNATIONAL OF BIOCHEMISTRY (1984) Enzyme Nomenclature. Academic Press, New York.

EPHRITIKHINE, G., A. LAMANT and R. HELLER (1980) Influence of sucrose on the charaterization of higher plant membranes by $\beta$–glucan synthetase activity and its relation to osmotic pressure. *Plant Sci. Lett.* 19, 55–64.

EVANS, A. and H. SMITH (1976) Spectrophotometric evidence for the presence of phytochrome in the envelope membranes of barley etioplasts. *Nature* 259, 323–325.

FARLEY, R.A. and L.D. FALLER (1985) The amino acid sequence of an active site peptide from the H,K–ATPase of gastric mucosa. *J. Biol. Chem.* 260,

3899-3901.

FELLE, H. (1982) Effects of fusicoccin upon membrane potential. Resistance and current-voltage characteristics in root hairs of *Sinapsis alba*. *Plant Sci. Lett.* 25, 219-225.

FELLE, H. (1988) Short-term pH regulation in plants. *Physiol. Plant.* 74, 583-591.

FEYERABEND, M. and E.W. WEILER (1989) Photoaffinity labeling and partial purification of the putative plant receptor for the fungal wilt-inducing toxin, fusicoccin. *Planta* 178, 282-290.

FURUYA, M. (ed) (1987) Phytochrome and Photoregulation in Plants. Academic Press, London.

FURUYA, M., J.H. FREER, A. ELLIS and K.T. YAMAMOTO (1981) Electrostatic binding of proteins and phytochrome to differently charged liposomes. *Plant Cell Physiol.* 22, 135-144.

FURUYA, M. and K. MANABE (1976) Phytochrome in mitochondrial and microsomal fractions isolated from etiolated pea shoots. In: SMITH, H. (ed) Light and Plant Development. *pp.* 143-155. Butterworths, London.

GALLAGHER, S., T.W. SHORT, P.M. RAY, L.H. PRATT and W.R. BRIGGS (1988) Light-mediated changes in two proteins found associated with plasma membrane fractions from pea stem sections. *Proc. Natl. Acad. Sci. USA* 85, 8003-8007.

GALLAGHER, S.R. and R.T. LEONARD (1982) Effect of vanadate. molybdate, and azide on membrane-associated ATPase and soluble phosphatase activities of corn roots. *Plant Physiol.* 70, 1335-1340.

GIANNINI, J.L. and D.P. BRISKIN (1987) Proton transport in plasma membranes and tonoplast vesicles from red beet (*Beta vulgaris* L.) storage tissue. *Plant Physiol.* 84, 613-618.

GIANNINI, J.L., L.H. GILDENSOPH and D.P. BRISKIN (1987) Selective production of sealed plasma membrane vesicles from red beet (*Beta vulgaris* L.) storage tissue. *Arch. Biochem. Biophys.* 254, 621-630.

GIBRAT, R., J-P. GROUZIS, J. RIGAUD, N. GALTIER and C. GRIGNON (1989) Electrostatic analysis of effects of ions on the inhibition of corn root plasma membrane $Mg^{2+}$-ATPase by the bivalent orthovanadate. *Biochim. Biophys. Acta* 979, 46-52.

GILMAN, A.G. (1987) G proteins: transducers of receptor-generated signals. *Ann. Rev. Biochem.* 56, 615-649.

GIMMLER, H. (1988) Erythrosine B - a specific inhibitor of plasmalemma ATPase in intact microalgae? *J. Plant Physiol.* 132, 545-551.

GOORMAGHTIGH, E., C. CHADWICK and G.A. SCARBOROUGH (1986) Monomers of the *Neurospora* plasma membrane $H^+$-ATPase catalyze efficient

proton translocation. *J. Biol. Chem.* 261, 7466–7471.

GOTTMANN, K. and E. SCHAFER (1983) Analysis of phytochrome kinetics in light-grown *Avena sativa* L. seedlings. *Planta* 157, 392–400.

GRAF, P. and E.W. WEILER (1989) ATP-driven $Ca^{2+}$ transport in sealed plasma membrane vesicles prepared by aqueous two-phase partitioning from leaves of *Commelina communis*. *Physiol. Plant.* 75, 469–478.

GREEN, P.J., S.A. KAY and N-H. CHUA (1987) Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the rbc-3A gene. *EMBO J.* 6, 2543–2549.

GRIFFITH, C.J., P.A. REA, E. BLUMWALD and R.J. POOLE (1986) Mechanism of stimulation and inhibition of tonoplast $H^+$-ATPase of *Beta vulgaris* by chloride and nitrate. *Plant Physiol.* 81, 120–125.

GRIMM, R., D. GAST and W. RUDIGER (1989) Characterization of a protein-kinase activity associated with phytochrome from etiolated oat (*Avena sativa* L.) seedlings. *Planta* 178, 199–206.

GRIMM, R., F. LOTTSPEICH and W. RUDIGER (1987) Heterogeneity of the amino acid sequence of phytochrome from etiolated oat seedlings. *FEBS Lett.* 225, 215–217.

GROSS, J. and D. MARME (1978) ATP-dependent $Ca^{2+}$ uptake into plant membrane vesicles. *Proc. Natl. Acad. Sci. USA* 75, 1232–1236.

GROSS, J., M. SEYFRIED, L. FUKSHANSKY and E. SCHAFER (1984) *In vivo* spectrophotometry. In: SMITH, H. and M.G. HOLMES (eds) Techniques in Photomorphogenesis. *pp.* 131–157. Academic Press, London.

HALL, J.L. (1971) Further properties of adenosine triphosphatase and $\beta$-glycerophosphatase from barley roots. *J. Exp. Bot.* 22, 800–808.

HALL, J.L. (1983) Plasma membranes. In: HALL, J.L. and A.L. MOORE (eds) Isolation of Membranes and Organelles from Plant Cells. *pp.* 55–81. Academic Press, London.

HALL, J.L. and A. DYMOTT (1980) Localization and properties of adenosine triphosphatase in wheat coleoptiles. In: SPANSWICK, R.M., W.J. LUCAS and J. DAINTY (eds) Plant Membrane Transport: Current Conceptual Issues. *pp.* 517–518. Elsevier/North-Holland Biomedical Press, Amsterdam.

HALL, J.L., A.J. KINNEY, A. DYMOTT, J.R. THORPE and D.A. BRUMMELL (1982) Localization and properties of ATPase activity in pea stems and wheat coleoptiles. *Histochem. J.* 14, 323–331.

HALL, J.L. and A.L. MOORE (eds) (1983) Isolation of Membranes and Organelles from Plant Cells. Academic Press, London.

HALLBERG, M. and C. LARSSON (1981) Compartmentation and export of $^{14}CO_2$ fixation products in mesophyll protoplasts from the $C_4$-plant *Digitaria*

*sanguinalis*. *Arch. Biochem. Biophys.* 208, 121–130.

HARPER, J.F., T.K. SUROWY and M.R. SUSSMAN (1989) Molecular cloning and sequence of cDNA encoding the plasma membrane proton pump ($H^+$–ATPase) of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 86, 1234–1238.

HART, J.W. (1988) Light and Plant Growth. Unwin Hyman Ltd., London.

