PROPERTIES OF MEMBRANE ASSOCIATED ATPases

AND THEIR RELATION TO CELL GROWTH

IN THE HYPOCOTYL OF

CUCUMIS SATIVUS L.

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ABSTRACT.

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PROPERTIES OF MEMBRANE ASSOCIATED ATPases AND THEIR RELATION TO CELL GROWTH IN THE HYPOCOTYL OF *Cucumis sativus* L.

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Isolation procedures were examined for the preparation of plasma membrane and tonoplast enriched fractions from dark grown hypocotyl tissue of *Cucumis sativus*. The charged silica bead method was used to prepare plasma membrane fractions, but was unsuccessful due to mitochondrial contamination. A conventional discontinuous sucrose gradient was used. ATPase characterization was made of the 25-30% and 34%-38% (w/w) interfaces. ATPase activity associated with the low density fraction was more nitrate-sensitive than the higher density interface. Both fractions showed a fairly high vanadate sensitivity. Azide inhibition and cytochrome c oxidase activity were both absent from these fractions. The Golgi marker IDPase, indicated that there was contamination in both of these fractions, although it showed a peak in a different region of the gradient. Separation of the endoplasmic reticulum appeared not to be possible with this gradient type, since markers were found to be associated with each of the gradient interfaces. SW25 and erythrosin B were found to inhibit ATPase activity to some extent in both these fractions. These two inhibitors were also found to inhibit medium acidification and hypocotyl growth, but not to affect tissue respiration.

IAA applied *in vitro* was found to have no effect on ATPase specific activity. However, if the tissue was given a pretreatment in IAA *in vivo*, stimulation of the membrane associated ATPase *in vitro* was observed. At low ATP concentrations ATP-dependent H^+ transport, as measured by the fluorescence quenching of quinacrine, was found to be stimulated by IAA.

Red and far red light treatments given *in vitro* to microsomal membrane fractions that had received a red light treatment *in vivo* prior to extraction, were found to cause only minor changes in ATPase specific activity. Neither red nor far red light had any effect on ATP-dependent H^+ transport using membrane vesicles.

ATP-dependent Ca^{2+} transport was examined in microsomal vesicles from the hypocotyl using the fluorescent probe chlorotetracycline. Ca^{2+} transport was found to have an optimum pH of 8.0 and was stimulated slightly by calmodulin and inhibited by calmodulin inhibitors and IAA. The local anaesthetics dibucaine and tetracaine were both inhibitory to transport.

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ABBREVIATIONS USED IN THE TEXT

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
B	Blue light
BSA	Bovine serum albumin
BTP	Bis-tris-propane (1,3-bis(tris[hydroxymethyl] methyl(amino)propane)
Calmodulin	Phosphodiesterase 3'-5'-cyclic nucleotide-activator from bovine brain
CAM	Crassulacean acid metabolism
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
CTC	Chlorotetracycline
D	Darkness
2,3-D	2,3-dichlorophenoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
DCCD	N,N'-dicyclohexylcarbodiimide
DES	Diethylstilbestrol
DIDS	4,4'-diisothiocyano -2,2'-stilbene disulphonic acid
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
IAA	Indole-3-acetic acid
IDPase	Inosine 5'-diphosphatase
EB	Erythrosin B
EGTA	Ethylene glycol-*bis*-(β-aminoethylether) N,N' tetraacetic acid
FC	Fusicoccin
FCCP	Carbonyl cyanide p-trifluoromethoxylphenyl hydrazone
FDA	Fluorescein diacetate
FR	Far-red light
GA1 & GA3	Gibberellic acid
GTP	Guanosine triphosphate
h	Hour/s
HEPES	N-2-Hydroxyethylpiperazine -N'-2-ethane sulphonic acid

KD	Kilodaltons
IAA	Anion of IAA
IDP	Inosine diphosphate
ITP	Inosine triphosphate
MES	2-(N-morpholino)ethane sulphonic acid
mRNA	Messenger ribonucleic acid
NA	Nicotinic acid
1-NAA	1-naphthyl acetic acid
2-NAA	2-napthyl acetic acid
NPA	1-N-naphthylphthalamac acid
PIPES	Piperazine-N,N'-bis (2-ethanesulphonic acid)
PAA	Phenylacetic acid
Pi	Inorganic phosphate
PD	Transmembrane electrical potential difference
P_{fr}	Far-red light absorbing form of phytochrome
P_r	Red light absorbing form of phytochrome
PMSF	Phenylmethylsulphonyl fluoride
PPase	Pyrophosphatase
Pi	Inorganic phosphate
PPi	Inorganic pyrophosphate
PTA	Phosphotungstic acid-chromic acid stain
PVP	Polyvinylpolypyrrolidone
R	Red light
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SHAM	Salicylhydroxamic acid
SITS	4-acetamido-4'-isothiocyano-2,2'.stilbene disulphonic acid
SW26	2,2,2-trichloroethyl 3,4-dichlorocarbanilate
TEMED	N,N,N',N',-tetramethylethylenediamine
TIBA	2,3,5-triido benzoic acid
TFP	Trifluoperazine
TCA	Trichloroacetic acid
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
UDP	Uridine diphosphate
UDPase	Uridine diphosphatase
UDPG	Uridine 5'-diphosphoglucose
Vanadate	Na$^+$-orthovanadate
W	White fluorescent light

SYMBOLS USED IN THE TEXT.

$\blacktriangle\psi$	membrane potential difference
$\blacktriangle$pH	pH gradient
φ	Phytochrome photostationary state
φ_c	Calculated φ

DECLARATION

Parts of this work have been published as:

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Browning: And yet to me it's all simple and easy as the rule of three! And to you?

Elizabeth: Well....not *quite* always. Sometimes there are passages.... (*she picks up the book*) I have marked one or two in your "Sordello" which rather puzzle me. Here, for instance... (*She opens the book and hands it to him*)

Browning: (taking the book): Oh,"Sordello"! Somebody once called it "a horror of great darkness" I've done my best to forget it. However, (*He reads the passage to himself, smiling; the smile fades; he passes his hand over his brow and reads it again. She watches him, covertly smiling. He mutters*) extraordinary.....But -but a passage torn from its context.....

(*He rises and goes to the window, as though to get more light on the subject, and reads the passage a third time. Elizabeth has some difficulty in suppressing her amusement. He turns to her with an expression of humorous chagrin.*

Elizabeth: Well?....

Browning: Well Miss Barrett - when that passage was written only God and Robert Browning understood it. Now only God understands it.

(*she laughs and he joins in*)

What do you say - shall we lighten this great darkness by pitching it on to the fire?

Elizabeth: (*indignantly*) No indeed! We shall do nothing of the kind! Please give me back the book

[*He does so*]

Such passages are only spots on the sun. I love "Sordello."

The Barretts of Wimpole Street. Rudolf Besier.

CHAPTER ONE

Mechanisms of H^+-transport across membranes and the influence of plant hormones, calcium and light.

1.1 Introduction.

Solute transport in plants may be classified into primary active, where movement is directly coupled to a metabolic reaction, secondary active, or passive processes (Nicholls 1982). It is now well established that H^+ gradients at the plasma membrane and tonoplast play a key role in plants in the coupled secondary transport of ions and solutes. This secondary transport is established by a biochemical mechanism that mediates the obligatory coupling of an exergonic biochemical process to the transport of protons across a biological membrane. The H^+ flux may be coupled directly or indirectly to the movement of ions or non-electrolytes across the membrane, and it is known to drive the uptake of amino acids, sugars, Na^+, Ca^{2+} and Cl^-, HPO_4^- and NO_3^- (e.g. Ratner & Jacoby 1976, Komor & Tanner 1980, Lüttge *et al.* 1981, Novacky & Ullrich-Eberius 1983). The coupling between the opposite fluxes of H^+ and of the compensating cations can be either strictly specific and obligatory, with transport occurring at the level of the same catalytic site (chemical coupling) or loose, non-specific, and driven by the transmembrane electrical potential charge induced by the electrogenic H^+ transport (electrical coupling).

The generation of the H^+ gradient is an active process and defined as the net movement of H^+ across a membrane in the opposite direction to that predicted from the prevailing passive driving forces of the H^+ concentration gradient and electrical potential difference (Marrè & Ballarin-Denti 1985). When H^+ pumps are working in a 'forward' direction i.e. converting chemical energy into a H^+ free energy gradient, the H^+ flux is from the side of the membrane at which adenine nucleotides and nicotinamide adenine dinucleotides interact with the pump (Mitchell 1976). At the plasma membrane, active H^+ efflux is from the cytoplasm to the apoplast. Much evidence has

accumulated since 1980 for the existence of ATP-dependent mechanisms which operate the electrogenic transport of protons in non-mitochondrial vesicles, so providing the chemical H^+ gradients within plants. The first H^+ gradient that was demonstrated in non-mitochondrial vesicles was based on the observation that the associated ATPase activity could be stimulated by protonophores and ionophores, which induce either a H^+/K^+ exchange or a depolarization of the membrane potential (Rungie & Wiskich 1973, Sze 1980). Further work on membrane preparations from material including tobacco callus, oat and corn roots, pea internodes, corn coleoptile and radish seedlings has shown that sealed vesicles are capable of building up a strictly ATP-dependent electrical PD, inside positive, associated with an accumulation of H^+ and thus with the formation of a proton gradient (Dupont *et al.* 1982a,b, Macri *et al.* 1982, Bennett & Spanswick 1983, Churchill & Sze 1983, de Michelis *et al.* 1983). The electrogenic H^+ transport demonstrated was specifically ATP-dependent and influenced by protonophores and PD-depolarizing agents. These characteristics, together with the optimum pH between 6.0 and 7.5, the insensitivity to oligomycin and the low activity of mitochondrial marker enzymes in these preparations all provided further evidence for the existence of proton transporting mechanisms which were different from those associated with mitochondria. The most widely accepted value for the H^+/ATP ratio of the ATP-driven H^+ pump is two (Slayman 1974, Raven & Smith 1976a,b), though values of three have also been suggested (Papa 1976).

Active H^+ transport using ATP hydrolysis of the high energy phosphate bond for the generation of the H^+ gradient is widespread in plants, but gradients driven by substrates other than ATP exist and are likely to participate in electron transport at the plasma membrane. Active H^+ transport with membrane-associated redox reactions as the energy source occurs in the plasma membrane of many prokaryotes, and in thylakoids of chloroplasts and cristae of mitochondria in eukaryotes. These pumps are associated with photochemical or thermochemical redox reactions. Transfer of one reducing

equivalent in the respiratory chain from $NADH_2$ to O_2 probably pumps three protons, while in photosynthesis both the non-cyclic and the cyclic pathway pumps two protons (Mitchell 1976, Raven & Smith 1976a,b).

The redox system in other eukaryotic membranes has been investigated by the capacity of cells and isolated membranes to carry out FC-stimulated reduction of exogenous ferricyanide which is associated with an increased H^+ extrusion; for example, in intact carrot cells (Craig & Crane 1981,1982) and maize roots (Federico & Giartosio 1983). These workers also demonstrated the presence of a cyanide-insensitive NADH-ferricyanide oxidoreductase activity in membrane fractions from maize. The operation of an exogenous NADH-oxidising system (O_2 as the final acceptor) at the plasma membrane of maize root protoplasts, with NADH oxidation associated with an increased rate of H^+/K^+ exchange, has also been reported (Lin 1982); this work further demonstrated the isolation from intact protoplasts of a plasma membrane bound NADH oxidising activity. These results are consistent with the view that a trans-membrane redox system transferring electrons from NADH to O_2 (or to an artificial acceptor e.g. ferricyanide) can provide the energy for electrogenic proton extrusion at the plasma membrane.

1.1.1 Properties of the plasma membrane and tonoplast associated ATPase.

Biochemical studies of the ATPase associated with the plasma membrane have shown that activity of this ATPase is inhibited by orthovanadate (Gallagher & Leonard 1982). Vanadate inhibits many phosphohydrolases that have a covalent phosphoenzyme intermediate in the mechanism of action by inhibiting its formation (Cantley *et al.* 1978). A phosphorylated intermediate has been reported in the plasma membrane ATPase from corn roots (Briskin & Leonard 1982) and red beet (Briskin & Poole 1983). Mg^{2+} or Mn^{2+} is specifically required for activity, and is greatly reduced if replaced by Ca^{2+} (Leonard & Hotchkiss 1976) and the basal Mg.ATPase activity is typically stimulated by alkali cations with the general sequence $K^+ > NH_4^+ > Rb^+ >$

$Na^+ > Cs^+ > Li^+$ (Hodges 1976). Anions do not affect the hydrolytic activity. The generally accepted pH optimum for this ATPase is 6.5. This ATPase is not inhibited by the mitochondrial ATPase inhibitor oligomycin (Hodges 1976), DCCD, DES and erythrosin B are all inhibitory of its activity, but not azide, molybdate or ouabain. Recently SW26 has been used as a potent inhibitor of the plasma membrane ATPase and proton transport activity (Blein *et al.* 1986). The other important characteristic of this membrane associated enzyme is its insensitivity to nitrate.

The ATPase associated with the tonoplast is typically sensitive to anions in the order $Cl^-, Br^- > I^- > HCO_3^-$, SO_4^{2-}. Organic anions such as acetate and malate are also stimulatory (Cambraia & Hodges 1980, O'Neill *et al.* 1983, Churchill & Sze 1984). Nitrate and chlorate inhibit the ATPase activity as does DIDS, an anion channel blocker (Cambraia & Hodges 1980, Bennett & Spanswick 1983). The enzyme is vanadate, azide and oligomycin insensitive (O'Neill *et al.* 1983). The characteristics of some of the tonoplast and plasma membrane associated ATPases reported by various workers are outlined in Table 1.1

1.1.2 ATP dependent H^+-pumps.

The pH gradient established by H^+-pumps across tightly sealed vesicle membranes has been monitored by the use of weak bases, such as the fluorescent amine quinacrine. The amine is lipid permeable in its neutral form and impermeable in its charged form. In the absence of a pH gradient, the concentration of the unprotonated and protonated forms inside a vesicle are equal to that in the outside medium. If a pH gradient is generated the concentration of the protonated species increases and becomes trapped within the vesicles, while the concentration of the neutral form remains relatively constant. A change in fluorescence results from the protonation of quinacrine which may be monitored. Quinacrine has been successfully used to measure pH gradients generated by H^+-ATPases in vesicles isolated from a variety of plant tissues (e.g. Hager *et al.* 1980, Dupont *et al.* 1982a,b, Bennett & Spanswick 1983).

Using this probe in particular, characterization of the H^+-ATPases have been made. At least two types of electrogenic H^+-ATPases have been demonstrated in microsomal vesicles from various plant tissues (Sze 1983, Churchill *et al.* 1983, de Michelis *et al.* 1983, Hager & Biber 1984, Chen & Sze 1984, Bennett *et al.* 1984, Scherer 1984c). One type is vanadate-insensitive and the other vanadate-sensitive and have both been separated on linear sucrose and dextran gradients. The vanadate insensitive H^+-ATPase is associated with low density vesicles and likely to be of tonoplast origin (Dupont *et al.* 1982a,b, Mandala *et al.* 1982, Churchill & Sze 1983, Bennett *et al.* 1984, Poole *et al.* 1984). The other is the vanadate-sensitive ATPase that carries out active transport of H^+- and is associated with vesicles of a high density which are thought to be of plasma membrane origin (Churchill *et al.* 1983, Bennett & Spanswick 1984, Scherer 1984c). The characteristics of these two pumps as reported by various workers from different tissues are outlined in Table 1.2. However, caution must be taken with vanadate insensitivity. Studies of H^+-ATPase activity have depended on the isolation of tightly sealed vesicles and, although the membrane ATPase shows strong sensitivity to vanadate, ATP-dependent H^+ transport has been reported to be less sensitive to vanadate (Hager & Helm 1981, Dupont *et al.* 1982b, Mettler *et al.* 1982, Poole *et al.* 1984). The vanadate-sensitive H^+ pump may be more difficult to detect in some plant tissues due to the formation of leaky vesicles or alternatively, tightly sealed vesicles with the substrate site to the interior. At least two classes of ATP-dependent proton pumps have been separated on linear sucrose and dextran gradients.

1.1.3 Plasma membrane associated H^+-ATPases.

These pumps are considered to be analogous to the Na^+, K^+-ATPase, Ca^{2+}-ATPase of the sarcoplasmic reticulum, and gastric H^+, K^+-ATPase of animal cells (Serrano *et al.* 1986). Antibodies raised against plasma membrane associated ATPase from *Saccharomyces cerevisiae* was found to inhibit maize coleoptile ATPase (90KD) from the plasma membrane (Clement *et al.* 1986). This suggests a wide

conservation of catalytic sites or overall conformation.

The plasma membrane-associated H^+-ATPase of fungi and plants catalyze an essentially irreversible reaction involving both ATP hydrolysis and H^+ translocation (Serrano 1984). The enzyme consists of a single catalytic subunit (Scarborough & Addison 1984), forming an aspartyl phosphate intermediate during ATP hydrolysis (Amoury *et al.* 1980). The phosphorylated protein is most stable between pH 2 and 3 (Briskin & Poole 1983) and at low temperatures, consistent with the presence of an unstable acyl phosphate bond (Bader *et al.* 1966, Bastide *et al.* 1973) and a feature common to those transport ATPases that form phosphorylated intermediates (Cocucci *et al* 1980, Amoury & Goffeau 1982).

The enzyme exists in two kinetically and conformationally distinct states during transport termed E_1 and E_2. Recent amino acid sequence data confirms that this class of H^+-ATPase is distinct from the F_oF_1 ion translocases (Serrano *et al.* 1986, Hager *et al.* 1986).

The catalytic subunit of the corn root plasma membrane associated ATPase has a molecular weight of about 95KD in SDS-PAGE and of about 110KD in PAU-PAGE (Gallagher & Leonard 1987). The subunit of the enzyme from oat roots (Serrano 1984), red beet (Briskin & Poole 1983) and tomato roots (Anthon & Spanswick 1986) also has a molecular mass of about 100KD in SDS-PAGE. For red beet, radiation inactivation analysis indicates that the molecular size of the native ATPase is larger than 228KD and probably exists in the plasma membrane as a dimer of catalytic subunits (Briskin *et al.* 1985). Cross-linking studies with solubilized tomato root plasma membrane ATPase indicate that the enzyme though may exist as a trimer (Anthon & Spanswick 1986).

In addition to the major ATPases described above, a Mg^{++} dependent KCl stimulated ATPase has been reported to be associated with the Golgi from corn coleoptiles (Chanson *et al.*1984). ATP-dependent H^+ transport was found to be associated with membranes of density 1.16g cm^{-3} and likely to be of Golgi origin (Chanson & Taiz 1985). The ATPase activity was found to be similar to that of the tonoplast associated ATPase in having an

optimum pH of 7.2, showing stimulation by chloride, inhibition by DES and DCCD and insensitivity to oligomycin and azide, and a strong specificity for Mg^{2+}ATP. However, the H^+ pump was found to be much less sensitive to nitrate and iodide and more sensitive to SITS and DIDS than the tonoplast H^+ Golgi pump. A further difference reported was that the Golgi pump was stimulated by valinomycin in the presence of KCl, whereas the tonoplast ATPase is not.

1.1.4 Mitochondrial, chloroplastic and prokaryotic plasma membrane-associated ATPase.

The F_1F_o-ATPase has been found to be associated with all these membranes. This ATPase catalyzes a freely reversible reaction but does not form a phosphorylated intermediate during ATP hydrolysis (Maloney 1982). The enzyme molecule consists of several distinct subunits and there is partitioning of function between these subunits, the F_1 sector functioning as an ATPase and the F_o sector as a H^+ channel. The mitochondrial ATPase complex is located in the inner mitochondrial membrane and consists of three components, an F_oF_1 and an oligomycin-sensitive conferring protein (OSCP) (Tzagoloff 1982).

The various subunits of the complex are encoded either by the nuclear or mitochondrial genome. In most organisms, the genes for the five F_1-subunits are encoded by the nuclear genome. These subunits are translated on cytosolic ribosomes yielding precursors with amino-terminal signals that facilitate transport into the mitochondrion (Neupert & Schatz 1981). In yeast, subunits 6, 8 and 9 of the F_o component are mitochondrial gene products while the other subunits are of nuclear origin (Yaffe & Schatz 1984). Subunit 6 from maize mitochondria has been sequenced (Dewey *et al.* 1985a) and the nucleotide and amino acid homology that exists between this subunit and that from mitochondria of eukaryotic organisms indicates that it is also encoded by a mitochondrial gene in this species. The situation in animal systems and in the fungi *Neurospora* and *Aspergillus* differs in that subunit 9 is encoded in the nucleus. Higher plant mitochondrial genomes contain a gene coding for subunit 9 (Dewey *et al.* 1985b). Maize and *Vicia faba* differ from

Table 1.1 Characteristics of the ATPase associated with the tonoplast and plasma membrane in plants. Percentages are inhibition values over the control at the inhibitor concentrations quoted. (NI - No inhibition).
(1) Hodges & Leonard 1974, (2) Leonard & Hotchkiss 1976, (3) Anderson & Ray 1978, (4) Dupont *et al.* 1982b, (5) Yoshida *et al.* 1986a, (6) Bennett *et al.*1984, (7) Mandala & Taiz 1985, (8) Sommarin *et al.* 1985, (9) Leigh & Walker 1980, (10) Sommarin *et al.* 1985, (11) Scherer & Fischer 1985, (12) Jochem *et al.* 1984, (13) Yoshida *et al.* 1983, (14) Briskin & Thornley 1985, (15) Cocucci 1986, (16) Memon *et al.* 1987, (17) Yoshida *et al.* 1986b, (18) Anthon & Spanswick 1986, (19) de Michelis & Spanswick 1986.