HENDRICKS, S.B. and H.A. BORTHWICK (1967) The function of phytochrome in regulation of plant growth. *Proc. Natl. Acad. Sci. USA* 58, 2125–2130.

HENDRICKS, S.B. and W.J. VAN DER WOUDE (1983) How phytochrome acts – perspectives on the continuing quest. In: SHROPSHIRE, W.Jr. and H. MOHR (eds) Photomorphogenesis. Encyclop. Plant Physiol. New Series vol. 16A, *pp.* 3–23. Springer–Verlag, Heidelberg.

HEPLER, P.K. and R.O. WAYNE (1985) Calcium and plant development. *Ann. Rev. Plant Physiol.* 36, 397–439.

HERSHEY, H.P., R.F. BAKER, K.B. IDLER, J.L. LISSEMORE and P.H. QUAIL (1985) Analysis of cloned cDNA and genomic sequences for phytochrome: complete amino acid sequences for two gene products expressed in etiolated *Avena*. *Nucleic Acid Res.* 13, 8543–8559.

HETHERINGTON, A. and A.J. TREWAVAS (1984) Binding of nitrendipine, a calcium channel blocker, to pea shoot membranes. *Plant Sci. Lett.* 35, 109–113.

HIGINBOTHAM, N. and W.P. ANDERSON (1974) Electrogenic pumps in higher plant cells. *Can. J. Bot.* 52, 1011–1021.

HILLMAN, W.S. (1966) Responses of *Avena* and *Pisum* tissues to phytochrome conversion by red light. *Plant Physiol.* 41, 907–908.

HILTON, J.R. and H. SMITH (1980) The presence of phytochrome in purified barley etioplasts and its in vitro regulation of biologically–active gibberellin levels in etioplasts. *Planta* 148, 312–318.

HILTON, J.R. and B. THOMAS (1985) A comparison of seed and seedling phytochrome in *Avena sativa* L. using monoclonal antibodies. *J. Exp. Bot.* 36, 1937–1946.

HILTON, J.R. and B. THOMAS (1987) Photoregulation of phytochrome synthesis in germinating embryos of *Avena sativa* L. *J. Exp. Bot.* 38, 1704–1712.

HODGES, T.K. (1973) Ion absorption by plant roots. *Adv. Agron.* 25, 163–207.

HODGES, T.K. (1976) ATPases associated with membranes of plant cells. In: LUTTGE, U. and M.G. PITMAN (eds) Transport in Plants. Encyclop. Plant Physiol. New Series vol. 2A, *pp.* 260–283. Springer–Verlag, Berlin/Heidelberg/New York.

HODGES, T.K. and R.T. LEONARD (1974) Purification of a plasma membrane–bound adenosine triphosphatase from plant roots. *Methods Enzymol.*

32, 392–406.

HODGES, T.K., R.T. LEONARD, C.E. BRACKER and T.W. KEENAN (1972) Purification of an ion–stimulated adenosine triphosphatase from plant roots: association with plasma membranes. *Proc. Nat. Acad. Sci. USA* 69, 3307–3311.

HODGES, T.K. and D. MILLS (1986) Isolation of the plasma membrane. *Methods Enzymol.* 118, 41–54.

HUG, D.H. (1981) Photoactivation of enzymes. In: SMITH, K.C. (ed) Photochemical and Photobiological Reviews. vol. 6, *pp.* 87–138. Plenum Press, New York/London.

HUG, D.H., P.S. O'DONNELL and J.K. HUNTER (1980) Light activation of enzymes. *Photochem. Photobiol.* 32, 841–848.

HUNT, R.E. and L.H. PRATT (1979) Phytochrome radioimmunoassay. *Plant Physiol.* 64, 327–331.

HUNT, R.E. and L.H. PRATT (1980) Radioimmunoassay of phytochrome content in green, light–grown oats. *Plant Cell Environ.* 3, 91–95.

JABBEN, M. and M.G. HOLMES (1983) Phytochrome in light–grown plants. In: SHROPSHIRE, W.Jr. and H. MOHR (eds) Photomorphogenesis. Encyclop. Plant Physiol. New Series vol. 16, *pp.* 704–722. Springer–Verlag, Heidelberg.

JAFFE, L.F. (1981) The role of ionic currents in establishing developmental pattern. *Phil. Trans. R. Soc. Lond.* B 295, 553–566.

JENKINS, G.I. (1988) Photoregulation of gene expression in plants. *Photochem. Photobiol.* 48, 821–832.

JONES, A.M. and P.H. QUAIL (1986) Quaternary structure of 124–kilodalton phytochrome from *Avena sativa* L. *Biochem.* 25, 2987–2995.

JORDAN, B.R. (1982) Evidence for the interaction of membrane lipids and the plant photoreceptor, phytochrome. In: WINTERMANS, J.F.G.M. and P.J.C. KUIPER (eds) Biochemistry and Metabolism of Plant Lipids. *pp.* 479–482. Elsevier Biomedical Press, Amsterdam.

JORDAN, B.R., M.D. PARTIS and B. THOMAS (1984) A study of phytochrome–membrane association using an enzyme–linked immunosorbent assay and western blotting. *Physiol. Plant.* 60, 416–421.

JORDAN, B.R., M.D. PARTIS and B. THOMAS (1986) The biology and molecular biology of phytochrome. *Oxford Surv. Plant Mol. Cell Biol.* 3, 315–362.

JOSE, A.M. (1977) Phytochrome modulation of ATPase activity in a membrane fraction from *Phaseolus. Planta* 137, 203–206.

JOSE, A.M. and E. SCHAFER (1979) Red/far–red modulation in vitro of enzyme activity in a membrane fraction from *Phaseolus aureus. Planta* 146, 75–81.

JOSE, A.M., D. VINCE-PRUE and J.R. HILTON (1977) Chlorophyll interference with phytochrome measurement. *Planta* 135, 119-123.

KAHR, M. and I.M. MOLLER (1976) Temperature response and effect of $Ca^{2+}$ and $Mg^{2+}$ on ATPases from roots of oats and wheat as influenced by growth temperature and nutritional status. *Physiol. Plant.* 38, 153-158.

KASAMO, K. (1986) Comparison of plasma membrane and tonoplast $H^+$-translocating ATPases in *Phaseolus mungo* L. roots. *Plant Cell Physiol.* 27, 49-59.

KATZ, A.M. (1981) Regulation of calcium transport in the cardiac sarcoplasmic reticulum by cyclic-AMP-dependent protein kinase. In: ROSEN, O.M. and E.G. KREBS (eds) Protein Phosphorylation. *pp.* 849-854. Cold Spring Harbour Laboratory, New York.

KELLY, J.M. and J.C. LAGARIAS (1985) Photochemistry of 124-kilodalton *Avena* phytochrome under constant illumination *in vitro*. *Biochem.* 24, 6003-6011.

KENDRICK, R.E and G.H.M. KRONENBERG (eds) (1986) Photomorphogenesis in Plants. Martinus Nijhoff Publishers, Dordrecht.

KETCHUM, K.A., A. SHRIER, and R.J. POOLE (1989) Characterization of potassium-dependent currents in protoplasts of corn suspension cells. *Plant Physiol.* 89, 1184-1192.

KIM, I-S., U. BAI and P-S. SONG (1989) A purified 124-kDa oat phytochrome does not possess a protein kinase activity. *Photochem. Photobiol.* 49, 319-323.

KIM, I-S., E.S. KIM and P-S. SONG (1983) Interactions of phytochrome with detergents and liposomes. *Biochim. Biophys. Acta* 747, 55-64.

KIM, I-S. and P-S. SONG (1981) Binding of phytochrome to liposomes and protoplasts. *Biochem.* 20, 5482-5489.

KIM, Y-S., D-K. MOON, J.R. GOODIN and P-S. SONG (1986) Swelling of etiolated oat protoplasts induced by cAMP and red light. *Plant Cell Physiol.* 27, 193-197.

KLEIN, A.O. (1980) Rhythms and timing - a review. In: De GREEF, J. (ed) Photoreceptors and Plant Development. *pp.* 579-598. Antwerpen Univ. Press, Antwerpen.

KLOTZ, M.G., A. BERCZI, L. ERDEI and B. LIEBERMANN (1989) Picomolar concentrations of tentoxin affect $Mg^{2+}$- and $Ca^{2+}$-ATPase activities of plasma membrane vesicles from roots of winter wheat seedlings. *Physiol. Plant.* 75, 405-410.

KLUCIS, E. and G.M. POLYA (1988) Localization, solubilization and characterization of plant membrane-associated calcium-dependent protein kinases. *Plant Physiol.* 88, 164-171.

KOCHIAN, L.V., J.E. SHAFF and W.J. LUCAS (1989) High affinity $K^+$ uptake

in maize roots. *Plant Physiol.* 91, 1202–1211.

KOLAND, J.G. and G.G. HAMMES (1986) Steady state kinetic studies of purified yeast plasma membrane proton–translocating ATPase. *J. Biol. Chem.* 261, 5936–5942.

KONOMI, K., H. ABE and M. FURUYA (1987) Changes in the content of phytochrome I and II apoproteins in the embryonic axes of pea seeds during imbibition. *Plant Cell Physiol.* 28, 1443–1451.

KRIEG, M., S.E. BRASLAVSKY and K. SCHAFFNER (1988) 124–kDa phytochrome in model membrane systems: association studies and covalent binding to preformed liposomes. *Photochem. Photobiol.* 47, 311–316.

KYLIN, A. and M. KAHR (1973) The effect of magnesium and calcium ions on adenosine triphosphatases from wheat and oat roots at different pH. *Physiol. Plant.* 28, 452–457.

LADROR, U.S. and R.E. ZIELINSKI (1989) Protein kinase activities in tonoplast and plasmalemma membranes from corn roots. *Plant Physiol.* 89, 151–158.

LAEMMLI, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

LAGARIAS, J.C. (1985) Progress in the molecular analysis of phytochrome. *Photochem. Photobiol.* 42, 811–820.

LAGARIAS, J.C. and D.M. LAGARIAS (1989) Self–assembly of synthetic phytochrome holoprotein *in vitro*. *Proc. Natl. Acad. Sci. USA* 86, 5778–5780.

LAGARIAS, J.C. and F.M. MERCURIO (1985) Structure function studies on phytochrome. *J. Biol. Chem.* 260, 2415–2423.

LAGARIAS, J.C. and H. RAPOPORT (1980) Chromopeptides from phytochrome. The structure and linkage of the $P_R$ form of the phytochrome chromophore. *J. Am. Chem. Soc.* 102, 4821–4828.

LAGARIAS, J.C., Y–S. WONG, T.R. BERKELMAN, D.G. KIDD and R.W. McMICHAEL Jr. (1987) Structure–function studies on *Avena* phytochrome. In: FURUYA, M. (ed) Phytochrome and Photoregulation in Plants. *pp.* 51–61. Academic Press, London.

LAM, E., P.J. GREEN, M. WONG and N–H. CHUA (1989) Phytochrome activation of two nuclear genes requires cytoplasmic protein synthesis. *EMBO J.* 8, 2777–2783.

LAMB, C.J. and M.A. LAWTON (1983) Photocontrol of gene expression. In: SHROPSHIRE, W.Jr. and H. MOHR (eds) Photomorphogenesis. Encyclop. Plant Physiol. New Series vol. 16A, *pp.* 213–257. Springer–Verlag, Heidelberg.

LARSSON, C. (1985) Plasma membranes. In: LINSKENS, H.F. and J.F. JACKSON (eds) Cell Components. Modern Methods of Plant Analysis. New Series vol. 1, *pp.* 85–104. Springer–Verlag, Berlin.

LARSSON, C., P. KJELLBOM, S. WIDELL and T. LUNDBORG (1984) Sidedness of plant plasma membrane vesicles purified by partitioning in aqueous two-phase systems. *FEBS Lett.* 171, 271-276.

LARSSON, C., S. WIDELL and M. SOMMARIN (1988) Inside-out plant plasma membrane vesicles of high purity obtained by aqueous two-phase partitioning. *FEBS Lett.* 229, 289-292.

LEE. H.C., J.G. FORTE and D. EPEL (1982) The use of fluorescent amines for the measurement of pH: applications in liposomes, gastric microsomes, and sea urchin gametes. In: NUCCITELLI, N. and D.W. DEAMER (eds) Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions. *pp.* 135-160. A.R. LISS, Inc., New York.

LEIGH, R.A. and R.A. WALKER (1980) ATPase and acid phosphatase activities associated with vacuoles isolated from storage roots of red beet (*Beta vulgaris* L.). *Planta* 150, 222-229.

LEONARD, R.T. (1984) Membrane-associated ATPases and nutrient absorption by roots. In: TINKER, P.B. and A. LAUCHI (eds) Advances in Plant Nutrition. vol. 1, *pp.* 209-240. Praeger Scientific, New York.

LEONARD, R.T. and C.W. HOTCHKISS (1976) Cation-stimulated adenosine triphosphatase activity and cation transport in corn roots. *Plant Physiol.* 58, 331-335.

LEONARD, R.T., G. NAGAHASHI and W.W. THOMSON (1975) Effect of lanthanum on ion absorption in corn roots. *Plant Physiol.* 55, 542-546.

LEVITAN, I.B. (1985) Phosphorylation of ion channels. *J. Membrane Biol.* 87, 177-190.

LEW, R.R., D.P. BRISKIN and R.E. WYSE (1986) $Ca^{2+}$ uptake by endoplasmic reticulum from Zucchini hypocotyls. *Plant Physiol.* 82, 47-53.

LINK, G. (1988) Photocontrol of plastid gene expression. *Plant Cell Environ.* 11, 329-338.

LISSEMORE, J.L. and P.H. QUAIL (1988) Rapid transcriptional regulation by phytochrome of the genes for phytochrome and chlorophyll a/b-binding protein in *Avena sativa*. *Mol. Cell. Biol.* 8, 4840-4850.

LUNDBORG, T., S. WIDELL and C. LARSSON (1981) Distribution of ATPases in wheat root membranes separated by phase partition. *Physiol. Plant.* 52, 89-95.