PLASMA MEMBRANE	TONOPLAST	TISSUE	REFERENCE
Density gcm^{-3}			
1.15-1.20		corn root	2
1.16-1.18		oat root	1
1.18		pea internode	3
1.17	1.11	zucchini hypocotyl	11
	1.06-1.07	mungbean	17
	1.1-1.12	corn root	4
Substrate specificity			
ATP>>UTP>ITP>IDP>AMP ATP>>CTP>ITP,		orchard grass	5
UTP,UDP>GDP,IDP>GTP ATP>>UTP>GTP>CTP,		cucumber root	16
ITP,UDP ATP>>CTP>UTP>UDP>		oat root	8
CDP>GTP>IDP ATP>>UDP>IDP>ITP>		wheat root	8
GTP>ADP>CTP		sugar beet	14
	ATP>>GTP>ITP>PPi>UTP	corn root	7
K_m ATP.Mg			
0.7mol m^{-3}		corn roots	19
0.75mol m^{-3}		sugar beet	5
Specific Activity (µmol Pi $mg^{-1}min^{-1}$)			
0.7		oat root	10
9.43		radish	15
1.2	7.41	cucumber root	16
0.15		wheat root	8
	1.18	corn root	7
	0.6	red beet	6
ATPase pH optimum			
6.5		corn root	2
5.8		cucumber root	16
	8.0	Red beet	9
	7.0	Mung bean	17

PLASMA MEMBRANE	TONOPLAST	TISSUE	REFERENCE
Cation/Anion sensitivity			
	$Mg^{2+} > Mn^{2+} > Ca^{2+}, Co^{2+}$	*Kalanchoë*	12
$K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$		corn root	2
	$Cl^- > HCO_3^-$	*Kalanchoë*	12
Nitrate sensitivity			
	100% 100mol m^{-3}	corn coleoptiles	7
89% 50mol m^{-3}		*Kalanchoë*	12
NI		cucumber root	16
1% 0.1mol m^{-3}		oat	8
53% 0.1mol m^{-3}		wheat	8
Vanadate sensitivity			
50% $60x10^{-3}$mol m^{-3}		cucumber root	16
52% 0.25mol m^{-3}		oat root	8
50% $22x10^{-3}$mol m^{-3}		corn coleoptile	17
91% $50x10^{-3}$mol m^{-3}		sugar beet	14
80% $50x10^{-3}$mol m^{-3}		red beet	6
49% $50x10^{-3}$mol m^{-3}	8% $50x10^{-3}$mol m^{-3}	orchard grass	13
DCCD sensitivity			
89% $100x10^{-3}$mol m^{-3}		red beet	6
17% 0.1mol m^{-3}		oat root	8
51% 0.1mol m^{-3}		wheat root	8
50% 0.1mol m^{-3}		corn root	2
83% 0.1mol m^{-3}		cucumber root	16
	100% $100x10^{-3}$mol m^{-3}	orchard grass	13
Azide sensitivity			
5% 5mol m^{-3}		tomato root	18
NI		sugar beet	14
	2% $100x10^{-3}$mol m^{-3}	corn coleoptile	7
SW26 sensitivity			
50% $8.5x10^{-3}$mol m^{-3}		corn hypocotyl	17
50% $14.5x10^{-3}$mol m^{-3}		sycamore cells	17
Erythrosin B sensitivity			
72% $100x10^{-3}$mol m^{-3}	73% $100x10^{-3}$mol m^{-3}	radish	15

both fungi and animals in that the mitochondrion codes for the F_1-α (58KD) subunit of the F_1 component (Hack & Leaver 1983, Boutry *et al.* 1983).

1.1.5 ATPase associated with the tonoplast.

This category of ATPase is associated not only with the tonoplast (Poole *et al.* 1984, Bowman & Bowman 1982, Uchida *et al.* 1985) but also with lysosomes (Schneider 1981, Ohkuma *et al.* 1982), chromaffin granules and synaptosomes (Cidon *et al.* 1983, Serrano *et al.* 1986), clathrin-coated vesicles (Xie *et al.* 1984) and endosomes (Galloway *et al.* 1983). In all cases the H^+-ATPase is insensitive to vanadate and azide, but reversibly inhibited by nitrate and stimulated by Cl^-. The 70KD and 60KD subunits of the endomembrane ATPase from chromaffin granules, adrenal medulla and higher plant tonoplast are immunologically cross-reactive with each other (Manolson *et al.* 1987) but not with the α- and β-subunits of mitochondrial F_oF_1, supporting the view that these are all members of one class (Bowman *et al.* 1986). The ATPase appears to be a multimer containing a 57KD and a 16KD subunit, with an associated 67KD polypeptide (Manolson *et al.* 1985).

Mandala & Taiz (1985) proposed that the functional molecular weight of the tonoplast ATPase is nearly 400KD, and that the molecule is composed of several polypeptides, including 72KD and 62KD proteins. The vacuolar ATPase of *Neurospora* was found to have associated polypeptides of molecular weights 70KD, 60KD and 16KD with a holoenzyme of molecular weight 520KD. The latter polypeptide bound [^{14}C]-DCCD, and may represent the proton channel of the ATPase (Bowman 1983). The 70KD and 60KD polypeptides have the characteristics of catalytic and regulatory subunits, respectively. The 16KD polypeptide is just visible with coomassie blue or silver stain on SDS gels but can be detected fluorographically after [^{14}C]-labelling with the lipophilic carboxyl reagent DCCD. This data suggests that the 16KD component is strongly hydrophobic and deeply embedded in the phospholipid bilayer.

1.1.6 Pyrophosphatase activity.

Inorganic pyrophosphatase activity is reported to be associated with tonoplast isolated from the storage roots of *Beta vulgaris* (Leigh & Walker 1980). More recently there have been reports of the presence of a Mg^{2+}-dependent PPase proton transport in sealed microsomal vesicles from maize roots (Dupont *et al.* 1982a), oat roots (Churchill & Sze 1983) and red beet (Bennett *et al.* 1984). H^+-translocating inorganic pyrophosphatase has been shown to be associated with low density tonoplast vesicles from red beet (Rea & Poole 1985) and oat roots (Wang *et al.* 1986). However, in maize coleoptiles, a PPi dependent pump is associated with tonoplast and Golgi rich fractions (Chanson *et al.* 1985). In maize roots, a PPi dependent pump was found associated with the tonoplast, but Golgi and endoplasmic reticulum markers were overlapping indicating that these membranes may similarly possess a PPi dependent pump (Chanson & Pilet 1987). The PPi driven pump is independent of the ATP-dependent pump (Rea & Poole 1985). Activity is inhibited by nitrate (Chanson *et al.* 1985) and is insensitive to vanadate and azide. The alkaline pyrophosphatase with a pH optimum of 8.5, is Mg^{2+}-dependent and reported to be further stimulated by salts of monovalent cations in the order K^+, Rb^+, NH_4^+ > Cs^+. Activity is greatly reduced by the replacement of Mg^{2+} with Mn^{2+} or Zn^{2+} and Ca^{2+}. Co^{2+} and Cu^{2+} do not support activity (Walker & Leigh 1981).

PPase catalyzes the electrogenic transport of H^+ into membrane vesicles generating a pH gradient and membrane potential. The enzymic hydrolysis of PPi to Pi is the simplest example of the utilization of a high energy phosphate bond and recent investigations have shown the presence of a high concentration of PPi in plant tissues (Smyth & Black 1984), which suggests that PPi-dependent proton pumps may be operative in plants. Consideration has been given as to why these two pumps co-exist (Rea & Sanders 1987). Although PPase and ATPase activity appears to be associated with the tonoplast, they may not both be associated with the same vacuole, but may reside in distinct sub-populations. However, the kinetics of the ATP- and PPi-dependent H^+ translocation point

Table 1.2. Characteristics of ATP-dependent proton transport associated with the plasma membrane and tonoplast. Percentages are inhibition values over the control at the inhibitor concentrations quoted. (1) de Michelis & Spanswick 1986, (2) Giannini & Briskin 1987, (3) Rasi-Caldogno *et al.* 1986, (4) Mandala & Taiz 1985, (5) Blein *et al.* 1985, (6) Rasi-Caldogno *et al.* 1985.

PLASMA MEMBRANE	TONOPLAST	TISSUE	REFERENCE
pH optimum			
6.5		corn root	1
6.5	7.75	red beet	2
6.56		radish	3
Mg^{2+} sensitivity			
100%		corn root	1
	100%	corn root	4
Cation/anion sensitivity			
$Mg^{2+}>Co^{2+}>Mn^{2+}>>$ $Zn^{2+}>>Ca^{2+}, Mg^{2+}Ca^{2+}$		corn root	1
$Cs^{+}>>Rb^{+}>Li^{+}>Na^{+}$		sycamore	5
$K>>Na>choline>BTP$		red beet	3
$I^{-}>>NO_3^{-}>Br^{-}>$ $ClO_3^{-}>SO_4^{2-}$	$I^{-}>Br^{-}>Cl^{-}>>$ $ClO_3^{-}>SO_4^{2-}$	red beet	2
Vanadate sensitivity			
74% $100x10^{-3}$mol m^{-3}		red beet	2
79% $100x10^{-3}$mol m^{-3}	0% $100x10^{-3}$mol m^{-3}	radish	6
Nitrate sensitivity			
+7% 100mol m^{-3}	69%100mol m^{-3}	red beet	2
Oligomycin sensitivity			
2% 2.5μg cm^{-3}		corn root	1
DCCD sensitivity			
9% $50x10^{-3}$mol m^{-3}		corn root	1
DES sensitivity			
65% $30x10^{-3}$mol m^{-3}		corn root	1
NaF sensitivity			
43% 10mol m^{-3}		corn root	1
50% 25mol m^{-3}	30%25mol m^{-3}	red beet	2

to co-residence. A stoichiometric determination has yet to be made, but a rate of ⩾2 would indicate the presence of two H^+ pumps within the same membrane. The tonoplast ATPase would act physiologically as a pump, while the H^+ it generated would be used to synthesize PPi which would possibly stabilize cytosolic PPi levels (Rea & Sanders 1987).

1.1.7 Role of H^+ pumps in cytosolic pH control.

There are few reported measurements of cytosolic pH in higher plant cells, but a slightly alkaline value is generally indicated e.g. *Zea* root cells, 7.1 (Brummer *et al.* 1985), *Zea* root tip, 7.2 (Roberts *et al.* 1981), *Zea* root hair, 7.3 (Brummer *et al.* 1984). Although cytosolic variation of 0.5 pH units has been reported (Raven & Smith 1974), it is clear that in both growing and non-growing cells the cytosolic pH is tightly regulated to allow efficient functioning of enzymes involved in metabolism, which are mostly very sensitive of pH changes.

A number of mechanisms have been postulated to explain the control of cytosolic pH. A contribution may be made by intracellular buffers, but as they are likely to be produced by reactions which consume or produce H^+ are unlikely to play a major role in the pH stat. Another mechanism proposed to maintain the cytoplasmic pH stat involves the concentration of carboxyl groups in the cytoplasm (Davies 1973a&b). This mechanism postulated that production of H^+ or OH^- by secondary metabolism was carried out to counter excess OH^- or H^+ production in primary metabolism. It was envisaged that a pH rise within the cytoplasm is counteracted by malate production from weak or neutral acid substrates by phosphoenolpyruvate carboxylase. Lowering of the cytoplasmic pH inhibits malic acid production but stimulates the malic enzyme, with production of pyruvate, CO_2 and OH^-. This maintains the cytoplasmic pH in the event of either acidification or alkalinization. Although this mechanism may participate in the maintenance of the pH stat, it is unlikely to be the only mechanism in operation.

The major control of cytoplasmic pH is likely to

require the transport of H^+ or OH^- between the cell and its bathing medium, and is likely to be fulfilled by the plasma membrane and tonoplast H^+ pumps *via* an energy dependent process (Raven & Smith 1974, Smith & Raven 1979). The contribution made to the cytoplasmic pH by the chloroplasts and mitochondria which similarly actively transport H^+ across their outer membranes is debatable. Alkalinization of the cell may be countered by H^+ re-entering by a carrier mediated uniport or as part of a carrier complex involving other solutes.

It has been argued that regulation of cytoplasmic pH has imposed a primary selective pressure on plants throughout their evolutionary history and may be responsible for the development of the H^+ pump. Growth with CO_2 or carbohydrate as the carbon source and NH_4^+ as nitrogen source leads to the production of excess H^+ in the cytoplasm which cannot be countered by the pH stat mechanism. Conversely, NO_3^- as the sole N source leads to the generation of OH^- in the cytoplasm (Raven & Smith 1976b). In most algae OH^- is disposed of entirely by excretion, while in others such as *Acetabularia* and in aquatic and terrestrial angiosperms, some of the OH^- is neutralised by the pH-stat. This mechanism was unlikely to be adequate for terrestrial plants and levels were maintained by the development of active H^+ efflux (Raven & Smith 1976a,c).

1.2 Mechanism of action of auxin in plant growth.

1.2.1 Cell wall rigidity.

Cytoplasmic and vacuolar expansion is limited by the rigidity of the plant cell wall, and the rate at which the wall yields and expands in response to turgor changes governs the rate of cell growth. For expansion to occur, the wall must undergo a loosening process and in both monocotyledons and dicotyledons, the outer epidermal cell layers appear to exercise greatest control over growth by being the first cells to expand.

In monocotyledons, the cell wall loosening mechanism is associated with the degradation of β(1-3)(1-4)glucan (Sakurai *et al.* 1979, Yamamoto *et al.* 1980) and depolymerization of an insoluble xyloglucan

(Inouhe *et al.* 1984). In dicotyledons, the loosening mechanism appears to be associated with the degradation of galactan (Nishitani & Masuda 1980) and depolymerization of a xyloglucan (Nishitani & Masuda 1981). Additionally, cell wall synthesis must occur during cell growth and promotion of synthesis by auxin has been reported in coleoptiles (Bonner 1934) and dicotyledonous stem segments (Christiansen & Thiman 1950). During auxin-induced growth in *Pinus pinaster* and various other gymnosperms, there are reports of a very active turnover of glucose-rich polysaccharide (Lorences & Zarra 1986) and synthesis of a high molecular weight polysaccharide (Lorences *et al.* 1987).

1.2.2 The acid growth theory.

Several theories have been proposed to explain the mechanism of rapid auxin-induced cell elongation and most attention has been paid to the acid growth theory of Rayle & Cleland (1970) and Hager *et al.* (1971). It suggests that auxin induces efflux of H^+ from the cytoplasm into the cell wall. The lowered pH of the wall solution causes either a direct breakage of acid-labile cell wall bonds or activates enzymes with acidic pH optima which cleave load-bearing bonds, allowing turgor-driven growth to take place. Breakage of these bonds has been correlated with changes in the composition of cell wall polysaccharides, which cause the cell wall loosening needed for wall extension (Masuda 1978, Taiz 1984).

The acid growth theory must fulfil the following criteria (Cleland 1980):

1. That cells excrete protons on application of auxin;
2. That exogenous protons must be able to substitute for auxin in causing cell wall loosening and growth;
3. Buffers at a neutral pH must inhibit auxin-induced growth;
4. Other agents which induce H^+ excretion must promote the stimulation of growth in the same manner as that caused by IAA.

Supportive evidence for this theory is provided by reports that exogenous auxin promotes acidification of the

bathing medium by hypocotyl and coleoptile segments (e.g. Marrè *et al.* 1973, Jacobs & Ray 1976, Mentze *et al.* 1977). Further evidence is provided by the demonstration that exogenous protons can substitute for IAA and rapidly promote elongation in various tissues including pea and sunflower (Bonner 1934, Ganot & Reinhold 1970, Rayle & Cleland 1970, 1972, Hager *et al.* 1971, Yamamoto et al 1974). Auxin stimulated growth is not suppressed by metabolic inhibitors such as cyanide and cycloheximide. Neutral buffers inhibit auxin-induced growth of *Avena* coleoptile and pea epicotyl sections (Hager *et al.* 1971, Durand & Rayle 1973, Jacobs & Ray 1975, Rayle & Cleland 1980). This suggests that auxin may promote growth by causing active acidification of the cell wall (Hager *et al.* 1971, Rayle & Cleland 1972), though the objection has been raised that neutral buffers may inhibit auxin uptake.

1.2.3 Criticisms and data not supporting the acid growth theory.

Many observations have been reported that do not support the acid growth theory, but some have subsequently been resolved. With regard to measurement of H^+ excretion, it has often proved difficult to detect auxin-induced movement of protons from the cytoplasm into the cell wall space. However, acidification of a bathing solution by isolated hypocotyl segments has been reported to occur equally readily in the presence of water and auxin. Using abraded pea and cucumber hypocotyls segments, Brummell *et al.* (1986) found that the pH of the bathing solution dropped from 6.5 to 5.0 in 60 min in the presence of water alone. With 10^{-2}mol m^{-3} IAA in the bathing medium, the pH dropped a further 0.1 pH unit. Similarly, soybean hypocotyl segments lowered the pH of a bathing solution to 5.3 in the presence or absence of auxin (Vanderhoef *et al.* (1977). Growth in these tissues was stimulated by IAA. This result may reflect an unsatisfactory method for the detection of H^+ excretion. Cleland & Rayle (1978) found that auxin caused a dramatic decrease in the pH compared with the water control and suggested that this difference was due to an acid pre-wash which saturates carboxyl groups in the wall of the tissue

(Cohen 1985).

The detection of H^+ excretion depends on the permeability of the cell wall and cuticle and the volume of the external bathing solution. The permeability of the cuticle to protons is very low, and is similar to that for cations e.g. K^+ (McFarlane & Berry 1974). Auxin-induced H^+ excretion is readily detected when microelectrodes are inserted into the tissue (Jacobs & Ray 1976) or strapped to the cell wall surface (Cleland & Rayle 1978). The impermeability of the cuticle has been overcome also by gentle abrasion with carborundum powder (Brummell *et al.* 1986) making it possible to detect H^+ movement into a bathing solution from hypocotyl tissue. The tissue/volume ratio must be kept as high as possible to optimize detection of H^+ extrusion into the bathing solution. Vanderhoef *et al.* (1977) used a very low tissue/volume ratio and non-abraded tissue, which probably explains the observed lack of acidification of the external medium. Measurement of medium acidification, though useful, is a very crude assay. Although it is assumed to measure efflux across the plasma membrane, it cannot take into account the influence of surrounding organelles and tissues. It has been reported that every cell has an equilibrium pH (Cleland 1975). Cortical cells have an equilibrium pH of 5.5-6.0 and the equivalent value for epidermal cells lies in the range of 4.5-5.0. In dicotyledonous tissues the control of auxin-induced growth occurs mainly in the epidermal and sub-epidermal layers. If cortical cells predominate in the tissue being examined, H^+ may be absorbed by the cortical area and so mask any H^+ excretion.

Some other experimental observations cannot be explained readily by the acid growth theory and include reports that auxin promotes growth in certain tissues, while an acidic pH has little or no such effect. Pope (1978) showed that oat coleoptiles when transferred from pH 3 to 7 showed little response to a second immediate exposure to acid whereas, if placed in auxin, tissue growth occurred. Pope (1978) also reported that on adding auxin to wheat coleoptile segments in a buffered solution at pH 3.4, further growth is induced, suggesting that

acidification only partially accounts for the growth-promoting action of auxin, and that additional components are involved. It has been shown that H^+ efflux is not directly related to growth (Ilan & Shapira 1976). For example, a large stimulation of growth occurs in the presence of auxin and sodium, but little acidification is detectable in sunflower hypocotyls. In the presence of NH_4^+, no significant change in acidification occurred but there is enormous stimulation of growth. Furthermore, under conditions of similar H^+ efflux, different growth responses are observed, indicating that H^+ efflux is not related directly to growth. Naphthyl acetate (NA) is taken up and hydrolyzed by esterases to acetic acid in coleoptile segments (Vesper & Evans (1979). Acid efflux can therefore be manipulated by changing the concentration of NA applied. The effects of auxin- and NA-induced efflux on growth were compared. With auxin, there was significantly more growth than in the presence of NA. Similarly, fusicoccin, when applied at a concentration that causes similar H^+ efflux to that caused by IAA, fails to induce growth of maize coleoptiles (Kutschera & Schopfer 1984). This indicates that although auxin induces acidification and therefore growth, further stimulation of growth involves an additional mechanism.

1.2.4 Development and interpretation of the biphasic response.

In early experiments auxin-stimulated growth was observed to be a biphasic response in auxin-depleted dicotyledonous stem segments floating on neutral buffer (e.g. Köhler 1956, dela Fuente & Leopold 1970, Philipson *et al.* 1973). Typically, there is an initial lag of only a few minutes, the growth rate then rises to a maximum, falls and rises again to a steady-state. Vanderhoef was the first to develop the biphasic concept and to show that the two phases were separable (Vanderhoef & Stahl 1975, Vanderhoef *et al.* 1976). Cytokinin inhibits auxin-induced growth in soybean hypocotyls by inhibiting the long-term response to auxin, but not the short-term response (Vanderhoef & Stahl 1975). This biphasic growth response has been confirmed in a variety of species including pea

(Barkley & Evans 1970, P.Penny *et al.* 1972), lupin (D.Penny *et al.* 1972), soybean (Vanderhoef & Stahl 1975) and cucumber (Kazama & Katsumi 1976). The biphasic growth response has been definitively demonstrated in abraded sunflower hypocotyls (Vesper 1984). A pH of an external solution was used which caused a similar growth response to that produced by 0.3×10^{-3} mol m^{-3} IAA. On summing the two components, additional promotion of elongation was obtained. This result indicates that, while IAA causes acidification and growth, it also stimulates growth directly by a separate mechanism. The initial phase of growth is thought to be due to rapid auxin stimulation of H^+ extrusion causing wall loosening, while the second phase involves a slower auxin promotion of other processes such as cell wall synthesis (Vanderhoef & Dute 1981).