MACARA, I.G. (1980) Vanadium - an element in search of a role. *Trends Biochem. Sci.* 5, 92-94.

MacKENZIE, J.M. Jr., R.A. COLEMAN, W.R. BRIGGS and L.H. PRATT (1975) Reversible redistribution of phytochrome within the cell upon conversion to its physiologically active form. *Proc. Natl. Acad. Sci. USA* 72, 799-803.

MADDY, A.H. and M.J. DUNN (1976) The solubilization of membranes. In: MADDY A.H. (ed) Biochemical Analysis of Membranes. *pp.* 177–196. Chapman and Hall, London.

MANABE, K. (1987) Properties of membrane-associated phytochrome in peas. In: FURUYA, M. (ed) Phytochrome and Photoregulation in Plants. *pp.* 209–214. Academic Press, London.

MANABE, K. and M. FURUYA (1975) Experimentally induced binding of phytochrome to mitochondrial and microsomal fractions in etiolated pea shoots. *Planta* 123, 207–215.

MANABE, K., A. NAGATINI, K.T. YAMAMOTO and M. FURUYA (1984) Detection of pelletable pea phytochrome by western blotting using monoclonal anti-pea-phytochrome antibodies. *Photochem. Photobiol.* suppl. 39, 86s.

MANDALA S. and L. TAIZ (1986) Characterization of the subunit structure of the maize tonoplast ATPase. *J. Biol. Chem.* 261, 12850–12855.

MARME, D. (1977) Phytochrome: membranes as possible sites of primary action. *Ann. Rev. Plant Physiol.* 28, 173–198.

MARME, D., J. BIANCO and J. GROSS (1976) Evidence for phytochrome binding to plasma membrane and endoplasmic reticulum. In: SMITH, H. (ed) Light and Plant Development. *pp.* 95–110. Butterworths, London.

MARME, D., J.M. MacKENZIE Jr., J. BOISARD and W.R. BRIGGS (1974) The isolation and partial characterization of a membrane fraction containing phytochrome. *Plant Physiol.* 54, 263–271.

MARRE, E. (1979) Fusicoccin: a tool in plant physiology. *Ann. Rev. Plant Physiol.* 30, 273–288.

MARRE, E. and A. BALLARIN-DENTI (1985) The proton pumps of the plasmalemma and the tonoplast of higher plants. *J. Bioenerg. Biomemb.* 17, 1–21.

MARRE, M.T., F.G. ALBERGONI, A. MORONI and E. MARRE (1989) Light-induced activation of electrogenic $H^+$ extrusion and $K^+$ uptake in *Elodea densa* depends on photosynthesis and is mediated by the plasma membrane $H^+$ ATPase. *J. Exp. Bot.* 40, 343–352.

MARSCHNER, H. (1986) Mineral Nutrition of Higher Plants. Academic Press, London.

McCARTY, D.R., K. KEEGSTRA and B.R. SELMAN (1984) Characterization and localizaton of the ATPase associated with pea chloroplast envelope membranes. *Plant Physiol.* 76, 584–588.

McCURDY, D.W. and L.H. PRATT (1986) Kinetics of intracellular redistribution of phytochrome in *Avena* coleoptiles after its photoconversion to the active, far-red-absorbing form. *Planta* 167, 330–336.

MEMON, A.R., Q. CHEN and W.F. BOSS (1989) Inositol phospholipids activate plasma membrane ATPase in plants. *Biochem. Biophys. Res. Comm.* 162, 1295–1301.

MENGEL, K. and S. SCHUBERT (1985) Active extrusion of protons into deionized water by roots of intact maize plants. *Plant Physiol.* 79, 344–348.

MILLS, D. and T.K. HODGES (1988) Characterization of plasma membrane ATPase from roots of *Atriplex nummularia*. *J. Plant Physiol.* 132, 513–519.

MITCHELL, P. (1976) Vectorial chemistry and the molecular mechanism of chemiosmotic coupling: power transmission by proticity. *Biochem. Soc. Trans.* 4, 399–430.

MORRE, D.J., A.O. BRIGHTMAN and A.S. SANDELIUS (1987) Membrane fractions from plant cells. In: FINDLAY, J.B.C. and W.H. EVANS (eds) Biological Membranes – a Practical Approach. *pp.* 37–72. IRL Press, Oxford/Washington DC.

MORRE, D.J. and T.J. BUCKHOUT (1979) Isolation of Golgi apparatus. In: REID, E. (ed) Plant Organelles. vol. 9, *pp.* 117–146. Ellis Horwood, Chichester.

MORRIS, S.J., E.K. SILBERGELD, R.R. BROWN and D.H. HAYNES (1982) Erythrosin B (USFD&C RED 3) inhibits calcium transport and ATPase activity of muscle sarcoplasmic reticulum. *Biochem. Biophys. Res. Comm.* 104, 1306–1311.

MORSE, M.J., R.C. CRAIN, G.G. COTE and R.L. SATTER (1989) Light–stimulated inositol phospholipid turnover in *Samanea saman* pulvini. *Plant Physiol.* 89, 724–727.

MORSE, M.J., R.C. CRAIN and R.L. SATTER (1987) Light–stimulated inositolphospholipid turnover in *Samanea saman* leaf pulvini. *Proc. Natl. Acad. Sci. USA* 84, 7075–7078.

MORSE, M.J., R.L. SATTER, R.C. CRAIN and G.G. COTE (1989) Signal transduction and phosphatidylinositol turnover in plants. *Physiol. Plant.* 76, 118–121.

NAGAHASHI, G., R.T. LEONARD and W.W. THOMSON (1978) Purification of plasma membranes from roots of barley. *Plant Physiol.* 61, 993–999.

NAGATANI, A., G.I. JENKINS and M. FURUYA (1988) Non–specific association of phytochrome to nuclei during isolation from dark–grown pea (*Pisum sativum* cv. Alaska) plumules. *Plant Cell Physiol.* 29, 1141–1145.

NAGY, F., S.A. KAY and N–H. CHUA (1988) Gene regulation by phytochrome. *Trends Genet.* 4, 37–42.

NAPIER, R.M. and H. SMITH (1987a) Photoreversible association of phytochrome with membranes. I. Distinguishing between two light–induced binding

responses. *Plant Cell Environ.* 10, 383–389.

NAPIER, R.M. and H. SMITH (1987b) Photoreversible association of phytochrome with membranes. II. Reciprocity tests and a model for the binding reaction. *Plant Cell Environ.* 10, 391–396.

NELSON, N. (1988) Structure, function, and evolution of proton–ATPases. *Plant Physiol.* 86, 1–3.

NEWMAN, I.A. (1981) Rapid electric responses of oats to phytochrome show membrane processes unrelated to pelletability. *Plant Physiol.* 68, 1494–1499.

NEWMAN, I.A. and W.R. BRIGGS (1972) Phytochrome–mediated electric potential changes in oat seedlings. *Plant Physiol.* 50, 687–693.

NEWMAN, I.A. and J.K. SULLIVAN (1976) Auxin transport in oats: a model for the electric changes. In: WARDLAW, I.F. and J.B. PASSIOURA (eds) Transport and Transfer Processes in Plants. *pp.* 153–159. Academic Press, London/New York.

OBERFELDER, R. (1989) Immunoblotting: comparison of detection methods. *Focus* 11, 1–5.

O'NEILL, S.D., A.B. BENNETT and R.M. SPANSWICK (1983) Characterization of a $NO_3^-$–sensitive $H^+$–ATPase from corn roots. *Plant Physiol.* 72, 837–846.

O'NEILL, S.D. and R.M. SPANSWICK (1984a) Characterization of native and reconstituted plasma membrane $H^+$–ATPase from the plasma membrane of *Beta vulgaris*. *J. Memb. Biol.* 79, 245–256.

O'NEILL, S.D. and R.M. SPANSWICK (1984b) Effects of vanadate on the plasma membrane ATPase of red beet and corn. *Plant Physiol.* 75, 586–591.

OTTO, V. and E. SCHAFER (1988) Rapid phytochrome–controlled protein phosphorylation and dephosphorylation in *Avena sativa* L. *Plant Cell Physiol.* 29, 1115–1121.

PARDO, J.M. and R. SERRANO (1989) Structure of a plasma membrane $H^+$–ATPase gene from the plant *Arabidopsis thaliana*. *J. Biol. Chem.* 264, 8557–8562.

PARK, M–H. and Q. CHAE (1989) Intracellular protein phosphorylation in oat (*Avena sativa* L.) protoplasts by phytochrome action. *Biochem. Biophys. Res. Comm.* 162, 9–14.

PARKER, M.W., S.B. HENDRICKS, H.A. BORTHWICK and N.J. SCULLY (1946) Action spectrum for the photoperiodic control of floral initiation of short–day plants. *Bot. Gazette* 108, 1–26.

PARR, A.J. and B.C. LOUGHMAN (1983) Boron and membrane function in plants. In: ROBB, D.A. and W.S. PIERPOINT (eds) Metals and Micronutrients: Uptake and Utilization by Plants. *pp.* 87–107. Academic Press, London.

PARTIS, M.D. and B. THOMAS (1985) Conformational changes in phytochrome determined by using monoclonal antibodies. *Biochem. Soc. Trans.* 13, 110.

PEDERSEN, P.L. and E. CARAFOLI (1987a) Ion motive ATPases. I. Ubiquity, properties, and significance to cell function. *Trends Biochem. Sci.* 12, 146–150.

PEDERSEN, P.L. and E. CARAFOLI (1987b) Ion motive ATPases. II. Energy coupling and work output. *Trends Biochem. Sci.* 12, 186–189.

PERLIN, D.S. and R.M. SPANSWICK (1981) Characterization of ATPase activity associated with corn leaf plasma membranes. *Plant Physiol.* 68, 521–526.

PETERSON, G.L. (1978) A simplified method for analysis of inorganic phosphate in the presence of interfering substances. *Anal. Biochem.* 84, 164–172.

PICK, U. (1981) The interaction of vanadate ions with the Ca-ATPase from sarcoplasmic reticulum. *J. Biol. Chem.* 257, 6111–6119.

PITMAN, M.G. (1970) Active $H^+$ efflux from cells of low-salt barley roots during salt accumulation. *Plant Physiol.* 45, 787–790.

POMEROY, M.K. and E.J. McMURCHIE (1982) A comparison of the properties of ATPase assotiated with wheat and cauliflower plasma membranes. *Plant Physiol.* 69, 1241–1246.

POOLE, R.J. (1974) Ion transport and electrogenic pumps in storage tissue cells. *Can. J. Bot.* 52, 1023–1028.

POOLE, R.J. (1978) Energy coupling for membrane transport. *Ann. Rev. Plant Physiol.* 29, 437–460.

POOLE, R.J. (1988) Plasma membrane and tonoplast. In: BAKER, D.A. and J.L. HALL (eds) Solute Transport in Plant Cells and Tissues. *pp.* 83–105. Longman, Harlow.

PRICE, C.A. (1983) General principles of cell fractionation. In: HALL, J.L. and A.L. MOORE (eds) Isolation of Membranes and Organelles from Plant Cells. *pp.* 1–24. Academic Press, London.

PRATT, L.H. (1978) Molecular properties of phytochrome. *Photochem. Photobiol.* 27, 81–105.

PRATT, L.H. (1979) Phytochrome: function and properties. In: SMITH, K.C. (ed) Photochemical and Photobiological Reviews. vol. 4, *pp.* 59–124. Plenum Press, New York/London.

PRATT, L.H. (1983) Assay of photomorphogenic photoreceptors. In: SHROPSHIRE, W.Jr. and H. MOHR (eds) Photomorphogenesis. Encyclop. Plant Physiol. New Series vol. 16A, *pp.* 152–177. Springer-Verlag, Heidelberg.

PRATT, L.H., M.M. CORDONNIER and J.C. LAGARIAS (1988) Mapping of antigenic domains on phytochrome from *Avena sativa* L. by immunoblot analysis of proteolytically derived peptides. *Arch. Biochem. Biophys.* 267, 723–735.

PRATT, L.H. and D. MARME (1976) Red light-enhanced phytochrome pelletability: a re-examination and further characterization. *Plant Physiol.* 58, 686-692.

QUAIL, P.H. (1974) Particle-bound phytochrome: spectral properties of bound and unbound fractions. *Planta* 118, 345-355.

QUAIL, P.H. (1978a) Irradiation-enhanced phytochrome pelletability in *Avena*: *in vivo* development of a potential to pellet and a role of $Mg^{2+}$ in its expression *in vitro*. *Photochem. Photobiol.* 27, 147-153.

QUAIL, P.H. (1978b) Irradiation-enhanced phytochrome pelletability in *Avena*: pigment release by $Mg^{2+}$ gradient elution. *Photochem. Photobiol.* 27, 759-765.

QUAIL, P.H. (1979) Plant cell fractionation. *Ann. Rev. Plant Physiol.* 30, 425-484.

QUAIL, P.H. (1983) Rapid action of phytochrome in photomorphogenesis. In: SHROPSHIRE, W.Jr. and H. MQHR (eds) Photomorphogenesis. Encyclop. Plant Physiol. New Series vol. 16A, *pp.* 178-212. Springer-Verlag, Heidelberg

QUAIL, P.H. (1984) Phytochrome: a regulatory photoreceptor that controls the expression of its own gene. *Trends Biochem. Sci.* 9, 450-453.

QUAIL, P.H. and W.R. BRIGGS (1978) Irradiation-enhanced phytochrome pelletability. *Plant Physiol.* 62, 773-778.

QUAIL, P.H., J.T. COLBERT, N.K. PETERS, A.H. CHRISTENSEN, R.A. SHARROCK and J.L. LISSEMORE (1986) Phytochrome and the regulation of the expression of its genes. *Phil. Trans. R. Soc. Lond.* B 314, 469-480.

RACUSEN, R.H. (1976) Phytochrome control of electrical potentials and intercellular coupling in oat coleoptile tissue. *Planta* 132, 25-29.

RACUSEN, R.H. and R.L. SATTER (1975) Rhythmic and phytochrome-regulated changes in transmembrane potential in *Samanea* pulvini. *Nature* 255, 408-410.