1.2.4.1 The initial growth phase and the mechanism of H^+ efflux.

In maize coleoptiles this initial phase of growth appears to be brought about by the loosening of the axial extensibility of the outer epidermal cell wall, which is under strong elastic tension in order to maintain the cell walls of the inner tissues in a compressed state (Kutschera & Schopfer 1986). Removal of the epidermis allows the inner tissues to extend spontaneously in water by about 5%, and it is proposed that auxin causes the same release of elastic tension.

With regard to the mechanism of auxin-induced H^+ efflux, the major candidate is the H^+-ATPase associated with the plasma membrane (Cleland & Lomax 1977). It is proposed that, in responsive cells, auxin stimulates growth by activating a plasma membrane-bound ATPase which drives the transport of protons from the protoplast into the cell wall space (Hager *et al.* 1971). Evidence for the involvement of the plasma membrane associated ATPase in auxin-stimulated H^+ efflux is available for oat coleoptiles (Jacobs & Taiz 1980) and pea and cucumber hypocotyls (Brummell *et al.* 1986). In the latter, vanadate was found to inhibit auxin-enhanced H^+ secretion in pea and cucumber tissues and auxin-stimulated growth in cucumber. Furthermore, direct stimulation of the plasma

membrane associated ATPase from *Cucurbita maxima* (Scherer 1981) and of ATP-dependent H^+ transport in pea root vesicles has been reported (Gabathuler & Cleland 1985).

The fungal phytotoxin, fusicoccin, parallels auxin in its ability to promote growth in epicotyl sections (Marrè et al. 1973), *Avena* coleoptiles (Cleland 1976a) and soybean hypocotyls (Cleland & Rayle 1977), and to stimulate H^+ excretion in *Phaseolus aureus* hypocotyls (Goldberg & Prat 1979). The following observations strongly suggest that the fusicoccin-sensitive electrogenic H^+ pump is located in the plasma membrane:

1. The demonstration of a high affinity FC-binding factor in plasma membrane-enriched preparations from maize coleoptiles (Dohrman *et al.* 1978, Pesci *et al.* 1979a,b, Ballio *et al.* 1980, Aducci *et al.* 1982);
2. The observation that FC hyperpolarizes the transmembrane PD across the plasma membrane, rather than that across the tonoplast (Goldsmith and Cleland 1978)
3. Circumstantial evidence for alkalinization of the cytoplasm by FC, as inferred by its effects in increasing cell sap pH (Guern *et al.* 1982).

Recently, it has been reported that fusicoccin stimulates ATP-dependent H^+ transport in plasma membrane vesicles from *Raphanus sativus* (Rasi-caldogno *et al.* 1986).

Ca^{2+} and K^+ are both required for strong auxin-induced H^+ efflux from pea internode sections, though growth can occur in the absence of these ions and has also been reported to slightly decrease growth in their presence (Terry & Jones 1981). Similarly H^+ efflux in maize coleoptiles is greatly reduced if Na^+ is substituted for K^+ in the incubation medium though there is no change in growth (Kutschera & Schopfer 1984). Using this system it was observed that treatment of coleoptile sections with a variety of sugars caused stronger H^+ efflux than treatment with auxin, but no change in growth. The authors concluded that induction of growth by IAA is independent of the secretion of protons, but that the results obtained reflect the two phases, medium acidification and cell wall synthesis. The former makes

only a small contribution to sustained growth and was probably undetected, but the observed medium acidification demonstrated that this phase does require K^+. The second phase requires IAA in the absence of K^+. This supports the view that, although medium acidification may be very transitory, it is brought about by a K^+-stimulated plasma membrane ATPase. The promotion of wall acidification by calcium may be related to the ability of calcium to greatly reduce the passive permeability to protons of lipid bilayer membranes assembled from root cell phospholipids (Rossignol & Grignon 1982).

1.2.4.2 The control of RNA levels by auxin during the second phase of growth.

When cucumber hypocotyls are given a vanadate pretreatment and then kept at pH 4.0, IAA causes growth even at this low pH, presumably by affecting some process other than acidification (Brummell 1986). There is now increasing evidence for auxin induced changes in translatable messenger RNA (mRNA), which occur well within the latent period of the second phase of the auxin response. Auxin-promoted growth is accompanied by a change in the pattern of newly synthesized proteins (Zurfluh & Guilfoyle 1980). A large increase in rRNA and mRNA in soybean hypocotyls within 15 min is reported to take place in pea within 20 min of treatment with IAA or 2,4-D (Theologis & Ray 1982). Promotion of activity of pre-existing β-glucan synthetase occurs within 15 min of treatment in pea (Ray 1973a,b) and increased transcription rates have been observed within 5 min of application of auxin in soybean plumules (Guilfoyle *et al.* 1984).

Correlation of the pattern of translation products with growth in responsive tissues has been studied, and it is reported that in actively growing soybean plumules, the level of translatable mRNA is low, while in the lower non-actively growing region of the hypocotyl, mRNA levels are increased (Baulcombe *et al.* 1981). No likely polypeptide candidates have been found to be synthesized within the latent period although auxin binding proteins may be candidates. Trewavas (1980) was unable to detect auxin-

binding activity in membrane preparations from artichoke, but, within 1 day of addition of 2,4-D, specific auxin receptors were detectable.

1.2.5 Alternative explanations for the biphasic growth response.

It has been found that in segments of lupin hypocotyl with the epidermis removed, the initial response to auxin is absent, but the more long-term response is still expressed (Pearce & Penny 1983). It is suggested that the primary response to auxin is the result of auxin-induced epidermal relaxation and the secondary phase is due to a slower response of the cortex. More recently, it has been found that on removal of the starch sheath, all tissues within the hypocotyl (including the stele) are acid responsive, although the cortex was most responsive and the outer cell layers the least (Pearce & Penny 1986). These results suggest that acid-induced elongation of intact segments may be controlled by the response of the least acid-responsive tissue, the outer cell layers. The effect of acidification in these cell layers alone might be sufficient for the whole hypocotyl to elongate, at least in the initial stages. However, it has not been possible to monitor changes of cell wall pH in the outermost layers in response to the pH of the bathing medium.

An alternative to the wall acidification theory was presented by Brummer & Parish (1983). Their hypothesis predicts that auxin may induce extension growth by lowering the cytoplasmic pH. This is said to occur by the binding of auxins to specific receptors on the endoplasmic reticulum and plasma membrane, which induces an increase in the cytoplasmic levels of free Ca^{2+}. The latter is taken up by mitochondria which secrete protons into the cytoplasm, and so stimulates the plasma membrane bound ATPase and H^+ efflux.

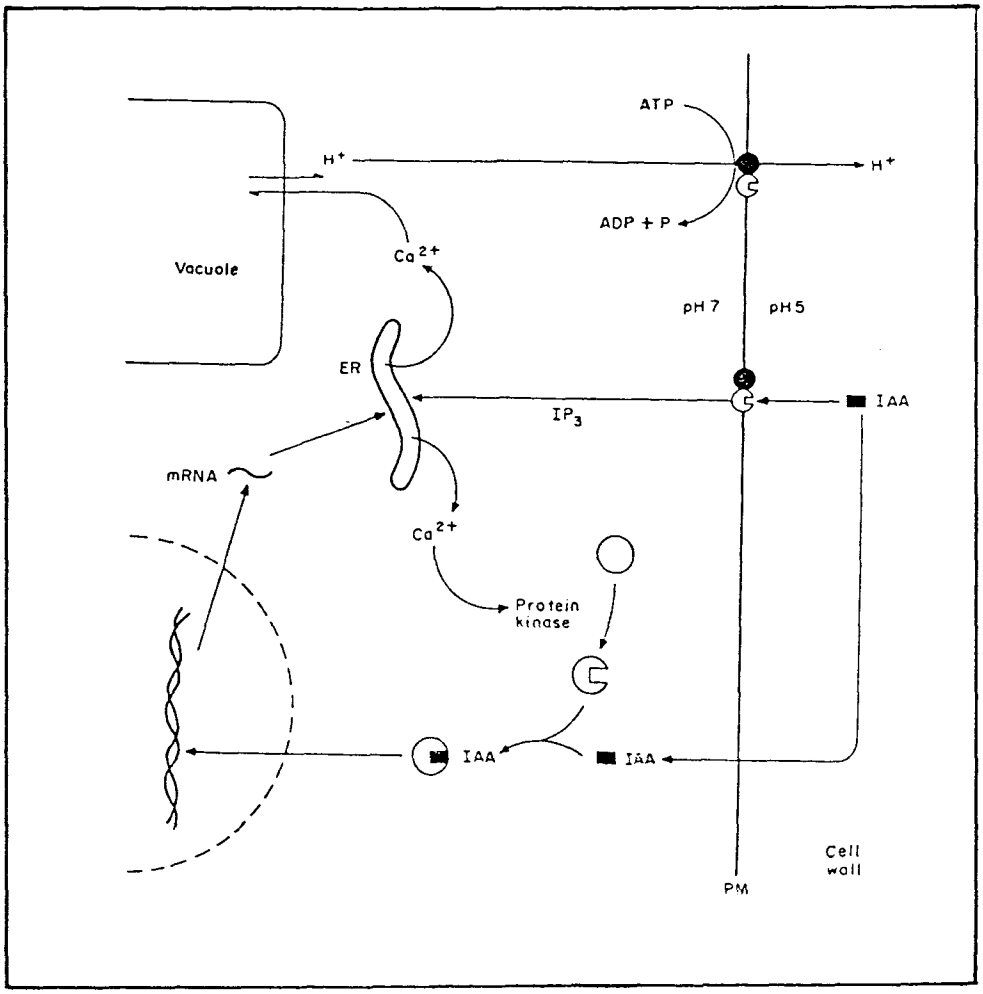
The acid growth theory and the hypothesis of Brummer and Parish all rely on the acidification of the protoplast being the trigger for growth. However, as noted earlier, a constant pH stat is operational in the cytoplasm and it is likely not to be adjusted for the purpose of wall

acidification which would require an acid environment for a long period of time. Taking this into account and although a rapid auxin-induced decrease in the cytosolic pH of maize coleoptile cell has been detected (Brummer et al. 1985), the effect was very small. The model does provide an explanation for several of the most rapid cellular effects of auxin and for the first growth response in isolated segments which is due to H^+ movement towards the apoplast.

An alternative to the acid growth theory has been proposed by Theologis (1986). Auxin stimulated H^+ excretion is thought to be associated with secretory vesicles rather than the plasma membrane, and to provide part of the energy requirement for the transport and incorporation processes. It is suggested that mRNA synthesis is the primary auxin event with all other events of growth resulting from this. Growth is then regulated by a protein product of these mRNA's which facilitates the fusion of secretory vesicles with the plasma membrane and thus the incorporation of new wall materials. But this model does not explain the biphasic growth response nor the direct effect of auxin on membrane bound ATPase activity (Scherer 1984a, Gabathuler & Cleland 1985).

A new model has been put forward which includes all the auxin-induced observations (Brummell & Hall 1987, Fig.1.1). The primary consequence of auxin binding to a receptor is envisaged to cause an increase in the cytoplasmic Ca^{2+} concentration *via* transport across the plasma membrane or *via* phosphoinositides, which mediate release of Ca^{2+} from internal stores such as the endoplasmic reticulum or vacuoles. This transient Ca^{2+} rise is thought to trigger a Ca^{2+}/H^+ antiport or Ca^{2+}-ATPase in the endoplasmic reticulum restoring the cytoplasmic Ca^{2+} status. The sequestration of Ca^{2+} *via* the antiport causes an increase in H^+ concentration which therefore stimulates the electrogenic ATP-driven H^+ extrusion across the plasma membrane. It is further suggested that the transient Ca^{2+} rise activates protein kinases which phosphorylate proteins in the cytoplasm and bind to IAA which move into the nucleus and activate the transcription of specific mRNA sequences.

Figure 1.1 Figure taken from Brummell & Hall 1987. Model for auxin action on growth in dicotyledons. Auxin binds to a receptor (⊕) on the plasma membrane, which may be closely associated with an H^+-transporting ATPase (●). *Via* a phosphoinositide intermediate such as inositol-1,4,5- triphosphate (IP_3), Ca^{2+} is released from the ER into the cytosol. The elevated cytosolic Ca^{2+} concentration is reduced back to its normal resting level by antiport of Ca^{2+} into the vacuole in exchange for H^+. The consequent decrease in cytosolic pH stimulated H^+-transporting ATPases on the plasma membrane to extrude the excess H^+. HOwever the transient increase in cytosolic Ca^{2+} levels stimulates protein kinase activities which by protein phosphorylation, convert an inactive protein factor (○) into an active state capable of binding auxin (⊕). The protein-auxin complex so formed acts to derepress certain regions of the genome and specific mRNA sequences are synthesized. It is suggested that the protein products of these mRNA's are involved in cell wall synthesis, and the resulting increased incorporation of new matrix wall components into the wall brings about wall loosening and growth.


ATP
H+
H+
ADP + P
Vacuole
Ca2+
pH 7
pH 5
ER
IAA
IP3
mRNA
Ca2+
Protein kinase
IAA
IAA
Cell wall
PM

1.3 Levels of calcium within plants: its maintenance and its control of specific enzymes and physiological processes.

1.3.1 Role of calcium within plants.

Measurement of whole plant calcium levels in calcicole species are found to reflect the calcium status of the soil, indicating that selective Ca^{2+} uptake does not occur in many species (Ingestad 1974). Land plants have been classified on the basis of their calcium tolerance indicating that many have evolved to fill a particular niche, while others occur over a range of soil calcium status's. Plants may be placed in the following categories (Ingestad 1974):

1. Plants that are intolerant of a high calcium status, the calcifuges.
2. Plants that are obligate and only grow under a high Ca^{2+} regime, the calcicoles.
3. Plants which are able to grow over both soil Ca^{2+} regimes.

Until recently no specific intracellular ion measurements were possible and it was thought that the plant calcium status was controlled primarily at the root and that the cytoplasmic calcium status varied enormously between these plant categories, depending on the soil calcium status. Cytoplasmic Ca^{2+} levels were measured initially in the algal cells of *Fucus* and *Chara* by microinjecting the Ca^{2+} indicator aequorin and a mean value of 10^{-4}mol m^{-3} was calculated (Williamson & Ashley 1982). Further measurements were made by inserting microelectrodes and by the use of calcium indicating dyes (Brownlee & Wood 1986). These investigations have shown that lower plants maintained free cytoplasmic calcium at 10^{-4}mol m^{-3}, whereas the recent use of calcium indicating dyes suggest that higher plant cytosolic Ca^{2+} concentrations are maintained in the region of 10^{-3}mol m^{-3} (Gilroy *et al.* 1986). Measurements of cytosolic Ca^{2+} concentrations in protoplasts have shown the stability of the Ca^{2+}-regulatory system. It is capable of maintaining a constant cytosolic Ca^{2+} status despite changes in the extracellular environment over the range of 10^{-4} - 10^{-1}mol m^{-3} (Gilroy *et al.* 1987).

In animals it is also found that the intracellular Ca^{2+} concentration is held very constant, and levels range from 10^{-5} to 10^{-3}mol m^{-3} despite an extracellular concentration of 1mol m^{-3} (Gilroy *et al.* 1987). In animal cells this control is accomplished by a variety of calcium pumping systems located within the plasmalemma and organelles, and together they operate a constant calcium-stat system.

In plants there is similarly a steep electrochemical gradient favouring Ca^{2+} movement from the external medium which may reach 1mol m^{-3} into the cell. To maintain the calcium stat, plants on the long term scale may pump Ca^{2+} back out into the apoplastic space, or they may store Ca^{2+} within the cell. Plants consisting of only a small number of cells and in intimate contact with water over their surface may utilize the former method satisfactorily, but in higher plants this method would not be as applicable. It has been found that Ca^{2+} sequestration within plants plays a major role in the maintenance of the calcium-stat (Macklon 1984) and this is achieved at tissue and intracellular levels. A mechanism used by calcicole plants is to maintain their cytosolic calcium stat in young expanding leaves by a precipitation mechanism, which involves the accumulation of large amounts of calcium in the older leaves. *Cucumis* sp. are obligate calcicoles, in that they cannot grow in the absence of calcium and the precipitation mechanism operates continuously; thus, if placed in a low calcium status, calcium is still removed from the cytoplasm to the older leaves and the young tissues suffer from Ca^{2+} deficiency (Ingestad 1974).

Many plants rely on a precipitation mechanism at the intracellular level with the deposition of Ca^{2+} in cytoplasmic organelles. Organelles such as mitochondria and chloroplasts provide limited storage capacity for Ca^{2+} and may function for peak loads only. The vacuole on the other hand is involved in longer term storage and has been reported to accumulate Ca^{2+} to a concentration of 0.1 - 10mol m^{-3} (Macklon 1984, Moore & Akerman 1984). Chloroplasts have been reported to contain 1 - $6x10^{-4}$mol m^{-3} total calcium.mg^{-1} chlorophyll (Portis & Heldt 1976), which is equivalent to a stromal concentration of about 4

- 23mol m^{-3}. The concentration is dependent in the long term on the age of the tissue (Bouthyette & Jagendorf 1981, Jones & Halliwell 1984) and in the short term upon illumination (Muto *et al.* 1982). Thylakoid membranes possess a high calcium-binding capacity and probably act as calcium sinks (Gross & Hess 1974, Prochaska & Gross 1975).

The inner layer of the plasma membrane is appressed to the cytoplasm and it therefore experiences a high concentration of ions on its exterior and a low concentration on its interior surface. Ca^{2+} is extruded back into the extracellular space by an active process at the plasma membrane and is also transported from the cytoplasm to non-actively growing tissues (Macklon 1984). Specific calcium transport mechanisms have been reported to be associated with vesicle membranes which accumulate Ca^{2+}, notably the tonoplast, and membranes involved in Ca^{2+} transport, the plasma membrane particularly. The endoplasmic reticulum may also be involved in Ca^{2+} transport but this has been reported to depend on the developmental state of the cell (Reiss & Herth 1985).

There have been several reports of active Ca^{2+} accumulation into membrane vesicles derived from plant cells. One type is a Ca^{2+}/Mg^{2+}-ATPase that is at least partially dependent on calmodulin and represents a "primary pump" similar to that known from many animals (Dieter *et al.* 1984, Moore *et al.* 1986). Movement of calcium *via* this ATP-coupled direct mechanism is probably associated with the endoplasmic reticulum and has been demonstrated in preparations from *Lepidium sativum* (Buckhout 1984) and cultured carrot cells (Bush & Sze 1986). This system is unaffected by ionophores, is sensitive to vanadate and insensitive to nitrate (Bush & Sze 1986). The affinity of the enzyme for calcium is relatively high, with half saturation being reached below 10^{-3}mol m^{-3} which suggests that the endoplasmic reticulum may help to maintain the low cytoplasmic Ca^{2+} concentration of unstimulated cells (Buckhout 1984, Bush & Sze 1986).

Although it has been pointed out that the plasma membrane calcium pump is likely to be ultimately the major

regulator of cytoplasmic free calcium (Clarkson 1986), until recently only suggestions have been made for the existence of a Ca^{2+}-ATPase associated with the plasma membrane (Dieter 1984, Dieter *et al.* 1984). ATP-dependent Ca^{2+} transport was reported in plasma membrane preparations from soybean hypocotyls, but no marker characterization was included (Kubowicz *et al.* 1982). But a recent report indicated that active calcium pumps are found in the plasma membrane and Golgi apparatus in preparations from pea roots (Butcher & Evans 1987a). ATP-dependent Ca^{2+} transport in these vesicles was found to be both vanadate-sensitive and protonophore-insensitive (Butcher & Evans 1987b) indicating the presence of a direct Ca^{2+}-ATPase.

Another established mechanism for active Ca^{2+} transport is a "secondary pump" that makes use of a H^+ gradient as a driving force and has been characterized as a Ca^{2+}/H^+ antiport with the use of protonophores (Rasi-Caldogno *et al.* 1982, Schumaker & Sze 1985, Bush & Sze 1986). The Ca^{2+} antiport has been demonstrated in vesicle preparations from cultured carrot cells (Bush & Sze 1986), pea root (Butcher & Evans 1987a) and maize roots (Zocchi 1988). It has been speculated that a Ca^{2+}/H^+ antiport in higher plant cells may operate also at the plasma membrane in a similar fashion to the antiport associated with plasma membrane vesicles from *Neurospora* (Sze 1985).

The plasma membrane and vacuolar membranes may also possess Ca^{2+}-sensitive ion channels allowing controlled influx since these membranes significantly bind Ca^{2+}-channel antagonizing drugs *in vitro*, such as verapamil and nifedipine (Hetherington & Trewavas 1984, Andrejauskas *et al.* 1985). These same drugs are known to inhibit physiological processes in plants (Hepler & Wayne 1985).

1.3.2 Regulatory roles associated with cytoplasmic calcium concentration.

In animals, calcium occupies a preeminent place in the cellular control system (Campbell 1983). A constant calcium stat is operative, although temporary elevations of cytoplasmic calcium to between 10^{-3} - 10^{-2}mol m^{-3} follow plasma membrane perturbation. These alterations of

calcium channel activity are used by cells as signals in eliciting a variety of predetermined responses. Changes in cytoplasmic calcium concentrations are sensed by calcium binding proteins in animals, most notably calmodulin and the calmodulin complex in turn modulates the activity of numerous enzymes (Gilroy *et al.* 1987).

A precise calcium stat is similarly maintained within the cytoplasm of plants and it is envisaged that changes in this calcium level have a role in the regulation of cellular processes. Many physiological processes appear to be regulated by calcium. This can be demonstrated by artificially modulating calcium levels with ionophores, blocking calcium entry channels with antagonists and by reducing the extracellular Ca^{2+} concentration. These treatments affect a diversity of plant processes which include cytokinin-induced bud formation in mosses, desmid morphogenesis, protoplast fusion, a multitude of phytochrome-triggered responses, gravitropism, secretion, leaf movements, guard cell swelling, polar growth, mitosis and cytoplasmic streaming (Hepler & Wayne 1985, Trewavas 1986) and the basipetal transport of auxin (deLa Fuente 1984).

Because of the low intracellular Ca^{2+} concentration (Gilroy *et al.* 1987a), it has an advantage over the more abundant cations in acting as a carrier of information. The intracellular calcium concentration can transiently change 100-fold from 10^{-4} to 10^{-2}mol m^{-3} without substantially disturbing the ionic milieu of the cell. In contrast, Mg^{2+} and Na^{+} would have to change from 1 to 100mol m^{-3} and K^{+} from 100 to 10^{3}mol m^{-3} to provide an equivalent increase and this would seriously disturb the osmotic and charge balance within the cell. The concept of a stimulus-response coupling by Ca^{2+} has developed and envisages that a physiological stimulus leads to a change in the flux rates of Ca^{2+} between external and internal pools in the cytoplasm. The resulting change in concentration of cytoplasmic free Ca^{2+} represents the signal required to regulate the activity of respective target enzymes.

In plants local variations in calcium ion concentration have been reported. From these studies two

classes of modulation have emerged (1) a sustained but localized ion gradient as seen in the tip-growing cell of *Fucus* (Brownlee & Wood 1986) or pollen tubes (Reiss & Nobiling 1986) and (2) a transient increase in Ca^{2+} levels as shown by lily cells undergoing mitotic progression (Keith *et al.* 1985) or *Chara* cells responding to an action potential (Williamson & Ashley 1982).

The calcium receptor calmodulin has been detected and isolated from a number of plant species in both membrane and soluble fractions (Allan & Trewavas 1985). Calmodulin from spinach has been sequenced and differs by only 13 residues from bovine calmodulin (Roberts *et al.* 1986) and the two are often interchangeable in eliciting a response. Apart from calmodulin, other calcium binding proteins have been detected, notably in the phloem (Sabnis & McEuen 1986) and carrot cells (Ranjeva *et al.* 1986) which may similarly act as signal transducers. The chloroplast stromal fraction has been found to have an affinity for Ca^{2+} several times greater than that for Mg^{2+} (Kreimer *et al.* 1987) and this data is consistent with the existence of at least two, probably independent classes of binding sites. The total number of binding sites varies between 90-155nmol.mg^{-1} protein. The trivalent cation La^{3+} has been used as a probe for Ca^{2+}-binding sites (Tew 1977, Evans 1983), and typically binds much more tightly to the protein than Ca^{2+} (Wang *et al.* 1981). The competitive and relatively tight binding of La^{3+} to the stromal proteins found by Kreimer *et al.* (1987) lends support to the idea that the binding of Ca^{2+} reflects the existence of specific binding sites.

Exogenous calmodulin has been reported to stimulate ATP-dependent Ca^{2+} transport in vesicle preparations from apple fruit (Fukumoto & Venis 1986) while such transport is strongly inhibited by calmodulin antagonists. In pea root vesicles, calmodulin was found to have no effect on ATP-dependent Ca^{2+} transport or ATPase activity (Butcher & Evans 1987b). A similar effect was found in maize grown in FR (Dieter and Marmé 1981). Perhaps calmodulin sensitivity should be considered to be a special feature present in some tissues in some circumstances, rather than a ubiquitous mode of regulation of cytoplasmic free

calcium in plants.

Although many mechanisms have been found to be triggered by fluctuations in cytoplasmic calcium concentrations which lead to physiological changes, examination has also been made of mechanisms that are likely to cause those initial fluctuations. Primary signals such as light and plant hormones have been examined as possible modulator of calcium movement into a vesicle system.

Auxin has been reported to stimulate proton release from the cytoplasm which caused wall loosening and extension (Evans 1985). Since Ca^{2+} at relatively high concentrations (1 - 10mol m^{-3}) inhibits growth (Tagawa & Bonner 1957), it was suggested that calcium might be interfering with proton release. But it was found that Ca^{2+} actually stimulated proton efflux (Cohen & Nadler 1976, Cleland & Rayle 1977). It was then proposed that H^{+} could be displacing Ca^{2+} from wall-binding sites which was supported by the long held view that Ca^{2+} cross links acidic polysaccharides in the wall which causes them to stiffen and retard growth (Tagawa & Bonner 1957, Taiz 1984). Subsequent measurements have found that the pectic linking theory by calcium does not appear to regulate growth (Cleland & Rayle 1977). Instead Cleland and Rayle (1977) favoured a mechanism in which Ca^{2+} interfered directly with the biochemical process of wall loosening. Many auxin-induced responses such as thinning of the plasma membrane (Morré & Bracker 1976) and induction of growth (Morré & Ersinger 1968) are all reversed by the addition of calcium. One mechanism by which this hypothesized Ca^{2+}-auxin antagonism might occur involves an auxin modulation of Ca^{2+} affinity for sites on the hypocotyl membrane. This has been demonstrated for Ca^{2+}-insulin antagonism in rat liver cell membranes (Shlatz and Marinetti 1972) and Ca^{2+}-anaesthetic antagonism in human erythrocyte membranes (Low *et al.* 1979). Thus auxin may elicit in plants a response similar to the "secretion-coupling" cascade involving Ca^{2+} in mammalian cells (Rasmussen 1970). Similarly auxin may bind to a surface receptor, which induces membrane hyperpolarization and a local loss of membrane Ca^{2+}. In vesicles prepared from

soybean which were loaded with calcium, loss was observed by the addition of 2,4-D and IAA whereas 2,3-D had no effect (Buckhout *et al.* 1980). Ca^{2+} may also enter the cytoplasm from the vacuole (Hertel *et al.* 1983) or endoplasmic reticulum (Brummer & Parish 1983) or, if Ca^{2+} channels are opened in the plasma membrane, from the cell wall. The influx of Ca^{2+} from the cell wall or its release from the plasma membrane may be related to the observed auxin effects on the thinning of auxin-treated plasma membrane (Morré & Bracker 1976). But how auxin causes a release of Ca^{2+} from the vacuole and endoplasmic reticulum if the auxin receptor is solely located on the plasma membrane is not yet clear. However, it has been suggested that the auxin stimulation of the turnover of phosphotidylinositol may be related to calcium release (Morré *et al.* 1984).

1.3.3 Role of calcium in phytochrome action.

Following the discovery that calcium and calcium binding proteins were of central importance in animals in modulating the activity of certain enzymes *via* growth regulators, Haupt & Weisenseel (1976) proposed that some phytochrome effects on cellular enzyme activities, cell growth and development could be mediated through Ca^{2+} and Ca^{2+}-dependent regulatory proteins. At that time there were no Ca^{2+} dependent regulator proteins in plants, but calmodulin was soon discovered and has now been reported in many plant species (reviewed in Cheung 1980) and is activated by small increases in the free Ca^{2+} concentration. In this form, it may stimulate activity of many regulatory enzymes.

The hypothetical scheme proposed by Haupt & Weisenseel (1976) and further adapted by Roux *et al.* (1986) postulated that the physiologically active form of phytochrome, P_{fr}, caused an increase in cytoplasmic Ca^{2+} concentration which activated calmodulin and in turn sequentially stimulated calmodulin induced enzymes. The initial step in this scheme, namely that phytochrome directly causes a perturbation of cytoplasmic Ca^{2+}, has yet to be reported, although many other reports have been made that are supportive of the ensuing stages (e.g. Roux 1983).

Before examining further the proposed links between phytochrome and calcium levels, a background to phytochrome is given. Phytochrome isolated from rye and oat by acrylamide gel electrophoresis has been found to consist of a single homogeneous protein. Rice *et al.* (1973) concluded that phytochrome probably has a molecular weight of 120KD. The appearance of highly purified phytochrome when examined under the electron microscope suggests that the 120KD subunit may be dumb bell in shape. One part of the molecule is proteinaceous, while the associated chromophore for absorption of visible light is a highly conjugated, non-proteinaceous prosthetic group. The chromophore is capable of existing in two forms, one of which predominantly absorbs R and the other FR.

The absorption spectrum of phytochrome was identified as being very similar to that of certain algal pigments and this led Borthwick *et al.* (1950) to suggest that the chromophore might be a linear tetrapyrrole, similar to that of phycocyanin. Siegelman & Hendriks (1960) later showed that the chromophore absorption spectrum from purified higher plants was similar to those of l-phycocyanin and allophycocyanin and that their chromophores are similar to the bile pigments of animals consisting of linear tetrapyrrole molecules. The extent of conjugation and the number of methylene bridges varies from pigment to pigment. The number of chromophores per phytochrome molecule is 1 per subunit. Recent evidence has shown that the molecular weight of the phytochrome molecule to be 125KD (Shropshire & Mohr 1983).

The photoconversion of P_r to P_{fr} does not occur in one direct step but *via* four short lived spectral intermediates which may participate in initiating phytochrome responses (Kendrick & Spruit 1972). P_{fr} is usually referred to as the physiologically active form of phytochrome, although many phytochrome responses do not appear to be a simple function of the concentration of P_{fr} (Smith 1981). P_{fr} has a higher binding affinity for biological membranes than P_r (Cedel & Roux 1980, Watson & Smith 1982) and P_{fr} alters the permeability of artificial membranes more than P_r. In the dark,

there is photoconversion of P_{fr} to P_r or to an unidentified form with no photoreversibility.

Supportive evidence for the involvement of calcium in phytochrome action has been made from early observations of fern spore germination by Wayne & Hepler (1985). For example, Ca^{2+} was found to mediate a phytochrome response in *Onoclea*, whereby R induced the germination of spores in the presence of $3x10^{-3}$mol m^{-3} Ca^{2+} but, without Ca^{2+}, germination failed to take place. Following R illumination, the intracellular Ca^{2+} concentration rose by 0.5mol m^{-3} which was FR-reversible. By preventing Ca^{2+} uptake with La^{3+}, germination of the spores was prevented in the presence of R. With the addition of the ionophore A23187, which increased the intracellular Ca^{2+} concentration germination of the spores took place in the dark. P_{fr} was thought not to transport Ca^{2+} directly, as under certain conditions the fern spores took up newly added Ca^{2+} even after P_{fr} had been photoconverted back to P_r.

Das & Sopory (1985) provide further evidence that there are intermediate steps between P_{fr} activation and Ca^{2+} transport. They found that a 1 min R illumination caused an increase in the rate of $^{45}Ca^{2+}$ uptake into corn protoplasts which is FR reversible if given immediately. The stimulation of $^{45}Ca^{2+}$ uptake is enhanced by introducing a 3 min dark period between the R irradiation and the addition of $^{45}Ca^{2+}$. The authors provide some indirect evidence that the intermediate steps include the breakdown of a membrane lipid, phosphatidyl-inositol-4,5,-bis phosphate, into diacylglycerol and inositol triphosphate. This membrane reaction is known to stimulate the uptake of Ca^{2+} in animal cells (Nishizuka 1984). It is believed that primary signals in plants alter the cytosolic calcium levels *via* phosphoinositides (Reddy & Poovaiah 1987). Thus it seems that phytochrome may promote Ca^{2+} entry into the cell *via* the plasma membrane, specifically by R in algae and oat cells. But there are numerous reports of phytochrome effects on cellular membranes other than the plasma membrane. For example, Roux *et al.* (1981) found evidence for the high affinity binding of phytochrome to mitochondrial

membranes, and showed that Ca^{2+} fluxes could be photoreversibly altered. R diminished the net uptake rate and FR restored the rate to the dark control. Upon treatment of mitochondria with ruthenium red, an inhibitor of active Ca^{2+} uptake in plants and without effect on respiratory activity or efflux (Russell & Wilson 1978), Ca^{2+} uptake was blocked. In the presence of ruthenium red, Ca^{2+} efflux was induced by R. Changes in Ca^{2+} flux were found to be photoreversible.

1.3.4 Calcium action on protein phosphorylation.

There is no evidence that small variations in cytosolic calcium directly activates any enzymes, but near the site of phytochrome action local variations could occur and these possibly affect membrane fluidity (Papahadjopoulos 1978). At the present time, it is thought that rather than Ca^{2+} itself and the Ca^{2+}-calmodulin complex mediating affects on a wide variety of enzymes, a response is mediated *via* phosphatase and kinase activity which may stimulate or inhibit a range of enzymes that may be involved in growth and development. Protein phosphorylation and dephosphorylation *via* kinase and phosphatase activity respectively are recognized as important control mechanisms by which the activity of key regulatory enzymes and receptor molecules are altered within cells in response to a wide variety of external stimuli (Cohen 1985). By transferring a phosphate moiety from nucleoside triphosphate to a specific site of the protein substrate, protein kinases can profoundly alter the structure and function of the substrate. The activity of many enzymes is regulated post-translationally by the controlled addition or removal of phosphate groups. In animal cells external physiological stimuli, such as hormones and growth factors, have been shown to modulate the phosphorylation level of likely regulatory enzymes, often through Ca^{2+} and calmodulin-dependent mechanisms. The only known factors that regulate the activity of protein kinases in plants are Ca^{2+}, Ca^{2+}-calmodulin, phospholipids, light and polyamines. Ca^{2+} and Ca^{2+}-calmodulin-dependent protein kinases have been demonstrated in a variety of plant tissues (Ranjeva *et al.*

1984, Veluthambi & Poovaaiah 1984, Poovaiah & Veluthambi 1986). But there is no evidence of Ca^{2+}-dependent protein phosphatases in plants.

A number of Ca^{2+}/calmodulin-dependent protein kinases have been identified in plants and include some that have been purified and partially characterized (Ranjeva *et al.* 1984). Except for NAD+3-oxidoreductase it is not known what proteins these kinases phosphorylate (Ranjeva *et al.* 1983). Poovaiah *et al.*(1987) demonstrated that the phosphorylation of several membrane and soluble proteins from corn coleoptiles is promoted by exogenous additions of Ca^{2+} and calmodulin to crude preparations of these proteins.

NAD kinase was the first to be discovered in plants and catalyzes the phosphorylation of NAD to NADP. Phytochrome activated by R increased the NADP/NAD ratio. The ability to manipulate NAD and NADP levels argues for a pivotal role of this enzyme in the regulation of anabolic and catabolic processes (Anderson & Cormier 1978).

Kleinsmith (1975) reviewed the early evidence for a link between phosphorylation and the regulation of gene expression in animals. More recently Ranjeva *et al.* (1984) summarized results and indicated that this model may apply also to plant cells. There have been many reports indicating that gene expression may be regulated by phytochrome (e.g. Apel 1981, , Colbert *et al.* 1983, Batschauer & Apel 1984, Tobin & Silverthorne 1985). The control of mRNA for the SSU of RuBisCo by phytochrome has received attention (Jenkins *et al.* 1983, Sasaki *et al.* 1983, Thompson *et al.* 1983). Exposure of radish seedlings to FR causes an increase in several translatable mRNA's, including that coding for the small subunit of ribulose 1,5 bisphosphate carboxylase. Irradiation with FR for 12 - 36h and R for 1 - 5 min caused an increase in the SSU mRNA concentration, which was prevented by a subsequent exposure to FR (Fourcroy *et al.* 1985, Fourcroy 1986). However, these experiments were based on translation *in vitro* and may not reflect the true cellular concentration of a given mRNA, as the biological activity of mRNA's is susceptible to modulation by post-translational mechanisms (Revel & Groner 1978).

Hydridization analysis with cloned cDNA-mRNA has shown the involvement of phytochrome in controlling the SSU mRNA level in *Lemna* (Stiekema *et al.* 1983), soybean (Berry-Lowe & Meagher 1985) and pea (Jenkins *et al.*1983). When the effect of R and FR on SSU mRNA accumulation in radish cotyledons was investigated with cDNA (Fourcroy 1986), it was found that phytochrome indeed exerts an effect at the transcriptional level and the action of the P_{fr} is transmitted within a few minutes of illumination with R. Data presented on gene activation in pea nuclei suggest that phosphorylation may play a role in this process (Datta *et al.* 1985). It was reported that R and Ca^{2+} stimulate phosphorylation of the same nuclear proteins and that FR and EGTA and calmodulin antagonists both negate the R treatment. Although no calmodulin was added, nuclei are reported to contain significant endogenous levels (Biro *et al.* 1984). Thus in addition to the proposal that phytochrome may mediate an effect in the cytoplasm by post translational modification, phytochrome may also modulate enzymes in the nucleus by the same process, which may be responsible for triggering the translation of other proteins.

1.4 Research Aims.

The initial aim of this study was to investigate the effect of plant growth regulators on membrane associated ATPase and H^+ pumping activities in purified membrane fractions isolated from the hypocotyl of *Cucumis sativus*. The intact system has been previously used to investigate the effect of growth regulators on medium acidification and growth (e.g. Brummell *et al.* 1986) and an initial survey of the sensitivity of microsomal ATPase to inhibitors was also made. Secondly it was proposed to investigate further the effect of R and FR on ATPase activity and medium acidification that was reported by Roth-Bejerano & Hall (1986b) using microsomal fractions prepared from hypocotyls of *Cucumis*. It was planned to verify these results and then to determine the specific membrane bound ATPase that was responsive to the light treatments.

With the recent interest in the involvement of calcium in mediating phytochrome and auxin responses, an initial examination was made of Ca^{2+}-ATPase activity in microsomal vesicles and characterization of active Ca^{2+} transport.

CHAPTER TWO

Materials and Methods.

2.1 Plant material.

Cucumber seeds (*Cucumis sativus*.L. Long Green Ridge) were soaked for 2 h and sown in moist vermiculite or perlite and grown in darkness at 25°C for 6 days, at which stage they had reached approximately 60mm in height. In the winter months a 20 min soak in 2% (v/v) sodium hypochlorite was given to the seeds prior to sowing to avoid damping off. Plants were watered with tap water unless otherwise indicated in the text.

2.2 Growth measurements.

Incubation of intact cucumber hypocotyl segments was carried out for 1 - 11 h in IAA concentrations of 1mol m^{-3} - 10^{-3}mol m^{-3} containing 5mol m^{-3} Tris-Mes buffer (pH 6.75 & 5.65). Segments were incubated in an aerated IAA solution in the dark on a gently shaking water bath at 30°C. Segments were incubated for the same period in buffer alone as a control. Homogenization of the tissue and extraction of membrane-enriched fractions from a sucrose gradient was carried out as outlined in section 2.4.3.

2.3 Membrane preparation.

2.3.1 Measurement of growth.

A pre-treatment was given by incubating hypocotyl sections in distilled water in the dark for 1 h. Ten mm segments of hypocotyl were cut with a fixed blade cutter to obtain segments of equal length. Replicate samples of 6 sections were incubated in the dark at 25°C in 5mol m^{-3} Tris-Mes buffer (pH 6.75) and 10^{-3}mol m^{-3} IAA. Growth was measured by the shadowgraph technique using green light (Cinemoid filter 24A) as described by Brummell & Hall (1986).

Replicates were not ordered, therefore data of individual increases of hypocotyl extension was not obtained over the time period. To determine the degree of

significance with the data taken, one-way analysis of variance (ANOVA) with Duncan's multiple range test was made using the statistical package 'Statistics for Social Scientists' (SPSSX) on the IBM mainframe 3090 computer. This was carried out on hypocotyl length at time 0 h and if no significant difference was found between each group of measurements, then the ANOVA test was applied at time 4 h. Hypocotyls were cut with a fixed blade cutter to obtain segments of equal length.

2.3.2 Measurement of growth with a linear displacement transducer.

Hypocotyl segments were auxin-depleted by a 2 h incubation in distilled water and darkness. A 5mm hypocotyl section was placed vertically in a perspex chamber surrounded by an aerated bathing medium. A linear displacement transducer (Model DF50, Sangamo Transducers, Bognor Regis) was vertically mounted on an adjustable microscope stand and a piston lowered onto the uppermost cut end of the tissue. The ferro-magnetic end was adjusted so that it lay within the solenoid of the transducer. A DC output from the transducer was linked to a BBC microcomputer and Epson RX-80 F/T printer. A basic transducer program was supplied by A. Parsons, University of York, Copyright P.C.T. (Peoples Centre for Tropistic Research) with modifications by P. Hillier to run off EPROM (1985). Elongation and growth rate were monitored over a 1 mm range. Readings were taken every 10 sec for 2 h.

2.3.3 Measurement of acidification by hypocotyl segments.

The basic method followed was similar to that used by Brummell (1986) and Brummell & Hall (1986). Carborundum powder was cleaned with 100mol m^{-3} HNO_3 for 1 h and then washed in distilled water. Plants (30-60mm in height) were selected and the upper region of the hypocotyl abraded with a slurry of carborundum powder (600 grade) to disrupt the cuticle and the tissue was rinsed in distilled water. Forty segments (13mm in length) were cut with a fixed distance razor blade cutter and after a 1.5 h auxin depletion pretreatment as described above, were placed in

a glass vial (12cm^3) containing 0.5mol m^{-3} K_2SO_4, 1.0mol m^{-3} $CaCl_2$, 10^{-3}mol m^{-3} IAA and 1.0mol m^{-3} sodium phosphate buffer (pH 6.5) in a total volume of 10cm^3. The vial was placed in a circulating water bath at 26°C and the medium was aerated throughout the experiment. The pH was recorded continuously using a Russel CWL pH electrode connected *via* a Philips PW 9421 pH meter (Pye Unicam Ltd.) with millivolt output to a chart recorder.

2.3.4 Preparation of IAA.

IAA solutions were prepared by heating the salt of IAA in distilled water to 70°C for a few minutes with constant shaking. Solutions were kept frozen.

2.4 Membrane preparation.

2.4.1 Tissue used.

All preparative procedures were carried out under white fluorescent light except in the phytochrome and growth experiments. Hypocotyls, 30-40mm in length, were excised below the cotyledons and weighed.

Different regions of hypocotyl were used in many experiments and were typically sampled as 10mm portions down the hypocotyl from the cotyledons. Epidermal tissue was prepared from hypocotyl sections by peeling the tissue off in longitudinal strips using fine forceps and the remainder used as cortical tissue. Cotyledon tissue was used in preliminary experiments.