RANDALL, S.K. and A.W. RUESINK (1983) Orientation and integrity of plasma membrane vesicles obtained from carrot protoplasts. *Plant Physiol.* 73, 385-391.

RANJEVA, R. and A.M. BOUDET (1987) Phosphorylation of proteins in plants: regulatory effects and potential involvement in stimulus/response coupling. *Ann. Rev. Plant Physiol.* 38, 73-93.

RAY, P.M. (1979) Maize coleoptile cellular membranes bearing different types of glucan synthetase activity. In: REID, E. (ed) Plant Organelles: Methodological Surveys B. Biochemistry. vol. 9, *pp.* 135-146. Ellis Horwood, Chichester.

RAY, P.M., T.L. SHININGER and M.M. RAY (1969) Isolation of $\beta$-glucan synthetase particles from plant cells and identification with Golgi membranes. *Proc. Natl. Acad. Sci. USA* 64, 605-612.

RAYLE, D.L. and R. CLELAND (1970) Enhancement of wall loosening and elongation by acid solutions. *Plant Physiol.* 46, 250-270.

REA, P.A. and R.J. POOLE (1985) Proton-translocating inorganic pyrophosphatase in red beet (*Beta vulgaris* L.) tonoplast vesicles. *Plant Physiol.* 77, 46-52.

REA, P.A. and D. SANDERS (1987) Tonoplast energizaton: two $H^+$ pumps, one membrane. *Physiol. Plant.* 71, 131-141.

ROBINSON, C., C. LARSSON and T.J. BUCKHOUT (1988) Identification of a calmodulin-stimulated ($Ca^{2+}$+$Mg^{2+}$)-ATPase in a plasma membrane fraction isolated from maize (*Zea mays*) leaves. *Physiol. Plant.* 72, 177-184.

ROLAND, J.C. (1978) General preparation and staining of thin sections. In: HALL, J.L. (ed) Electron Microscopy and Cytochemistry of Plant Cells. *pp.* 1-62. Elsevier/North Holland, Amsterdam.

ROTH-BEJERANO, N. and J.L. HALL (1986a) Characterizaton of a light-modulated ATPase activity in a microsomal fracton from cucumber hypocotyls. *J. Plant Physiol.* 122, 315-327.

ROTH-BEJERANO, N. and J.L. HALL (1986b) Photoregulation of proton extrusion by cucumber hypocotyls and the role of ATPase activity. *J. Plant Physiol.* 122, 329-336.

ROTH-BEJERANO, N. and R.E. KENDRICK (1979) Effects of filipin and steroids on phytochrome pelletability. *Plant Physiol.* 63, 503-506.

ROUX, S.J. (1986) Phytochrome and membranes. In: KENDRICK, R.E. and G.H.M. KRONENBERG (eds) Photomorphogenesis in Plants. *pp.* 115-134. Martinus Nijhoff Publishers, Dordrecht.

ROUX, S.J. (1987) Phytochrome interactions with purified organelles. In: FURUYA, M. (ed) Phytochrome and Photoregulation in Plants. *pp.* 193-207. Academic Press, London.

ROUX, S.J., R.O. WAYNE and N. DATTA (1986) Role of calcium ions in phytochrome responses: an update. *Physiol. Plant.* 66, 344-348.

RUBINSTEIN, B., K.S. DRURY and R.B. PARK (1969) Evidence for bound phytochrome in oat seedlings. *Plant Physiol.* 44, 105-109.

RUBINSTEIN, B. and A.I. STERN (1986) Relationship of transplasmalemma redox activity to proton and solute transport by roots of *Zea mays*. *Plant Physiol.* 80, 805-811.

RUDIGER, W. (1987) Phytochrome: the chromophore and photoconversion. *Photobiochem. Photobiophys.* Suppl. 217-227.

SANDELIUS, A.S., R. BARR, F.L. CRANE and D.J. MORRE (1986a) Redox reactions of plasma membranes isolated from soybean hypocotyls by phase partition. *Plant Sci.* 48, 1-10.

SANDELIUS, A.S., C. PENEL, G. AUDERSET, A. BRIGHTMAN, M. MILLARD and D.J. MORRE (1986b) Isolation of highly purified fractions of plasma membrane and tonoplast from the same homogenate of soybean hypocotyls

by free-flow electrophoresis. *Plant Physiol.* 81, 177-185.

SANDSTROM, R.P., A.H. DeBOER, T.L. LOMAX and R.E. CLELAND (1987) Latency of plasma membrane $H^+$-ATPase in vesicles isolated by aqueous phase partitoning. *Plant Physiol.* 85, 693-698.

SCALLA, R., A. AMORY, J. RIGAUD and A. GOFFEAU (1983) Phosphorylated intermediate of a transport ATPase and activity of protein kinase in membranes from corn roots. *Eur. J. Biochem.* 132, 525-530.

SCARBOROUGH, G.A. and R. ADDISON (1984) On the subunit composition of the *Neurospora* plasma membrane $H^+$-ATPase. *J. Biol. Chem.* 259, 9109-9114.

SCHALLER, G.E. and M.R. SUSSMAN (1988) Phosphorylation of the plasma-membrane $H^+$-ATPase of oat roots by a calcium-stimulated protein kinase. *Planta* 173, 509-518.

SCHERER, G.F.E. (1984) Subcellular localization of $H^+$-ATPase from pumpkin hypocotyls (*Cucurbita maxima* L.) by membrane fractionation. *Planta* 160, 348-356.

SCHNEIDER, D.L. (1987) The proton pump ATPase of lysosomes and related organelles of the vacuolar apparatus. *Biochim. Biophys. Acta* 895, 1-10.

SCHOPFER, P. (1977) Phytochrome control of enzymes. *Ann. Rev. Plant Physiol.* 28, 223-252.

SCHUMAKER, K.S. and H. SZE (1985) A $Ca^{2+}/H^+$ antiport system driven by the proton electrochemical gradient of a tonoplast $H^+$-ATPase from oat roots. *Plant Physiol.* 79, 1111-1117.

SERLIN, B.S. and S.J. ROUX (1984) Modulation of chloroplast movement in the green alga *Mougeotia* by the $Ca^{2+}$ ionophore A23187 and by calmodulin antagonists. *Proc. Natl. Acad. Sci. USA* 81, 6368-6372.

SERLIN, B.S. and S.J. ROUX (1986) Light-induced import of the chromoprotein, phytochrome, into mitochondria. *Biochim. Biophys. Acta* 848, 372-377.

SERLIN, B.S., S.K. SOPORY and S.J. ROUX (1984) Modulation of oat mitochondrial ATPase activity by $Ca^{2+}$ and phytochrome. *Plant Physiol.* 74, 827-833.

SERRANO, E.E., E. ZEIGER and S. HAGIWARA (1988) Red light stimulates an electrogenic proton pump in *Vicia* guard cell protoplasts. *Proc. Natl. Acad. Sci. USA* 85, 436-440.

SERRANO, R. (1984) Plasma membrane ATPase of fungi and plants as a novel type of proton pump. *Curr. Top. Cell. Reg.* 23, 87-126.

SERRANO, R. (1985) Plasma Membrane ATPase of Plants and Fungi. CRC Press Inc., Boca Raton, Fla.