2.4.2 Preparation of protoplasts.

The technique followed was similar to the method used by Polenko & Maclachlan (1984). Hypocotyls (3-4 g) were cut longitudinally into at least 4 sections and placed in an isolation buffer containing 33mol m^{-3} Mes-Tris buffer (pH 5.5), 125mol m^{-3} KCl and 200mol m^{-3} sorbitol at room temperature. The material was rinsed in isolation buffer and placed in a further 2.5cm^3 isolation buffer with the addition of 0.67% (w/v) BSA, 2.67% (w/v) cellulase "Onozuka" R-10, 0.67% (w/v) hemicellulase, 1.33% (w/v) β-glucuronidase, and 6.67% (w/v) Macerozyme R-10. Cellulase and macerozyme were obtained from Yakult Pharmaceutical Industry Co. Ltd., 8-21 Shingikan-Cho, Nishinomiya, Japan.

The material was vacuum-infiltrated for 2 min and left at room temperature for 3 h. The macerate was strained through a zinc gauze of mesh size 75µm with gentle agitation. The brei was washed through with wash buffer containing 200mol m^{-3} sorbitol, 150mol m^{-3} KCl and 33mol m^{-3} Mes-Tris buffer (pH 5.5) with an osmotic potential of 515.3 mol m^{-3}/Kg (Measurements of vapour pressure were made on a Wescor 5100C vapour pressure osmometer).

Protoplasts were pelleted by centrifugation at 170*g* for 10 min at room temperature in a bench centrifuge, and washed three times with wash buffer. After the final rinse, protoplasts and debris were resuspended in the wash buffer with 4% (w/v) ficoll. An equal volume of buffer containing 6% (w/v) ficoll was carefully laid over the 4% (w/v) interface in a centrifuge tube, followed by an equal volume of wash buffer without ficoll. The discontinuous gradient was centrifuged at 220*g* for 20 min. Intact protoplasts concentrated at the 4% buffer interface, and debris pelleted at the bottom of the gradient or accumulated at the 4% - 6% interface. Protoplasts were immediately removed with a Pasteur pipette and positively charged microbeads prepared in wash buffer ($1cm^3$) were added. The mixture was gently mixed by inversion and centrifuged at 100*g* for 2 min and the supernatant discarded. Protoplasts were washed three times to remove unbound microbeads in the wash buffer and dextran sulphate (1.0 $mgcm^{-3}$) in wash buffer was added to neutralise the exposed surfaces of the microbeads. The protoplasts were washed three times and resuspended in wash buffer. Lysis was achieved by adding 10-20 volumes of a buffer with an osmotic potential of 280mol m^{-3}/Kg (33mol m^{-3} Tris-Mes buffer (pH 7.0), 1mol m^{-3} DTT, 150mol m^{-3} KCl and 0.1% (W/V) BSA) or 50.5 mol m^{-3}/Kg, 33mol m^{-3} Tris-Mes buffer (pH 7.0), 1mol m^{-3} DTT, 1mol m^{-3} $MgCl_2$ and 0.1% (W/V) BSA). The lysate was centrifuged at 1000*g* for 10 min and the supernatant retained. Finally, the bead-plasma membrane pellet was washed three times in wash buffer.

Fluorescein diacetate (FDA) was used to determine the viability of protoplasts. A solution containing 5 $mgcm^{-3}$ FDA in acetone was diluted to 0.01% (v/v) in wash buffer. Calcafluor white 0.1% (w/v) in wash buffer was used to

determine the extent of cell wall degradation. Protoplasts were examined under a Vicker's fluorescence microscope with excitation filter BG12 and suppression filter K510.

2.3.3 Preparation of microsomal fraction.

Tissue was immediately ground in an ice-cold mortar and pestle. A ratio of 1cm^3 homogenization medium to 1 g fresh weight plant tissue was used. The medium consisted of 25mol m^{-3} Tris-Mes (pH 7.3) containing 250mol m^{-3} mannitol/sucrose, 2mol m^{-3} EGTA and 1mol m^{-3} DTT. The homogenate was filtered through 4 layers of muslin and centrifuged at 7000g in a Sorvall SS34 rotor for 10 min at 4°C. The supernatant was decanted and centrifuged using a Sorvall A-841 rotor for 40 min at 104,000g at 4°C. The pellet was reannealed in a resuspension medium containing 5mol m^{-3} Tris-Mes buffer (pH 7.3) containing 250mol m^{-3}, mannitol/sucrose, 2mol m^{-3} EGTA and 1mol m^{-3} DTT and referred to as the microsomal fraction and was used in a number of preliminary experiments.

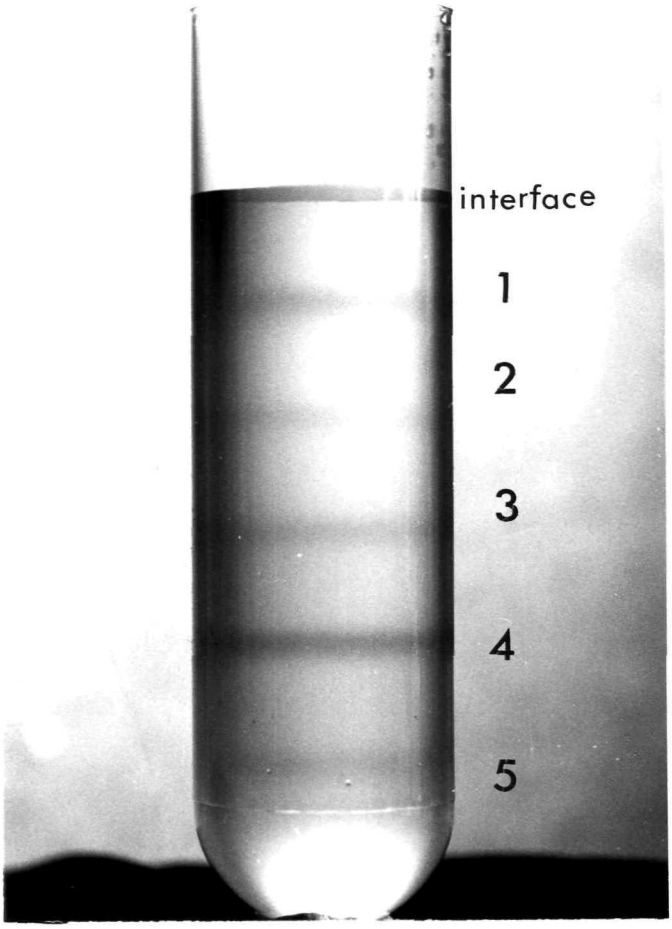
2.4.4 Preparation of purified membrane fractions.

The resuspended microsomal fraction was laid on the surface of a six step discontinuous sucrose gradient consisting of 4.5cm^3 each of 45%, 38%, 34%, 30%, 25%, 20% (w/w) sucrose, in 2mol m^{-3} Tris-Mes buffer (pH 7.0) and 1mol m^{-3} DTT (Fig.2.1). The gradient was centrifuged at 77,000g for 2 h at 4°C in a Sorvall AH-629 swing-out rotor. Membrane fractions were removed from the gradient interfaces with a Pasteur pipette and are designated 1 - 5 and were either used directly in assays or re-pelleted by centrifugation for 30 min at 104,000g and resuspended in the 5mol m^{-3} Tris-Mes buffer (pH 7.3).

2.4.5 Preparation of membrane vesicles for H^+ transport

Following removal of membranes from a gradient or after resuspension of a microsomal pellet, reannealing was carried out to increase the proportion of tightly sealed membrane vesicles. In this procedure a few grains of DTT and BSA were added to the mixture and the resuspension drawn through a pipette eighty times at 37°C.

Figure 2.1 Six step sucrose gradient composed of 4.5cm^3 each of 20%, 25%, 30%, 34%, 38%, and 45% (w/w) sucrose plus 1mol m^{-3} DTT and 2mol m^{-3} Tris-Mes buffer (pH 7.0) prior to loading of microsomal fraction.


interface
1
2
3
4
5

2.4.6 High concentration potassium chloride or iodide washes.

This washing procedure was carried out by resuspending the microsomal pellet to excess in 150mol m^{-3} KCl, 250mol m^{-3} sucrose and 1mol m^{-3} DTT (Dupont & Hurkman 1985). The mix was left on ice for 20 min and centrifuged at 104,000*g* for 30 min.

Alternatively, the microsomal pellet was resuspended in 5cm^3 of KI wash solution, left on ice for 20 min and repelleted by centrifugation at 104,000*g* for 30 min. The resuspension medium contained 250mol m^{-3} KI, 250mol m^{-3} mannitol, 2mol m^{-3} EGTA, 2mol m^{-3} $MgSO_4$, 2mol m^{-3} ATP, 10% (v/v) glycerol, 1mol m^{-3} PMSF, 2mol m^{-3} DTT, 0.5% (w/v) BSA and 25mol m^{-3} BTP-Mes buffer (pH 7.6).

2.4.7 Preparation of intact endoplasmic reticulum vesicles for ATP-dependent Ca^{2+} transport.

The homogenization procedure and preparation of purified membranes was carried out as outlined but with the following modifications. Hypocotyls were ground in the homogenization medium without EGTA and with the inclusion of 5mol m^{-3} $MgCl_2$. The microsomal pellet was resuspended in the resuspension medium without EGTA and with 5mol m^{-3} $MgCl_2$. Purified membrane fractions were prepared using the 20 - 45% sucrose gradient steps, which also contained 3mol m^{-3} $MgCl_2$. Gradient interfaces were removed and resuspended to excess in 20mol m^{-3} EGTA, 5mol m^{-3} Tris-Mes buffer (pH 7.3), 1mol m^{-3} DTT and centrifuged for 40 min at 104,000*g*. Pellets were resuspended in the resuspension medium without the addition of EGTA.

2.5 Enzyme and protein assays.

ATPase activity was assayed in triplicate at 37°C for 1 h using 10-30 µg protein per assay and by determining the Pi released from ATP, during which period phosphate released was linear. The assay mixture (1cm^{-3}) consisted of 20mol m^{-3} Tris-Mes buffer (pH 6.75), 10^{-1}mol m^{-3} ammonium molybdate, 2mol m^{-3} ATP, 2mol m^{-3} $MgSO_4$ and 50mol m^{-3} KCl. When nitrate inhibition was measured KNO_3 replaced KCl. The reaction was stopped by the addition 0.2cm^3 of $5x10^3$ mol m^{-3} sulphuric acid followed by 1.2cm^3

H_2O. Phosphate released was determined by the method of Sumner (1944) which involved colour development by the further addition of 0.2cm^{-3} 10% (w/v) ammonium molybdate and 0.4cm^{-3} of 8% (w/v) ferrous sulphate. The fraction was given a low speed spin for 5 min to remove acid precipitated protein and the absorbance was measured after 10 min at 620nm. For ATPase assays in which Triton X-100 was incorporated, SDS was added to a final concentration of 1.5% (w/v) to eliminate interference (Peterson 1978).

Mg^{2+}-stimulated specific ATPase activity is that value calculated in the presence of the standard assay minus that activity calculated in the presence of the standard mixture without magnesium. Mg^{2+}.K^+-stimulated activity is the value calculated in the presence of the standard assay mixture minus magnesium and potassium.

Specific pyrophosphatase activity was measured in the same way as ATPase except that Tris-Mes buffer (pH 8.0), 10^{-1}mol m^{-3} ammonium molybdate, 2mol m^{-3} $MgSO_4$ and 2mol m^{-3} pyrophosphate was used. The Mg^{2+} and K^+-stimulated pyrophosphatase activity was calculated in the same way as the Mg^{2+}.K^+ stimulated ATPase activity, but values were halved to account for the 2 Pi released per PPi.

Latent IDPase activity was determined by measuring the difference in activity in the presence and absence of 0.1% (w/v) digitonin (Chanson *et al.* 1984). The assay mixture contained 2.5mol m^{-3} IDP, 2.5mol m^{-3} $MgSO_4$, 50mol m^{-3} KCl, 25mol m^{-3} Tris-Mes buffer (pH 6.5) and 10-30µg membrane preparation and was incubated at 37°C for 20 min.

NADH-cytochrome c reductase assays were performed at room temperature by measuring the reduction of cytochrome c at 550nm. The assay mixture contained 40mol m^{-3} potassium phosphate buffer (pH 7.5), 1mol m^{-3} NADH, 10mol m^{-3} KCN, 30x10^{-3}mol m^{-3} cytochrome c, 0.01cm^3 antimycin and 7 - 10 µg protein per 1.0cm^3 (Hodges & Leonard 1974).

Cytochrome c oxidase assays were performed at room temperature by monitoring the reduction of reduced cytochrome c at 550nm (Tolbert *et al.* 1968). Protein (0.1 - 1.0 mg) was incubated with digitonin (0.15cm^3 of 4% (w/v)) for 1 min at 25°C and placed into a reaction mixture containing 0.75cm^3 sodium phosphate buffer (1 mol m^{-3}, pH 7.0) and reduced cytochrome c (5x10^{-2}mol m^{-3}).

Cytochrome c was reduced by the addition of few crystals of sodium dithionite and then aerated to remove excess or until the absorbance at $_{550}/_{565}$nm was equal to 9-10.

Glucan synthetase II was assayed at high UDPG concentrations and in the absence of Mg^{2+}. The basic procedure used was that of Ray (1973a,b). Aliquots ($100mm^3$) of the density gradient fractions was added to $40mm^3$ of 50mol m^{-3} Tris (pH 8) containing 17.5 nCi of UDP-[^{14}C] glucose and 0.5mol m^{-3} unlabelled UDP-glucose. After incubation at 25°C for 15 min, the reaction was stopped by the addition of $1cm^3$ 70% (v/v) ethanol, $50mm^3$ 50mol m^{-3} $MgCl_2$ and $150mm^3$ boiled crude microsomal fraction (obtained from 4 g fresh weight hypocotyl tissue/cm^3 suspension) in order to improve the recovery of labelled products. The mixture was boiled for 1 min and after standing overnight at 4°C was centrifuged for 5 min at 1000*g*. The pellet was washed four times with 70% (v/v) ethanol to remove all unreacted radioactive substrate together with ethanol-soluble by-products. It was then re-suspended in $10cm^3$ scintillation fluid. Radioactivity was measured by liquid scintillation spectrometry using a Beckman counter.

Protein was assayed by the binding of Coomassie blue using BSA as standard. The mixture was incubated at room temperature for 10 min, and the absorbance read at 595nm.

2.6 Measurement of H^+-pumping activity.

H^+-pumping activity was assayed by measurement of the fluorescence quenching of quinacrine at a final concentration of $5x10^{-3}$mol m^{-3} as described by Sze (1983). The assay contained 50mol m^{-3} KCl, 5mol m^{-3} $MgCl_2$, 200mol m^{-3} mannitol, 50mol m^{-3} Cl-BTP, 0.35mol m^{-3} EGTA, 15mol m^{-3} Hepes-BTP buffer (pH 6.75) and 100 µg protein in a total volume of $1.5cm^3$. ATP-dependent proton influx was initiated by addition of 5mol m^{-3} ATP-BTP. Quenching was reversed by the addition of $10mm^3$ gramicidin at a final concentration of $5x10^{-3}$mol m^{-3} or $10mm^3$ ammonium chloride at a final concentration of 10mol m^{-3}. When determining H^+-transport in the presence of nitrate, KNO_3 replaced KCl in the medium and the Cl^- concentration was kept constant by adding a further 50molm^{-3} Cl-BTP. Initial fluorescence

assays were carried out using a Varian DMS 90 fitted with a Varian total fluorescence accessory. The excitation wavelength was 420nm and the emission wavelength was of the order 475nm. In later experiments, fluorescence was measured with a LS-3 Perkin Elmer spectrophotometer, using excitation wavelength of 420nm and slit width 4mm and emission wavelength of 490nm and slit width 5mm. A constant temperature of 25°C was maintained, and a reaction volume of 0.5cm^3 was used. 1 volt DC output was linked to a Pharmacia chart recorder at 1 - 2mV sensitivity. Comparison was made of the total quenches.

2.6.1 Preparation of ATP-BTP (Hydrogen form).

50W Dowex (15cm^3) was mixed with 5% (v/v) sulphuric acid, and filtered through a buchner funnel with three washings. 200mol m^{-3} ATP was mixed with the hydrogen form of Dowex. The mixture was filtered on ice, and rinsed through with less than an equal volume of distilled water. The ATP was finally buffered with BTP (pH 6-7) and made up to volume and stored at -20°C.

2.7 Ca^{2+} transport assay.

Hypocotyls from seven-day-old *Cucumis sativus* cv. Perfection seedlings were ground with an ice-cold pestle and mortar in a buffered media consisting of 25mol m^{-3} Tris-Mes (pH 7.3) containing 250mol m^{-3} mannitol, 10mol m^{-3} EGTA and 1mol m^{-3} DTT. The microsomal fraction was prepared as described previously and the 104,000*g* pellet resuspended in 5mol m^{-3} Tris-Mes buffer (pH 7.3) containing 250mol m^{-3} sucrose and 1mol m^{-3} DTT. The vesicles were reannealed at 37°C for 5 min as previously described.

A modified homogenization medium was used after heavy watering with tap water which consisted of 250mol m^{-3} mannitol, 1 mol m^{-3} DTT, 13mol m^{-3} EGTA and buffered with 25mol m^{-3} Mes-KOH (pH 7.0). The microsomal pellet was resuspended in 5mol m^{-3} Mes-KOH buffer (pH 7.0) containing 250mol m^{-3} mannitol and 1mol m^{-3} DTT. Individual aliquots (0.02 - 0.28cm^3) with the addition of glycerol at a final concentration of 10% (v/v) were frozen in liquid nitrogen for up to 7 days. Samples were thawed by immersion in a

water bath at 37°C for a few seconds and used immediately. The microsomal fraction was either used immediately or purified further by layering onto the 20%, 25%, 30%, 34%, 38%, 45% (w/w) sucrose gradient as described earlier.

A fluorescence assay was used which contained 250mol m^{-3} mannitol, 25mol m^{-3} Mes-BTP (pH 7.5), 50mol m^{-3} KCl, 1mol m^{-3} $MgCl_2$, $30x10^{-3}$mol m^{-3} $CaCl_2$, $25x10^{-3}$mol m^{-3} chlorotetracycline and 1mol m^{-3} ATP-BTP (pH 7.0) in a total volume of 0.5cm^3 (Lew *et al.* 1986). The relative fluorescence was recorded using a Perkin-Elmer LS-3 fluorescence spectrophotometer at constant temperature (25°C) with excitation and emission wavelengths of 310 and 560nm respectively (Gimble *et al.* 1982, Lew *et al.*1986). Following equilibration, the reaction was started by addition of 5mm^3 ATP. The relative fluorescence values recorded were obtained by setting the fluorimeter to 100 'Fixed scale' after the addition of ATP. No multiplicative procedure was employed. The chart recorder was set at 1-2 mV. Quenching was reversed by the addition of 5mm^3 A23187 in DMSO to give a final concentration of the ionophore of 7$\mu g cm^{-3}$ and 0.66% (v/v) DMSO. In all experiments, each run was repeated at least twice and the whole experiment two times and a representative trace is illustrated. Solutions of the phenothiazines were prepared in water. Solutions of fluphenazine were stored in the dark.

2.7.1 IAA pre-treatment of intact tissue.

Hypocotyls prior to vesicle preparation for the Ca^{2+} assays were incubated in 5mol m^{-3} Tris-Mes buffer (pH 6.75) and 10^{-3}mol m^{-3} IAA in a total volume of 200cm^3, aerated vigorously in a gently shaking water bath at 30°C for 2 h. IAA was absent from the control.

2.7.2 Calcium determination in whole plant tissue.

5 g of hypocotyl sections or cotyledons were ground to a very fine suspension with a pestle and mortar in 25cm^3 deionized water with the addition of a small quantity of fine sand. The macerate was centrifuged at 10,000*g* for 10 min and the supernatant decanted. Prior to calcium measurement, the releasing agent, lanthanum

chloride was added to the supernatant to give a final concentration of 2.87mol m^{-3}.

Calcium concentrations were measured using a Pye Unicam atomic absorption spectrophotometer SP9 set on absorption mode at 422.7nm with an acetylene/air flame. Standard solutions of calcium were prepared in deionized water over a concentration range of 0.249 - 1.494mol m^{-3} with 2.87mol m^{-3} lanthanum, and a calibration curve fitted. Samples were run sequentially with an uptake volume of 3cm^3 per minute. Five readings were taken at 2 sec intervals, and a mean calculated. A read-out of calcium concentration calculated from the standard was given. Calcium ion concentration in the tissue was calculated by relating the actual weight of the hypocotyl that was suspended in the 10,000*g* supernatant.

2.8 Extraction of acidic auxins.

Hypocotyl segments (96 g fresh weight) were excised and ground in 160cm^3 80% (v/v) pre-cooled acetone. The resulting macerate was stirred for 48 h at 3°C in darkness and filtered through 4 layers of muslin. Acetone was removed from the filtrate by evaporation at 40°C *in vacuo* using a rotary evaporator. The aqueous concentrate was filtered and the eluent adjusted to pH 8.0. The mix was partitioned three times with freshly distilled diethylether and the neutral ether extracts discarded.

The pH of the mix was adjusted to 2.5 for extraction of the acidic hormones and partitioned 3 times with freshly distilled diethyl ether. The ether extracts were combined and concentrated in a rotary evaporator *in vacuo* at room temperature to yield an acid ether extract and stored at 0°C in the dark.

Prior to HPLC, the concentrate acid ether extract was dissolved in 1.2cm^3 absolute ethanol, sonicated and centrifuged at 10,000*g*. 1cm^3 aliquots of the supernatant were analysed. PAA in ether was run as a standard.

2.9 Red /Far red light experiments.

2.9.1 light source.

A complete black-out was achieved within the darkroom which was fitted with a dark lock between two doors. One

safelight was used, consisting of a single 40W Grolux fluorescent tube wrapped in one layer each of Cinemoid 46, 1 and 20 and two layers of 24A. Extraneous sources of light from the ultracentrifuge, water-bath and oven were either blacked out or covered with green cinemoid 24A.

Red and far red light sources were contained within a light-tight box of internal dimensions 420 x 420 x 440mm and lined with aluminium foil and fitted with a fan to draw air from the base over the lamps and leaving through the roof. Fluorescent and incandescent tubes were mounted alternately in a bank in the roof, each in a separate chamber with its own filter combination. The red source was provided by 4-15W Grolux tubes (400mm each in length) with one layer of 3mm red Plexiglass. Aluminium foil was wrapped around 20mm of each tube end to contain FR emission. The far red source was provided by 10-30W Crompton tubes (each 150mm in length) each with 3 layers of 3 mm blue Plexiglass and 1 layer of deep amber cinemoid 33. The filter combinations used by Roth-Bejerano & Hall (1986b) were also used, their red light source being obtained by filtering the Grolux tubes with one layer of Cinemoid 14. The far red source was from the incandescent tubes and filtered through a single layer of red Plexiglass and 2 layers of green cinemoid 24A. A 150W slide projector with Balzer filters mounted in a black perspex slide holder which fitted into the slide compartment perpendicular to the source were also used to provide a R and FR source. The wavelengths of filter used were 661nm, ϕ32mm and 741nm, ϕ32mm.

Seedlings were grown to the 6 day stage in complete darkness and not exposed to any safelight during watering. Material was transferred from the growth room to the dark room without exposure to any light/safe-light. Trays of plants were light-treated at a distance of 300mm. Microsomal fractions were illuminated at the same distance and immersed in either a large volume of water or ice. Microsomal fractions were also illuminated with the Balzer filter combination. To eliminate extraneous light from the projector, a cardboard tube was mounted on the end of the projector lens and microsomal fractions contained in a test tube were illuminated by inserting the latter

through the cardboard tube 5o mm from the lens. Light treatments were also given to fractions in a fluorescent quinacrine mixture, but in this case the cuvette was placed 20-50mm from the end of the lens and extraneous light cut out by covering the cuvette with black polythene.

2.9.2 Preparation of microsomal fractions.

Intact plants were irradiated *in vivo* with R for 30 min, or hypocotyl sections were placed in homogenization medium and irradiated under identical conditions. The homogenization medium was the same as that used in the ATPase standard homogenization procedure except that 5mol m^{-3} H_3BO_3 was incorporated. The material was ground under R and a microsomal pellet was prepared as described previously. Rotors were loaded and unloaded in the dark room. The resuspended pellets were irradiated again either with R for 10 min in the light box or with the Balzer filters or FR for 30 min. ATPase assays were carried out as described previously for 1 h at 37°C in complete darkness. Exposure to white light was only made after the reaction was stopped with acid.

2.10 Preparation of material for electron microscopy.

For general structural observations, small pieces of hypocotyl, cotyledon or microsomal pellets were fixed in 1% (v/v) glutaraldehyde and 4% (v/v) formaldehyde in 50mol m^{-3} cacodylate buffer (pH 7.0) for 2 h at room temperature. The tissue was washed in the above buffer and post-fixed overnight in 1% (v/v) OsO_4 in 50mol m^{-3} cacodylate buffer (pH 7.0). The tissue was rinsed in distilled water and dehydrated for 30 min in a series of 30%, 50%, 70%, 95% and 100% (v/v) absolute ethanol followed by propylene oxide before embedding in Spurr's resin and sectioned for light microscopy. For electron microscopy, gold sections were post-stained in uranyl acetate for 2 h followed by lead citrate for 5 min. Sections were cut using a Diatome diamond knife on a Reichart Ultra-Cut 2 and examined under a Jeol JEM-1200 EX electron microscope at 60KV.

2.10.1 ATPase localization.

Thin slices of hypocotyl were fixed in ice-cold 1% (v/v) glutaraldehyde and 4% (v/v) formaldehyde in 50mol m^{-3} cacodylate buffer (pH 7.0) for 90 min. The slices were washed thoroughly for 2 h in ice-cold buffer before staining for 1-2 h at 30°C in a medium containing 2mol m^{-3} ATP, 2mol m^{-3} $Mg(NO_3)_2$, 3mol m^{-3} $Pb(NO_3)_2$ in 50mol m^{-3} Tris-Hepes buffer (pH 7.0). The slices were rinsed in buffer before post-fixation in 1% (v/v) OsO_4, dehydrated as above and embedded in Spurr's resin and sectioned for electron microscopy. Controls consisted of slices incubated in the medium in the absence of ATP, or in the presence of 1mol m^{-3} vanadate.

2.11 Preparation of gradient gels.

Gels were poured to establish a gradient of 3.24% - 24.53% with a 3.24% loading gel. Glass plates (82 mm x 108 mm) with side spacers and top combs of 2 mm thickness were taped together and heated to 150°C for 12 h to contract the tape and establish a tight seal. These were placed in a pouring tank with a basal outlet which was covered with 10 mm link chain to prevent turbulence. The gradient pourer consisted of two glass tubes of internal diameter 40 mm joined at the base with tubing. A glass stirrer was placed in one tube from which an exit tube adjoined the pouring tank. A 'light' acrylamide solution was placed in the column with the exit tube and a 'heavy' acrylamide solution placed in the other. Light and heavy solutions were prepared as follows:

	Volume of light acrylamide	Volume of heavy acrylamide
H_2O	91.5cm^3	11.5cm^3
4X Buffer	35cm^3	25.5cm^3
Acrylamide	11.25cm^3	55cm^3
Ammonium persulphate	1cm^3	0.8cm^3
TEMED	10mm^3	0

The acrylamide mixture contained 40% (w/v) acrylamide, 1% (w/v) Bis. 4X buffer in a final volume of 500cm^3 contained 12.1g Tris, 4.0g glycine, 10cm^3 20% (w/v) Triton X-100. All solutions were cooled to 4°C before use. The 'light' solution was degassed for 5 min with a tap water vacuum pump, the TEMED added and placed into one arm of the gradient mixer and the 'heavy' acrylamide mixture in the other. Initially, 25cm^3 of the light solution alone was run into the pouring tank. The adjoining tap between the two columns was opened, the light solution stirred and the gradient gradually run into the tank. At the end of the procedure, the acrylamide gradient was pushed up into the plates by 200cm^3, 50% (w/v) sucrose plus bromophenol blue. The gels were left overnight to set and stored in distilled water in the dark at 4°C.

2.11.1 Running of gels.

Plate gels were placed vertically in a millipore electrophoresis tank which was kept at 4°C and the running buffer was circulated through a cooling coil. Up to four plates were placed just below the surface of the running buffer which contained 6.05g Tris, 2.0g glycine and 5cm^3 20% (w/v) Triton X-100 in a final volume of 250cm^3. The microsomal pellet was solubilized in 0.2cm^3 (20%-2% v/v) Triton X-100 and left on ice for 20min. The mixture was spun at 100,000*g* for 30min and the supernatant removed. 20mm^3 of solubilized protein and 20mm^3 4X buffer with bromophenol blue plus a few grains of sucrose were mixed and introduced into the well of the plate. The gels were run at 25 mA (140 volts) for 4 h.

2.11.2 Gel staining.

Gels were stained for protein using a mixture of 1.25g coomassie brilliant blue R250 in 227cm^3 100% methanol and 46cm^3 glacial acetic acid, made up to 500cm^3 with distilled water, poured over the gel and left for 2 h. Destaining was carried out for 24 h with 5 - 6 changes using a mixture of 50cm^3 (100%) methanol and 75cm^3 glacial acetic acid and made up to 1000cm^3 with distilled water.

For ATPase gels were washed in distilled water and given two 30 min rinses in 40mol m^{-3} Tris-Mes buffer (pH 6.5) on ice. The gels were then incubated for 3 h at 37°C in a medium containing 40mol m^{-3} Tris-Mes buffer (pH 6.5), 2mol m^{-3} $Mg(NO_3)_2$, 3.5mol m^{-3} $Pb(NO_3)_2$, and 2mol m^{-3} ATP. Following incubation the gels were washed with distilled water for 8h at 4°C, stained with fresh 1% (v/v) ammonium sulphide for 2 min and rinsed in distilled water (100cm^3) and stored at 4°C. To determine the effect of inhibitors on ATPase activity, gels were preincubated in the presence of the inhibitors and the inhibitor was present in the incubation medium. The effect of pH was examined by pre-incubation in various pH solution and incubating at final pH values.

2.12 Source of chemicals and equipment.

Balzers filters were obtained from Balzers Aktiengesellschaft Für Hochvakuumtechnik und Dünne schichten Balzers, Fürstentum Liechtenstein.

Beckman Ready-Solv E P from Beckman RIIC Ltd., High Wycombe, Bucks., U.K.

Charged silica microbeads were a kind gift from Dr B. Jacobson, University of Massachusetts, Amherst, Massachusetts, U.S.A.

Chemicals from BDH Ltd., Poole Dorset, U.K. and Sigma, Rochford, Illinois, U.S.A.

Cinemoid filtered were obtained from Strand Lighting (U.K. Sales), P.O. Box 51, Great West Road, Brentford, Middlesex, U.K.

Coomassie Blue G-250 based reagent (Protein assay reagent) from Pierce Chemical Company, Rochford, Illinois, U.S.A.

Cucumis sativus L.cv. Long Green Ridge seeds were obtained from Clause (U.K.) Ltd., Charvil Farm, New Bath Rd., Charvil, Reading. RG10 9RU U.K.

Cucumis sativus L. cv. Perfection seeds were obtained from Nutting & Speed, Station Rd., Long Stanton, Cambridge. CB4 5DU U.K.

Plexiglass was a kind gift from Dr. B. Thomas, Glasshouse Crops Research Institute, Littlehampton, U.K.

Radioactive sugars UDP-[^{14}C] glucose was obtained from Amersham International Plc, Amersham, Bucks., U.K.

SW26 was a kind gift from Dr. J-P Blein, Institut National de la Recherche Agronomique, France.

CHAPTER THREE.

Plant membrane separation and characterization of ATPases associated with the plasma membrane and tonoplast.

3.1 Introduction.

The plasma membrane is the outer permeability barrier of the plant cell and carries out several important functions including transport of ions and other solutes, hormone binding, and cell wall synthesis. It is thus generally concerned with the regulation of the cytoplasmic environment. The tonoplast plays a major role in the regulation of the cytoplasmic milieu, both osmotically and ionically by regulation *via* the vacuole. Although some of these functions may be examined using intact tissue and protoplasts or isolated vacuoles, membrane fractions of tonoplast and plasma membrane are used to study the enzymes involved in these processes. Such investigations require preparations of membrane fractions in sufficient quantities and of the highest purity, the latter usually being the limiting step.

Protoplasts have been used as a source of plasma membrane, since some of the difficulties encountered when whole tissues are used are eliminated. If wall digestion is complete, clearly there will be no contamination by cell wall components, though there may be some hydrolysis of the outer surfaces of the plasma membrane (Hall 1983). Considerable expertise is required in the production and maintenance of protoplasts through the isolation and purification stages. Since protoplast preparation entails considerable physical and osmotic stress, the validity of regarding protoplasts as still possessing a normal metabolism has been called into question (Burgess 1978).

The use of traditional centrifugal methods to isolate plasma membrane from protoplasts, apart from being time-consuming, generally provides low yields and results in contamination of the fraction with cytoplasmic components (Goffeau & Slayman 1981). This has led to the

development of novel methods which depend on the ease with which specific markers may be bound to the plasma membrane surface. Bound markers can facilitate both isolation and identification of plasma membrane after protoplast lysis. Concanavalin A has been used extensively for this purpose since it has an affinity for the outer plasma membrane surface (Randall & Ruesink, 1983). Other types of surface binding agents such as diazotized [^{125}I]-iodosulphanilic acid (Galbraith & Northcote 1977, Perlin & Spanswick 1980) and [^{125}I]-labelled myelome protein J539 have also been used successfully (Schibeci *et al.* 1982).

Isolation of plant vacuoles directly from protoplasts typically yields tonoplast fractions of high purity. For example, protoplasts have been isolated from *Kalanchoë daigremontiana* by digesting leaves with cellulase and pectolyase and vacuoles isolated after lysis with DEAE-dextran. Separation was made in one centrifugation step at 1200*g* for 15 min on a discontinuous ficoll gradient (Jochem *et al.* 1984).

Positively charged silica microbeads (diameter 10 - 50nm) have been used to isolate relatively pure sheets of plasma membrane from erythrocytes, HeLa cells, *Dictylostelium discoideum* and yeast protoplasts. The procedure relies on the ionic binding of beads applied at high density to the exposed, negatively charged surfaces of intact wall-free cells (Jacobson 1977, Kalish *et al.* 1978, Chaney & Jacobson 1983). Cell binding is followed by a neutralization step, either by the addition of polyacrylate or by a pH shift. Neutralization prevents the binding of negatively charged proteins or cell organelles upon lysis to regions of the bead surface not coated with membrane. Following lysis, plasma membrane-coated beads are washed to remove contaminating material and purified. The high density of the microbeads (2.5 g per cm^3) enables the rapid separation of coated plasma membrane sheets from cellular organelles by low speed centrifugation. Polenko & Maclachlan (1984) have reported that charged silica microbeads may be used to facilitate the preparation of purified plasma membrane sheets from stable protoplasts isolated from the apical regions of etiolated *Pisum sativum* seedlings.

3.1.1 Separation of plant membranes with associated ATPases.

Isolation of intact subcellular organelles is often possible from animal systems and usually only requires a gentle homogenization technique to break the plasmalemma. Plant tissues on the other hand require a greater force to break the cell wall and this may concomitantly disrupt the cytoplasmic membranes. Whole plant material is routinely homogenized with a pestle and mortar or polytron blender (e.g. Lew *et al.* 1985) at 4°C in a buffered medium (pH 7.0-8.0) containing a variety of protectants against phenolics, fatty acids and protease activity (e.g. EGTA, SHAM, PMSF, DTT, PVP and BSA). With such procedures the characteristic differences in size between cellular membranes is reduced and satisfactory separation of membranes and organelles becomes very difficult. In addition, the plasma membrane is closely appressed to the cell wall and substantial quantities may be lost when the wall fraction is removed during the differential centrifugation stage.

Organelle size, density and surface properties are utilized in various fractionation techniques to separate cell membrane components. The procedures typically used include phase partitioning (Larsson & Anderson 1979), free flow electrophoresis (Morré *et al.* 1983), and less commonly affinity column chromatography (Schroeder *et al.* 1982). Separation may be made from whole tissue macerates, protoplasts or vacuoles.

Differential centrifugation however is the most widely used method to separate membrane fractions on the basis of their size and density. A filtered macerate is typically centrifuged at low speed (250 - 3000*g*) to remove wall debris, plastids and nuclei. Soluble hydrolases and secondary metabolites are removed as quickly as possible by differential centrifugation or by loading the unpelleted homogenate directly onto a gradient or by gel filtration using a sepharose 4B column. The subcellular components pass in the void volume and soluble hydrolytic enzymes are retarded by the column.

Microsomal fractions obtained by differential centrifugation are used by various workers to study

membrane associated enzymic activities. However, these fractions may be further purified with step and linear density gradients (Quail 1979). Various gradient compositions have been used by different workers and the choice depends on the membrane being isolated and the tissue type. Four major classes of gradient have been used;

1. Sugars: sucrose, sorbitol, mannitol, dextran and ficoll;
2. Salt solutions: caesium chloride;
3. Colloidal silica suspensions: Ludox, Percoll;
4. Iodinated compounds: urograffin, renograffin, metrizamide and nycodenz.

In spite of the osmotic effects of sucrose, it is still the most widely used material mainly because of its cheapness.

Additives are incorporated into the gradient that act as protectants, while others are used to stabilize and influence membrane distribution. Buffers and sulphydryls may help stabilize membrane activity, especially when prolonged centrifugation is used. Rough endoplasmic reticulum is maintained by the addition of Mg^{2+} to the gradient. EDTA and EGTA are added when smooth endoplasmic reticulum is required.

Complete separation of subcellular components on a density gradient occurs when all the membranes are at their equilibrium density (isopycnic condition). Typically when sucrose gradients are used, centrifugation is undertaken for 1.5 - 2 h at 80,000g to 85,000g. This centrifugation time and g force has been reported to be ineffective in bringing membranes to equilibrium (Leonard & Vanderwoude 1976). Sharper resolution was reported with a 15 h centrifugation time; this was not improved upon with further centrifugation.

Phase partitioning has recently received much attention and the technique separates membrane vesicles according to their surface properties rather than their size and density as in gradient centrifugation. Preparations of membrane vesicles with uniform surface properties and sidedness are obtained; thus the technique is unsuitable when vesicles are required for proton

pumping, as typically plasma membrane vesicles are sealed for the most part right-side-out (Larsson *et al.* 1984). Phase partitioning is easily scaled up and chlorophyll-free preparations of plasma membranes from leaves of barley and spinach for example, have been obtained by partitioning in an aqueous dextran-polyethylene glycol two phase system (Kjellbom & Larsson 1984). An aqueous two-polymer phase system has been used for purification of plasma membranes from *Dactylis glomerata* L. (Yoshida *et al.* 1983), wheat and oat roots (Sommarin *et al.* 1985, Bérczi & Møller 1986), *Cucumis* (Memon *et al.* 1987), barley roots and leaves (Körner *et al.* 1985).

3.1.2 Assessment of membrane purity by markers.

Various biochemical, histochemical and analytical methods have been employed to assist in the identification of membrane fractions. The biochemical characteristics of the ATPases associated with the plasma membrane and tonoplast are outlined in the General Introduction. In addition, an ATPase associated with isolated secretory vesicles from a liquid suspension culture of *Avena sativa* has been reported (Binari & Racusen 1983) and a H^+-translocating ATPase associated with the Golgi membranes of corn coleoptiles has been demonstrated (Chanson *et al.* 1984, Chanson & Taiz 1985). Isolation of a highly purified Golgi complex has been made from sycamore suspension-cultured cells and the association of a H^+-translocating ATPase has been demonstrated (Skowkat & Akazawa 1986). Acidification was prevented by ionophores indicating that the proton pump was electrogenic in nature. No specific ATPase inhibitors have been found, but the ATPase was not inhibited by sodium vanadate, potassium nitrate, oligomycin or sodium azide. Both the ATPase and the H^+-translocating ATPase were inhibited by N',N'-dicyclohexylcarbodiimide.

Almost certainly there are several different plant associated glucan synthetases as a variety of polyglucans are found. One of these enzymes, glucan synthetase II has been used as a plasma membrane marker (Ray 1979). Estimation of the recovery of plasma membrane using glucan synthetase II activity may indicate relatively high

levels, but often represents an over estimate of the recovery, as the assay is not specific for plasma membrane-bound glucan synthetase (Quail 1979, Ray 1979).

While the tonoplast- and mitochondrial-associated ATPase is insensitive to vanadate, the mitochondrial enzyme is alone sensitive to azide and oligomycin (Churchill et al. 1983, O'Neill *et al.* 1983). The associated ATPase is also sensitive to erythrosin B (Cocucci 1986). Mitochondrial membranes are typically located with the cytochrome c oxidase assay. Activity is associated with the inner membranes of the mitochondria and is extensively used as a marker for this organelle type. Enzyme activity is assayed by measuring the initial rate of aerobic oxidation of reduced cytochrome c (Yonetani 1967). This assay requires solubilization of the outer membrane for full activity, since cytochrome c cannot permeate the outer membrane. Triton X-100 (Bomhoff & Spencer 1977) or digitonin (Tolbert 1974) are typically used to avoid detergent interference with the enzyme.

IDPase activity was initially located in the Golgi apparatus of plants by histochemical methods (Dauwalder *et al.* 1969) and later biochemically by Ray *et al.* (1969). There are also high levels of soluble activity and the enzyme has also been found in the vacuole, ER and purified nuclear membranes. However, the IDPase associated with the Golgi apparatus has been distinguished from other activities by its change in pH optimum from 6 to 7 when the membranes are stored at 5°C for 2-3 days. Latent IDPase is typically measured as the difference in activity the presence and absence of 0.1% digitonin at pH 6.5 (Chanson *et al.* 1984). Latent UDPase has been used as a marker for the Golgi apparatus and is similarly assayed with digitonin (Chanson *et al.* 1984, Chanson & Taiz 1985). Glucan synthetase 1 is also used as a marker for the the Golgi apparatus and there is good correlation between GS1 and latent IDPase of membranes isolated from sucrose gradients (Chanson *et al.* 1984). Activity is measured at a low concentration of UDPG and high Mg^{2+} concentrations (10mol m^{-3}).

The endoplasmic reticulum has been identified by the markers NADH- and NADPH-cytochrome c reductase.

Appreciable amounts of NADH-cytochrome c reductase activity are also present in mitochondrial membranes, but may be specifically inhibited by the inclusion of antimycin A into the assay.

3.2 Results.

3.2.1 Plasma membrane isolation from protoplasts.

Hypocotyl tissue yielded approximately 100,000 protoplasts g^{-1} FW with the standard procedure used and protoplasts from a typical isolation are illustrated (Fig.3.1).

The cell wall status of the protoplasts was assessed with calcafluor white and 75% of the protoplasts contained no wall material. FDA was used to assess protoplast integrity and 81% of the protoplasts fluoresced.

Following lysis of beaded protoplasts and purification, plasma membrane 'sheets' were removed from a gradient and examined under the electron microscope (Fig.3.2a,b & 3a,b). Both large sheets of membrane with silica beads attached and membrane fractions with no attached beads were seen. Mitochondria with attached beads or with beads in close association were observed (Fig.3.3b).