SERRANO, R. (1988) Structure and function of proton translocating ATPase in plasma membranes of plants and fungi. *Biochim. Biophys. Acta* 947, 1-28.

SERRANO, R. (1989) Structure and function of plasma membrane ATPase. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 40, 61–94.

SHANKLIN, J., M. JABBEN and R.D. VIERSTRA (1987) Red light–induced formation of ubiquitin–phytochrome conjugates: identification of possible intermediates of phytochrome degradation. *Proc. Natl. Acad. Sci. USA* 84, 359–363.

SHIINA, T., R. WAYNE, H.Y. LIM TUNG and M. TAZAWA (1988) Possible involvement of protein phosphorylation/dephosphorylation in the modulation of $Ca^{2+}$ channel in tonoplast–free cells of *Nitellopsis*. *J. Membrane Biol.* 102, 255–264.

SHIMAZAKI, Y., M.M. CORDONNIER and L.H. PRATT (1983) Phytochrome quantitation in crude extracts of *Avena* by enzyme–linked immunosorbent assay with monoclonal antibodies. *Planta* 159, 534–544.

SHROPSHIRE, W.Jr. and H. MOHR (eds) (1983) Photomorphogenesis. Encyclop. Plant Physiol. New Series vol. 16. Springer Verlag, Heidelberg.

SHULL, M.M. and J.B. LINGREL (1987) Multiple genes encode the human $Na^+,K^+$–ATPase catalytic subunit. *Proc. Natl. Acad. Sci. USA* 84, 4039–4043.

SILBERGELD, E.K. (1981) Erythrosin B is a specific inhibitor of high affinity $^3H$–ouabain binding and ion transport in rat brain. *Neuropharmacology*. 20, 87–90.

SINGH, B.R., J–K. CHOI, I–S. KIM and P–S. SONG (1989) Binding of 124–kilodalton oat phytochrome to liposomes and chloroplasts. *Physiol. Plant.* 76, 319–325.

SLAYMAN, C.L. (1987) The plasma membrane ATPase of *Neurospora*: a proton–pumping electroenzyme. *J. Bioenerg. Biomemb.* 19, 1–20.

SMITH, H. (1981) Evidence that $P_{fr}$ is not the active form of phytochrome in light–grown maize. *Nature* 293, 163–165.

SMITH, H. (1982) Light quality, photopercerption, and plant strategy. *Ann. Rev. Plant Physiol.* 33, 481–518.

SMITH, H. (1983) Is $P_{fr}$ the active form of phytochrome? *Phil. Trans. R. Soc. Lond.* B 303, 443–452.

SMITH, H. (1984) Definition and scope of photomorphogenesis. In: SMITH, H. and M.G. HOLMES (eds) Techniques in Photomorphogenesis. *pp.* 1–4. Academic Press, London.

SMITH, H., J.J. CASAL and G.M. JACKSON (1990) Reflection signals and the perception by phytochrome of the proximity of neighbouring vegetation. *Plant Cell Eviron.* 13, 73–78.

SOMMARIN, M., T. LUNDBORG and A. KYLIN (1985) Comparison of K,MgATPases in purified plasmalemma from wheat and oat.– Substrate

specificities and effects of pH, temperature and inhibitors. *Physiol. Plant.* 65, 27–32.

SOMMARIN, M. and A.S. SANDELIUS (1988) Phosphatidylinositol and phosphatidylinositol phosphate kinases in plant plasma membranes. *Biochim. Biophys. Acta* 958, 268–278.

SONG, P–S. (1988) The molecular topography of phytochrome: chromophore and apoprotein. *J. Photochem. Photobiol., B: Biology* 2, 43–57.

SPANSWICK, R.M. (1981) Electrogenic ion pumps. *Ann. Rev. Plant Physiol.* 32, 267–289.

SUKOW, W.W., H.E. SANDBERG, E.A. LEWIS, D.J. EATOUGH and L.D. HANSEN (1980) Binding of the Triton X series of nonionic surfactants to bovine serum albumin. *Biochem.* 19, 912–917.

SUMNER, J.B. (1944) A method for the colourimetric determination of phosphorous. *Science* 3, 413–444.

SUNDOVIST, C. (1974) The pool size of protochlorophyllide during different stages of greening of dark grown wheat leaves. *Physiol. Plant.* 30, 143–147.

SUNDQVIST, C. and S. WIDELL (1983) Binding of phytochrome to plasma membranes in vitro. *Physiol. Plant.* 59, 35–41.

SUSSMAN, M.R. and J.F. HARPER (1989) Molecular biology of the plasma membrane of higher plants. *The Plant Cell* 1, 953–960.

SUSSMAN, M.R. and T.K. SUROWY (1987) Physiology and molecular biology of membrane ATPases. *Oxford Surv. Plant Mol. Cell Biol.* 4, 47–70.

SZE, H. (1983) Proton–pumping adenosine triphosphatase in membrane vesicles of tabacco callus. *Biochim. Biophys. Acta* 732, 586–594.

SZE, H. (1985) $H^+$–translocating ATPases: advances using membrane vesicles. *Ann. Rev. Plant Physiol.* 36, 175–208.

TAKEDA, K., A.C. KURKDJIAN and R.T. KADO (1985) Ionic channels, ion transport and plant cell membranes: potential applications of the patch–clamp technique. *Protoplasma* 127, 147–162.

TANADA, T. (1978) Boron – key element in the actions of phytochrome and gravity? *Planta* 143, 109–111.

TAZAWA, M. and T. SHIMMEN (1982) Control of electrogenesis by ATP, $Mg^{2+}$, $H^+$, and light in perfused cells of *Chara*. *Curr. Top. Memb. Trans.* 16, 49–67.

TERRY, M.J., J.L. HALL and B. THOMAS (1989) Purification of plasma membrane from wheat leaves and characterization of the associated vanadate–sensitive $Mg^{2+}$–ATPase activity. *J. Plant Physiol.* 134, 756–761.

THOMAS, B., N.E. CROOK and S.E. PENN (1984a) An enzyme–linked immunosorbent assay for phytochrome. *Physiol. Plant.* 60, 409–415.

THOMAS, B., M.D. PARTIS and B.R. JORDAN (1986) Immunological approaches to the study of phytochrome. In: WANG, T.L. (ed) Immunology in Plant Science. *pp.* 171–195. Cambridge Univ. Press, Cambridge.

THOMAS, B., S.E. PENN, G.W. BUTCHER and G. GALFRE (1984b) Discrimination between the red- and far-red-absorbing forms of phytochrome from *Avena sativa* L. by monoclonal antibodies. *Planta* 160, 382–384.

THOMAS, B. and S.E. TULL (1981) Photoregulation of $K^+$-ATPase *in vitro* by red and far red light in extracts from cucumber hypocotyls. *Z. Pflanzenphysiol. Bd.* 102, 283–292.

THOMPSON, W.F. (1988) Photoregulation: diverse gene responses in greening seedlings. *Plant Cell Environ.* 11, 319–328.

TOBIN, E.M. and J. SILVERTHORNE (1985) Light regulation of gene expression in higher plants. *Ann. Rev. Plant Physiol.* 36, 569–593.