Cytochrome c oxidase assays were carried out on the bead membrane fraction and the lysed protoplast fraction. Activity was always detected in the bead membrane fraction but not in the latter. A variety of techniques were used in an attempt to achieve separation of mitochondria from the plasma membrane. These included: sonication; lysis in a solution with an osmotic pressure of 130mmol/Kg containing 33mol m^{-3} Hepes-KOH buffer (pH 7.0), 33mol m^{-3} $CaCl_2$, 1mol m^{-3} DTT and 0.1% (w/v) BSA; isolation in the presence of cytochalasin D ($4.9x10^{-2}$mol m^{-3}); and lysis followed by incubation in 33mol m^{-3} EGTA at pH 8.6 for 10 min prior to centrifugation. In each case significant levels of cytochrome c oxidase activity remained with the bead plasma membrane sheets. ATPase activity was detected in membrane fractions and a specific activity of 53 µmol Pi h^{-1} mg^{-1} protein in the presence of Mg^{2+} was obtained.

Figure 3.1. Light micrograph of protoplasts prepared from the hypocotyl of *Cucumis*. Magnification x100.

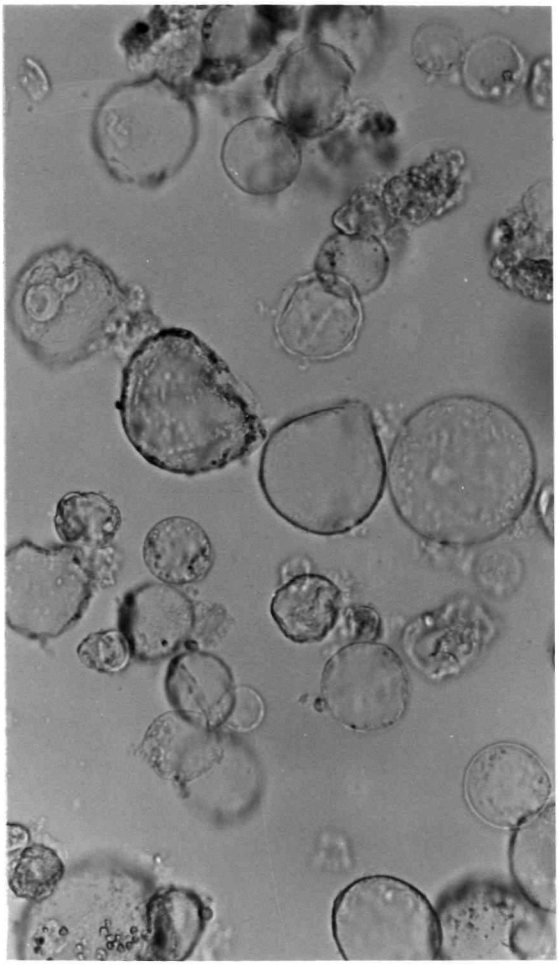
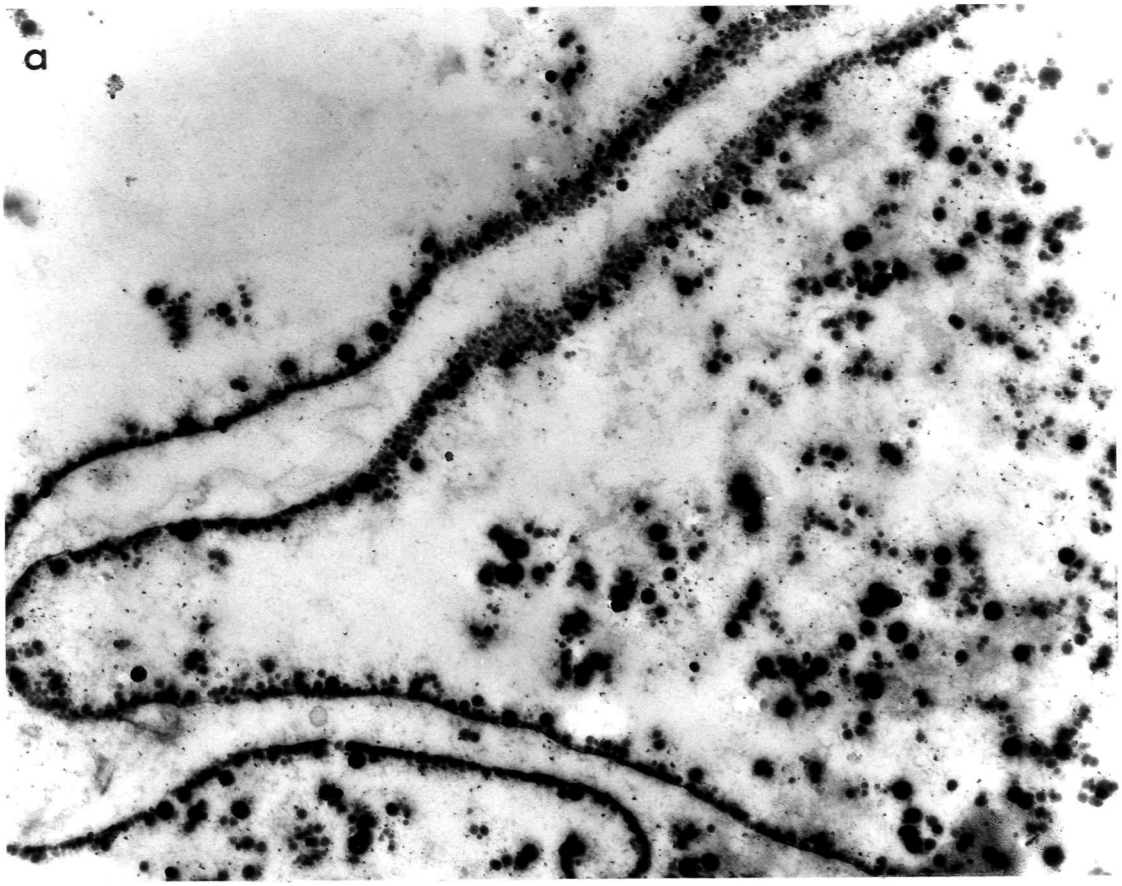


Figure 3.2a, b. Electron micrographs of plasma membrane sheets prepared from protoplasts that had been incubated with charged silica micro-beads, lysed and purified on a ficoll gradient. Magnification (a) 20K, (b) 5K.


a

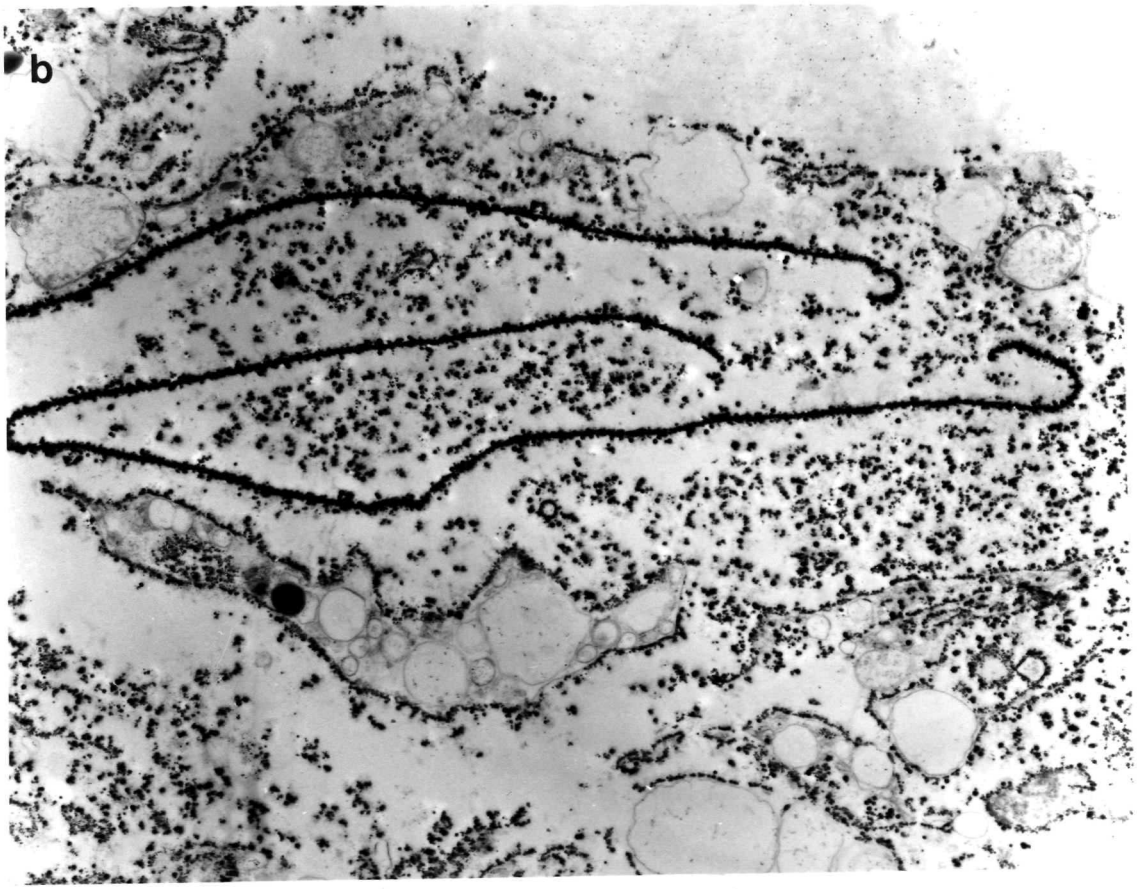
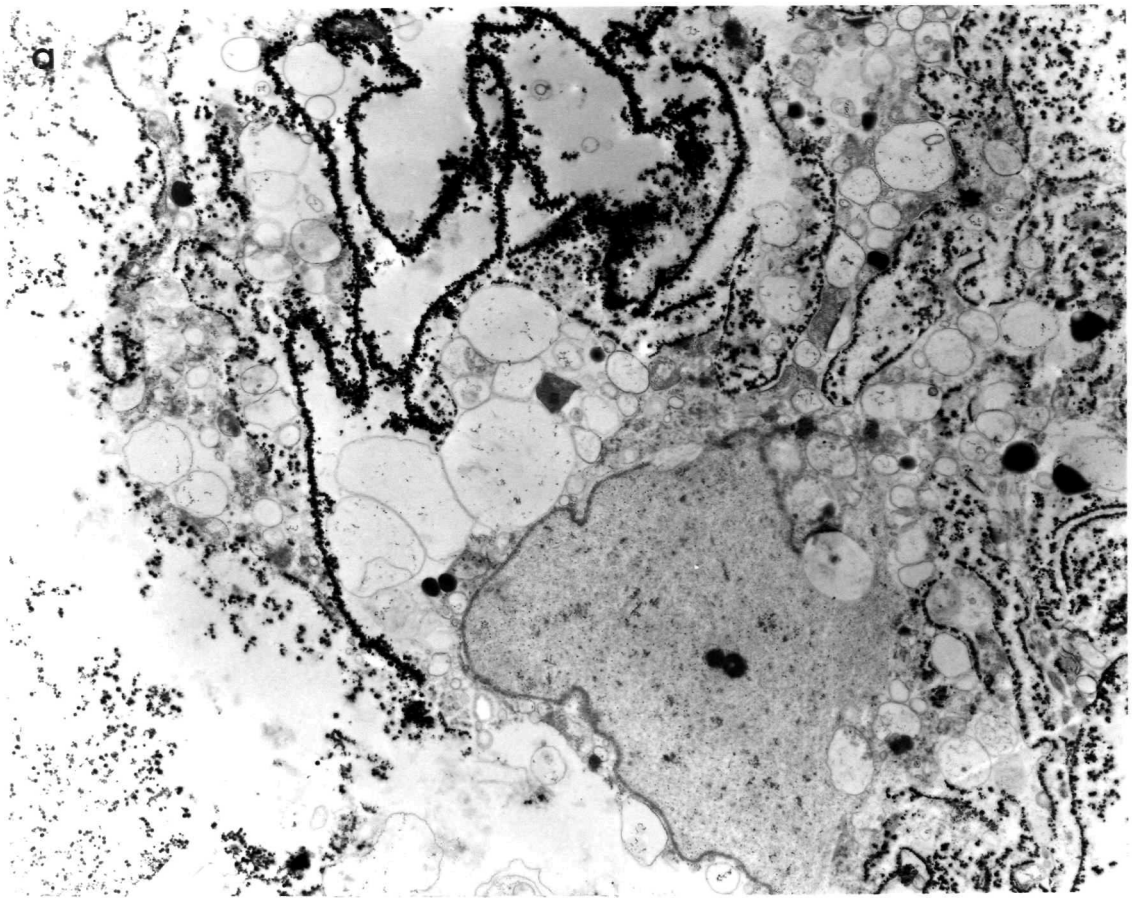
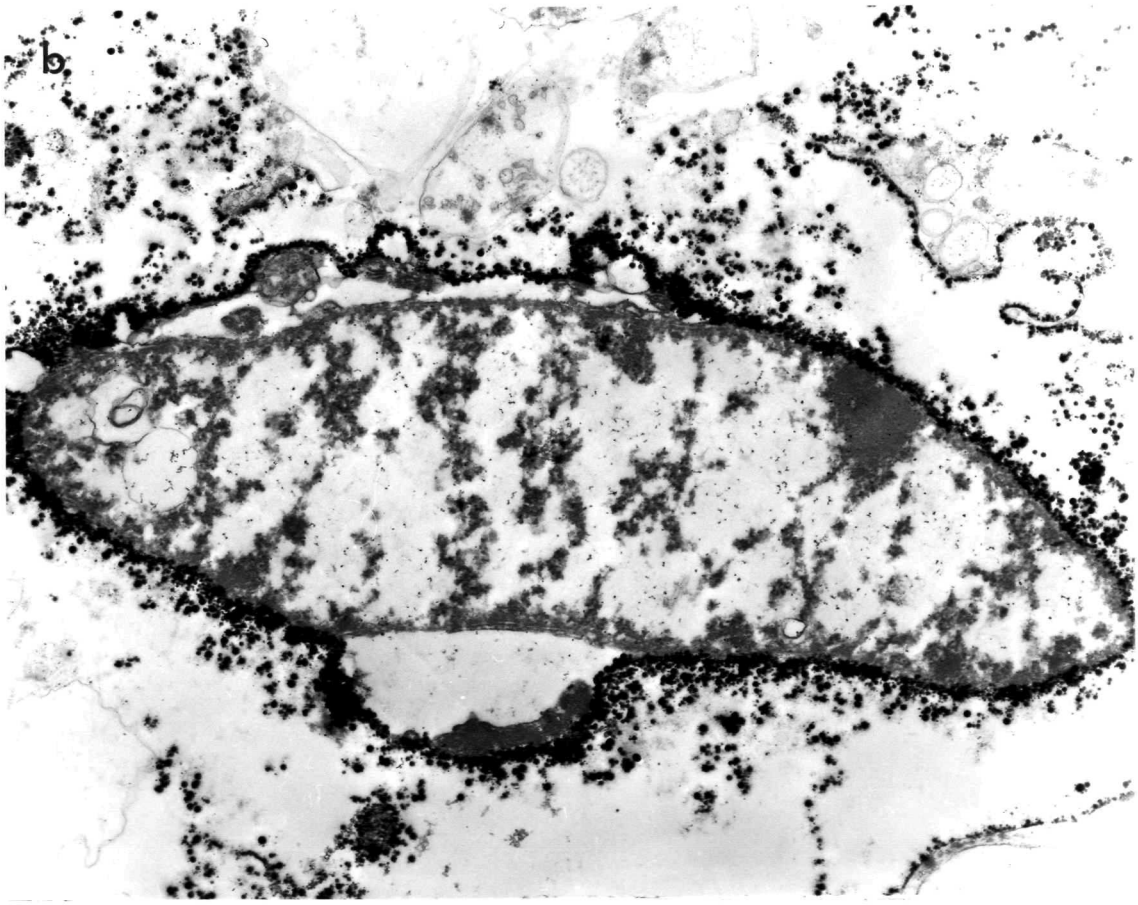

b

Figure 3.3a,b. Electron micrographs of plasma membrane sheets prepared from protoplasts that had been incubated with charged silica micro-beads, lysed and purified on a ficoll gradient. Magnification (a) 4K, (b) 7.5K.


a


b

3.2.2 General characterization of the microsomal-associated ATPase prepared from hypocotyl tissue.

The activity of the magnesium-dependent microsomal ATPase was found to be sensitive to pH, with an optimum at 7.0. In the presence of 50mol m^{-3} KCl, a similar optimum was found. The percentage stimulation by K^+ varied with pH from about 60% at pH 6.0 to 15% at pH 7.0 and rose again at pH 7.5 to 35%. Manganese was found to partially replace magnesium but calcium at 0.8mol m^{-3} only achieved 20% of the Mg^{2+}-ATPase activity, even when assayed in the presence of 2mol m^{-3} magnesium. The fraction was found to contain a nitrate-sensitive component showing up to 30% inhibition at 100mol m^{-3} KNO_3, a vanadate-sensitive component showing 60% inhibition at a concentration of $50x10^{-3}$mol m^{-3} and an azide-inhibitable component. The microsomal fraction was sensitive to monovalent cations, with potassium, sodium and lithium showing maximum stimulation at 20mol m^{-3} and all becoming inhibitory at 100mol m^{-3}. Calcium was found to be a potent inhibitor of this ATPase activity.

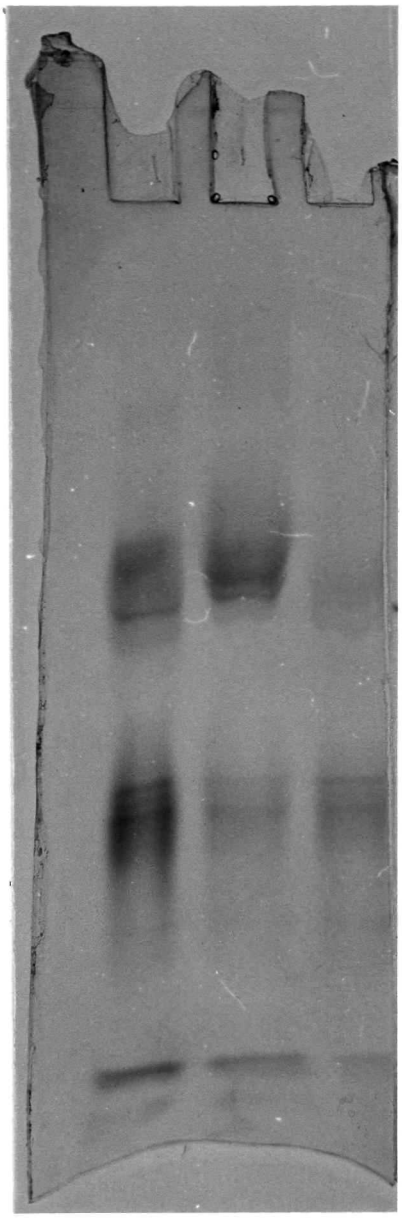
Detergent solubilized proteins from a microsomal fraction were separated by gel electrophoresis and stained for ATPase (Fig.3.4). Characteristically a low molecular weight band was observed near the base of the gel and up to 10 distinct bands were seen in the upper regions. The origin of these bands was examined by incorporating ATPase inhibitors into the incubation medium. In the presence of molybdate the band number was reduced, but with the addition of nitrate and vanadate no significant differential staining was observed. Gels were also run of proteins solubilized from the sucrose gradient interfaces, but no differential banding patterns were observed.

3.2.3 Discontinuous sucrose gradient separation of microsomal membranes.

3.2.3.1 Gradient types.

Several different gradient compositions were tested to achieve optimal separation of membranes. These were: 20-45% (w/w) sucrose; 20-45% (w/w) sucrose, 1mol m^{-3} DTT and 2mol m^{-3} EGTA; 20-45% (w/w) sucrose, 1mol m^{-3} DTT and

Figure 3.4. ATPase stained Triton gel run with a solubilized microsomal membrane fraction prepared from the hypocotyls of *Cucumis*. The microsomal pellet was solubilized in 20% (v/v) Triton X-100 for 15 min on ice. The gel was run for 4½ h. ATPase staining was carried out as described in "Materials and Methods".



2mol m^{-3} EDTA; 20-45% (w/w) sucrose, 2mol m^{-3} Tris-Mes buffer (pH 7.0) and 1mol m^{-3} DTT. Gradient fractions were examined for their ATPase sensitivity to nitrate, vanadate and azide. The latter composition gave the best separation and longer centrifugation times gave no further separation of the nitrate- or vanadate-sensitive ATPase.

Mg^{2+} dependent ATPase activity was found at each of the sucrose interfaces with numbers 3 and 4 typically exhibiting the highest specific activity ($\cong$ 10-12x10^{-3} mol m^{-3} $Pi.h^{-1}.mg^{-1}$ protein) (Fig.3.5). Pyrophosphatase activity was examined in the five interfaces at pH 8.0 and highest specific activity was found in interface 2.

3.2.3.2 Distribution of nitrate- and vanadate-sensitive ATPase.

Nitrate- and vanadate-sensitive ATPase activities were used to assess the distribution of tonoplast and plasma membranes within the gradient (Fig.3.6a&b). The inhibitor results indicated that the gradient produced an interface that was enriched in tonoplast (interface 1) but contained significant levels of plasma membrane. Interface 4 was enriched in ATPase of plasma membrane origin and contained little ATPase of tonoplast origin.

DCCD caused differential inhibition of the K^+ stimulated ATPase activity at the 5 interfaces of the sucrose gradient (Fig.3.7) with 100x10^{-3} mol m^{-3} and 10x10^{-3} mol m^{-3} concentrations causing a marked inhibition in interfaces 1 and 2.

Glucan synthetase II activity was examined in the microsomal fraction and each of the gradient interfaces (Fig.3.8). The experiment was carried out once and highest specific activity was found associated with interfaces 2 - 4 of the gradient.