TOGNOLI, L. and E. MARRE (1981) Purification and characterization of a divalent cation-activated ATP-ADPase from pea stem microsomes. *Biochim. Biophys. Acta* 642, 1–14.

TOKUHISA, J.G., S.M. DANIELS and P.H. QUAIL (1985) Phytochrome in green tissue: spectral and immunochemical evidence for two distinct molecular species of phytochrome in light-grown *Avena sativa* L. *Planta* 164, 321–332.

TOKUHISA, J.G. and P.H. QUAIL (1989) Phytochrome in green-tissue: partial purification and characterization of the 118-kilodalton phytochrome species from light-grown *Avena sativa* L. *Photochem. Photobiol.* 50, 143–152.

TOKUTOMI, S., K.T. YAMAMOTO and M. FURUYA (1981) Photoreversible changes in hydrophobicity of undegraded pea phytochrome determined by partition in an aqueous two-phase system. *FEBS Lett.* 134, 159–162.

TRETYN, A., R.E. KENDRICK and M.E. BOSSEN (1990) The effect of a calcium-channel antagonist, nifedipine and agonist, Bay K-8644, on the phytochrome-controlled swelling of etiolated wheat protoplasts. *Physiol. Plant.* 78, 230–235.

TU, S-I. and J.N. BROUILLETTE (1987) Metal ion inhibition of corn root plasma membrane ATPase. *Phytochem.* 26, 65–69.

TU, S-I. and B.J. SLIWINSKI (1985) Mechanistic investigation on the temperature dependence and inhibition of corn root plasma membrane ATPase. *Arch. Biochem. Biophys.* 241, 348–355.

VAN ETTEN, R.L., P.P. WAYMACK and D.M. REHKOP (1974) Transition metal ion inhibition of enzyme-catalyzed phosphate ester displacement reactions. *J. Am. Chem. Soc.* 96, 6782–6785.

VARA, F. and R. SERRANO (1982) Partial purification and properties of the proton-translocating ATPase of plant plasma membranes. *J. Biol. Chem.* 257,

12826-12830.

VARA, F. and R. SERRANO (1983) Phosphorylated intermediate of the ATPase of plant plasma membranes. *J. Biol. Chem.* 258, 5334-5336.

VERBELEN, J-P., L.H. PRATT, W.L. BUTLER and K. TOKUYASU (1982) Localization of phytochrome in oats by electron microscopy. *Plant Physiol.* 70, 867-871.

VIERSTRA, R.D. (1987) Ubiquitin, a key component in the degradation of plant proteins. *Physiol. Plant.* 70, 103-106.

VIERSTRA, R.D., M.M. CORDONNIER, L.H. PRATT and P.H. QUAIL (1984) Native phytochrome: immunoblot analysis of relative molecular mass and in-vitro proteolytic degradation for several plant species. *Planta* 160, 521-528.

VIERSTRA, R.D. and P.H. QUAIL (1983) Purification and initial characterization of 124-kilodalton phytochrome from *Avena*. *Biochem.* 22, 2498-2505.

VIERSTRA, R.D. and P.H. QUAIL (1986) Phytochrome the protein. In: R.E. KENDRICK and G.H.M. KRONENBERG (eds) Photomorphogenesis in Plants. *pp.* 35-60. Martinus Nijhoff Publishers, Dordrecht.

VIRGIN, H.I. (1981) The physical state of protochlorophyll(ide) in plants. *Ann. Rev. Plant Physiol.* 32, 451-463.

WAGNER, G. and R. ROSSBACHER (1980) X-ray microanalysis and chlorotetracycline staining of calcium vesicles in the green alga *Mougeotia*. *Planta* 149, 298-305.

WATSON, P.J. and H. SMITH (1982a) The use and misuse of calcium carbonate as an aid to the spectrophotometric assay of phytochrome in vitro. *Planta* 154, 115-120.

WATSON, P.J. and H. SMITH (1982b) Integral association of phytochrome with a membranous fraction from etiolated *Avena* shoots: red/far-red photoreversibility and in vitro characterization. *Planta* 154, 121-127.

WATSON, P.J. and H. SMITH (1982c) Integral association of phytochrome with a membranous fraction from *Avena* shoots: in vivo characterization and physiological significance. *Planta* 154, 128-134.

WAYNE, R. and P.K. HEPLER (1984) The role of calcium ions in phytochrome-mediated germination of spores of *Onoclea sensibilis* L. *Planta* 160, 12-20.

WAYNE, R. and P.K. HEPLER (1985) Red light stimulates an increase in intracellular calcium in the spores of *Onoclea sensibilis*. *Plant Physiol.* 77, 8-11.

WEISENSEEL, M.H. and H.K. RUPPERT (1977) Phytochrome and calcium ions are involved in light-induced membrane depolarization in *Nitella*. *Planta* 137, 225-229.

WIDELL, S. (1987) Purification of plasmalemma with aqueous polymer two-phase partition. *Physiol. Plant.* 69, 727-730.

WIDELL, S. and C. LARSSON (1981) Separation of presumptive plasma membranes from mitochondria by partition in an aqueous polymer two-phase system. *Physiol. Plant.* 51, 368-374.

WIDELL, S. and C. LARSSON (1983) Distribution of cytochrome b photoreductions mediated by endogenous photosensitizer or methylene blue in fractions from corn and cauliflower. *Physiol. Plant* 57, 196-202.

WIDELL, S. and C. SUNDQVIST (1984) Binding of phytochrome to plasma membranes in vivo. *Physiol. Plant.* 61, 27-34.

WONG, Y-S., H-C. CHENG, D.A. WALSH and J.C. LAGARIAS (1986) Phosphorylation of *Avena* phytochrome *in vitro* as a probe of light-induced conformational changes. *J. Biol. Chem.* 261, 12089-12097.

WONG, Y-S., R.W. McMICHAEL Jr. and J.C. LAGARIAS (1989) Properties of a polycation-stimulated protein kinase associated with purified *Avena* phytochrome. *Plant Physiol.* 91, 709-718.

YAMAMOTO, K.T. (1987) Structure-function relationship of pea phytochrome as deduced from amino-terminal sequence analysis of phytochrome chromopeptides. In: FURUYA, M. (ed) Phytochrome and Photoregulation in Plants. *pp.* 63-81. Academic Press, London.

YAMAMOTO, K.T. and M. FURUYA (1975) Photoreversible binding *in vitro* of cytosolic phytochrome to particulate fraction isolated from pea epicotyls. *Planta* 127, 177-186.

YUNGHANS, H. and M.J. JAFFE (1972) Rapid respiratory changes due to red light or acetylcholine during the early events of phytochrome-mediated photomorphogenesis. *Plant Physiol.* 49, 1-7.

ZHERELOVA, O.M. (1989) Protein kinase C is involved in regulation of $Ca^{2+}$ channels in plasmalemma of *Nitella syncarpa*. *FEBS Lett.* 242, 330-332.

ZOCCHI, G. (1985) Phosphorylation-dephosphorylation of membrane proteins controls the microsomal $H^+$-ATPase activity of corn roots. *Plant Sci.* 40, 153-159.