3.2.3.3 Response of ATPase to monovalent cations and anions.

Another potential marker for the plasma membrane and tonoplast is the differential response of the ATPases to monovalent anions and cations. Interface 1 was found to be relatively unaffected by the chloride cations of rubidium, sodium and lithium (Fig.3.9). Ammonium was

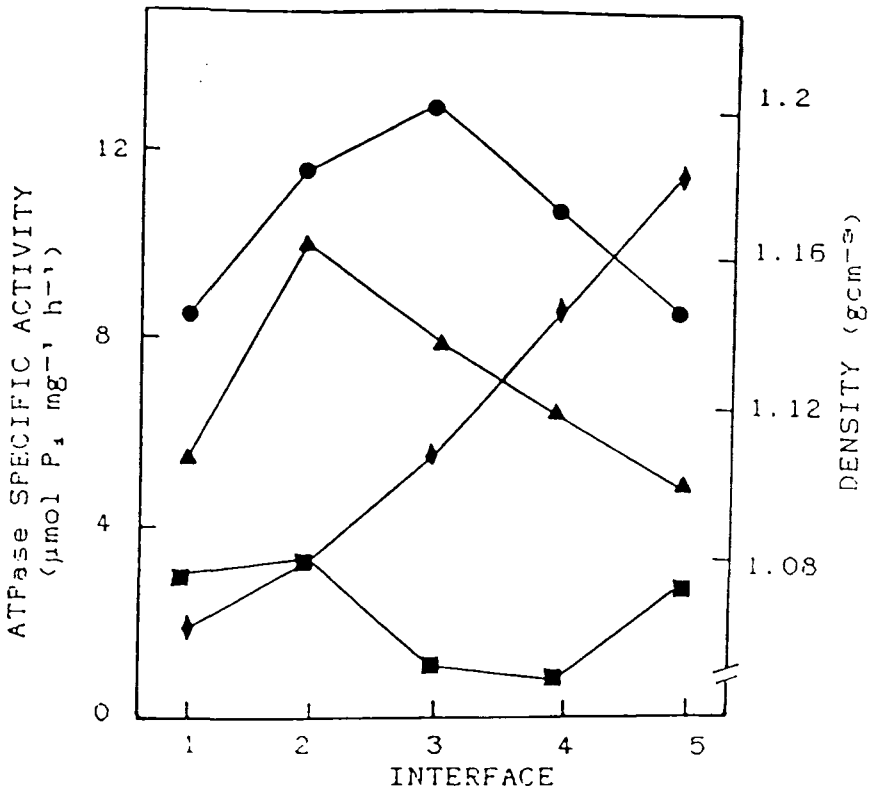


Figure 3.5. ATPase specific activity associated with interfaces 1 - 5 of a 25%, 30%, 34%, 38%, 45% (w/w) sucrose gradient. The standard ATPase assay was used and both specific ATPase activity (●) and KCl stimulated ATPase activity (■) were plotted. Pyrophosphatase specific activity associated with each of the gradient interfaces is also shown (▲) and the density (◆) of each gradient interface.

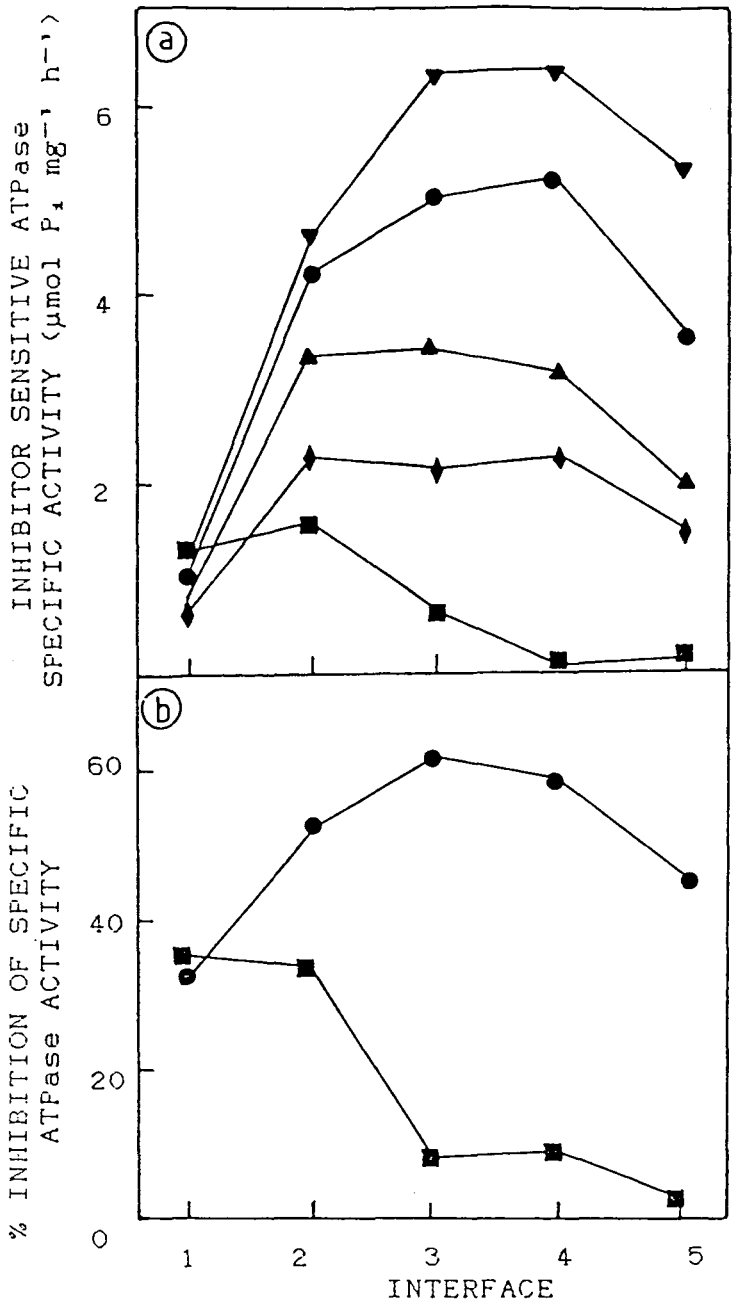


Figure 3.6a. Vanadate- and nitrate-sensitive ATPase specific activity associated with each of the gradient interfaces. The standard ATPase assay was used with the incorporation of $100x10^{-3}$mol m^{-3} vanadate (▼), $50x10^{-3}$mol m^{-3} vanadate (●), $25x10^{-3}$mol m^{-3} vanadate (▲) and $12.5x10^{-3}$mol m^{-3} vanadate◆and 50mol m^{-3} nitrate (■).

Figure 3.6b. Percentage inhibition of ATPase activity by vanadate and nitrate. The standard ATPase assay was used and activity compared to that obtained with the incorporation of $50x10^{-3}$mol m^{-3} vanadate (●) and 50mol m^{-3} nitrate (■).

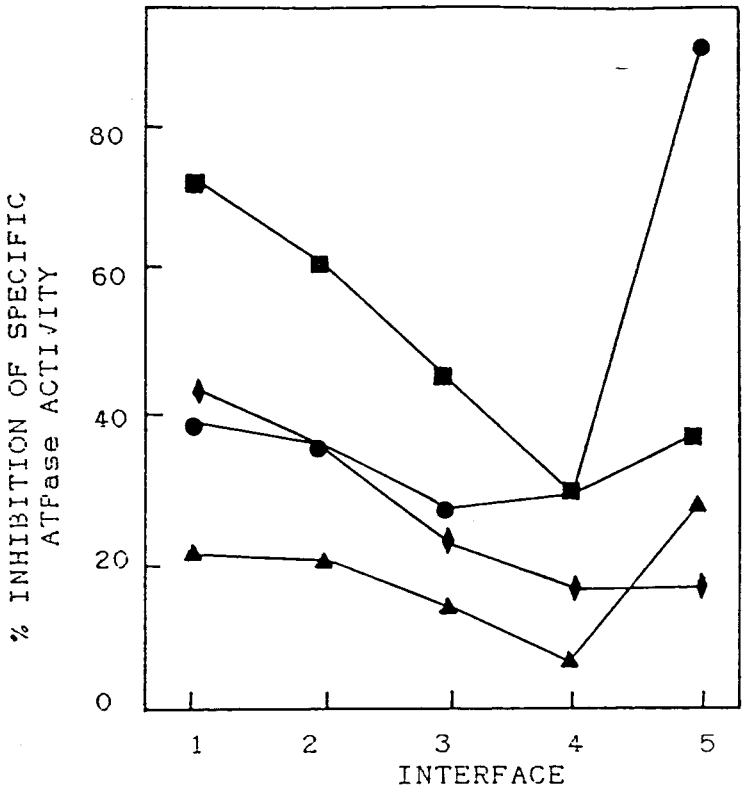


Figure 3.7. Percentage inhibition of ATPase activity by DCCD and azide in each of the gradient interfaces. The standard ATPase assay mixture was used with the incorporation of $100x10^{-3}$mol m^{-3} DCCD (■), $10x10^{-3}$mol m^{-3} DCCD (♦), 1mol m^{-3} azide (▲), 1mol m^{-3} azide at an assay pH of 8.5 (●).

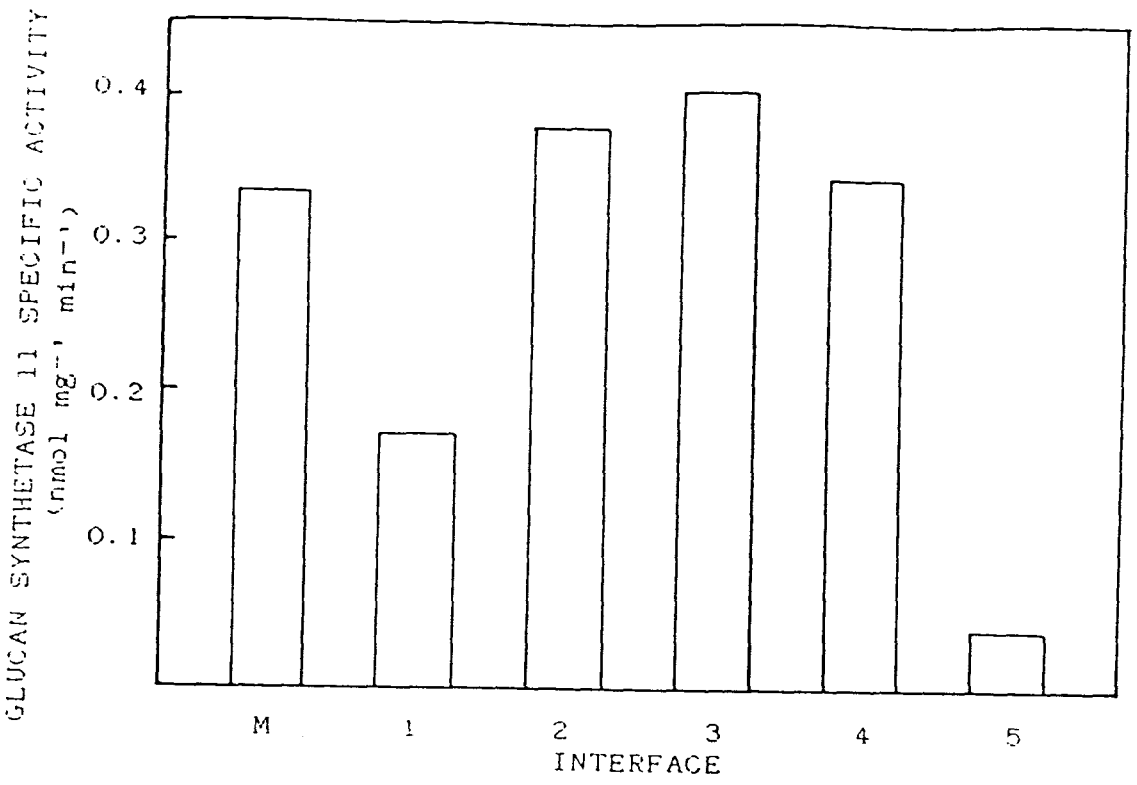


Figure 3.8. Specific glucan synthetase II activity in (M) a microsomal fraction, and in each of the gradient interfaces (1 - 5). The standard assay was used as described in "Materials and Methods". The results are the data from one experiment, each bar being the mean of three assays.

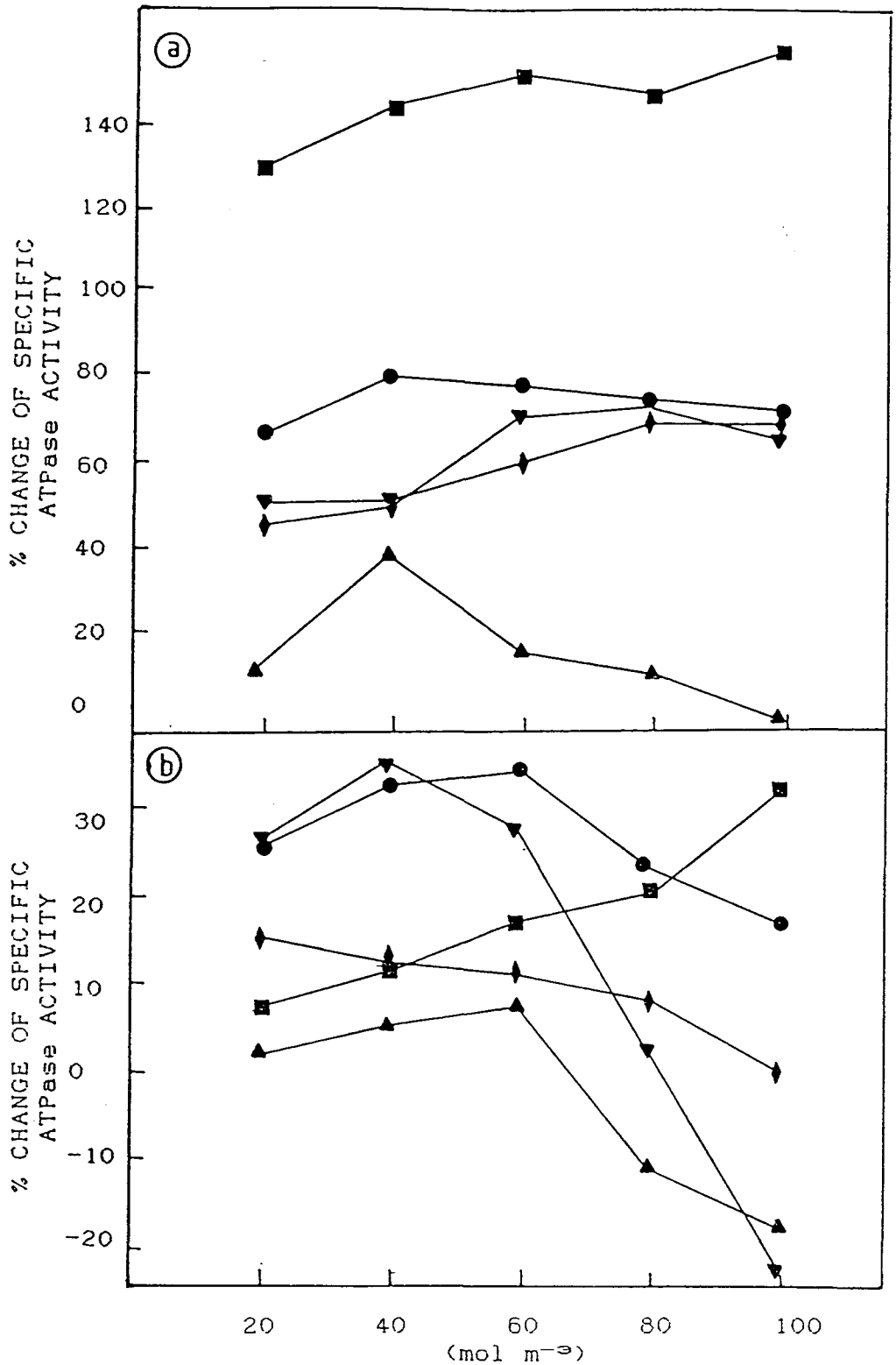


Figure 3.9a & b. Effect of cations on percentage stimulation/inhibition of Mg^{2+}-dependent ATPase activity associated with interface 1 (Fig.3.8a) and interface 4 (Fig.3.8b). The standard ATPase assay was used with KCl being replaced by the cations of chloride, NH_4^+ (■), Na^+ (●), Rb^+ (▼), K^+ (◆), Li^+ (▲).

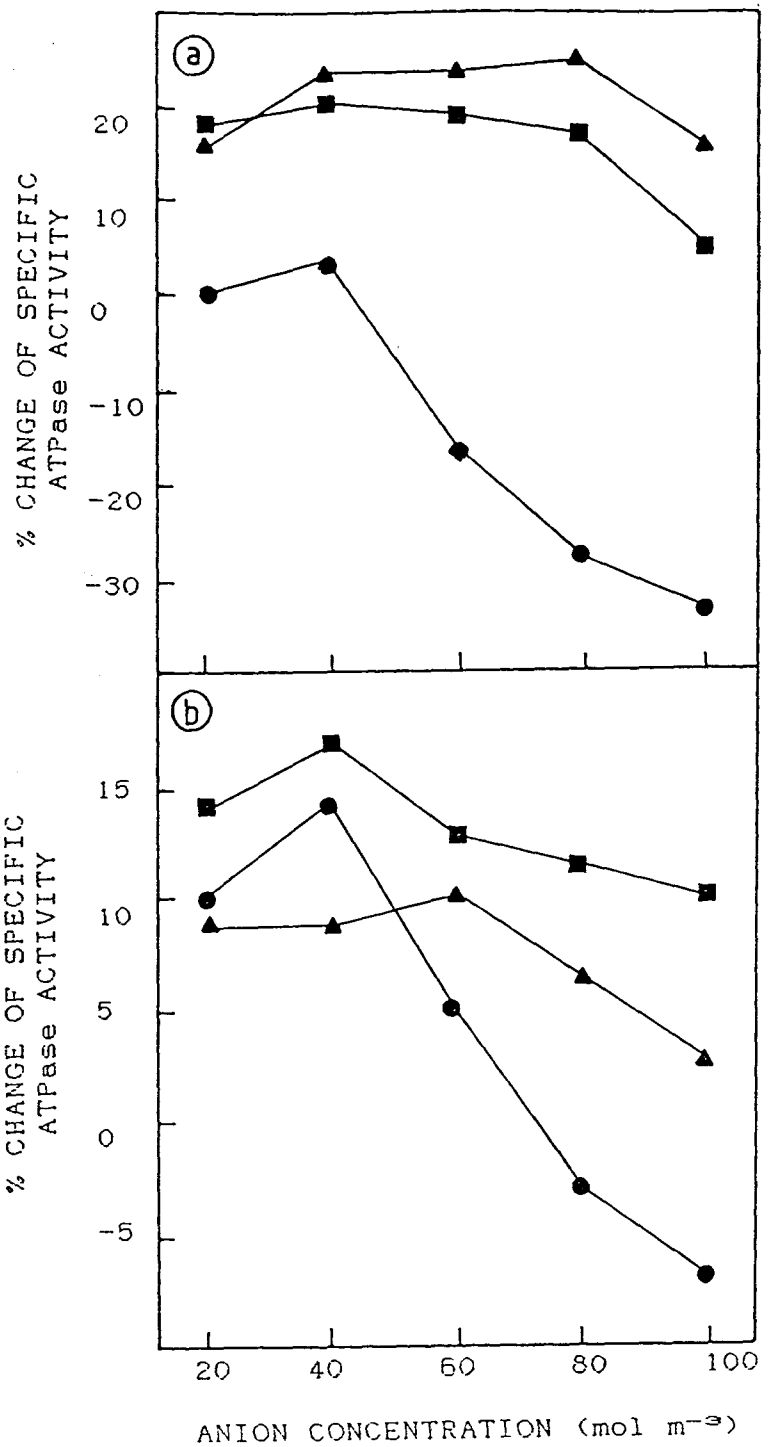


Figure 3.10a & b. Effect of anions on percentage stimulation/inhibition of Mg^{2+}-dependent ATPase activity associated with interface 1 (Fig.3.9a) and interface 4 (Fig.3.9b). The standard ATPase assay was used with KCl being replaced by the anions of potassium, Cl^- (▲), Br^- (■) and I^- (●).

stimulatory over the other cations. Stimulation of the plasma membrane-enriched region at 80-100 mol m^{-3} was observed in the order $NH_4^+ > K^+ > Li^+ > Rb^+$. Ca^{2+} was found to be a potent inhibitor at concentrations ranging from 5 - 50mol m^{-3} (data not shown). KCl and KBr were nearly equally stimulatory, with 80mol m^{-3} causing up to 36% excitation of the specific ATPase activity from interface 1. KBr, KCl, and KI at 40mol m^{-3} (Fig.3.10) enhanced the ATPase activity associated with interface 4 by up to 16%. At 100mol m^{-3} Br^-, Cl^-, and I^- decreased activity. The ATPase associated with interface 1 showed very marked stimulation by Cl^- and Br^- (≈30%) at 40-80mol m^{-3}. I^- was stimulatory at 40mol m^{-3} and became inhibitory at 100mol m^{-3}.

Interfaces 1 and 4 showed different responses to KCl when examined over a pH range (Fig.3.11a,b). Highest potassium-stimulated ATPase activity was observed at pH 7.0 (Fig.3.11a). Percentage stimulation of activity by potassium chloride showed the same trend.

3.2.3.4 Distribution of other markers.

Cytochrome c oxidase and sodium azide inhibition of ATPase activity have been used to locate the position of the mitochondrial membranes. The majority of the mitochondrial-associated ATPase was found in interface 5 and a small proportion in the uppermost interface. Figure 3.7 indicates the very different pH optimum for this ATPase, showing marked inhibition by azide at pH 8.5 and little inhibition of activity occurring at pH 6.75. ATPase assays at pH 6.75 in interfaces 1-4 therefore were not markedly affected by mitochondrial ATPase contamination. Low levels of cytochrome c oxidase were detected only in interface 5.

The Golgi membrane marker, latent IDPase, showed a central distribution within the gradient, with highest specific activity lying at interface 3 (Fig.3.12).

Cytochrome c reductase distribution indicated endoplasmic reticulum to be present in all of the gradient interfaces, with a higher proportion lying at interface 2 (Fig.3.12).

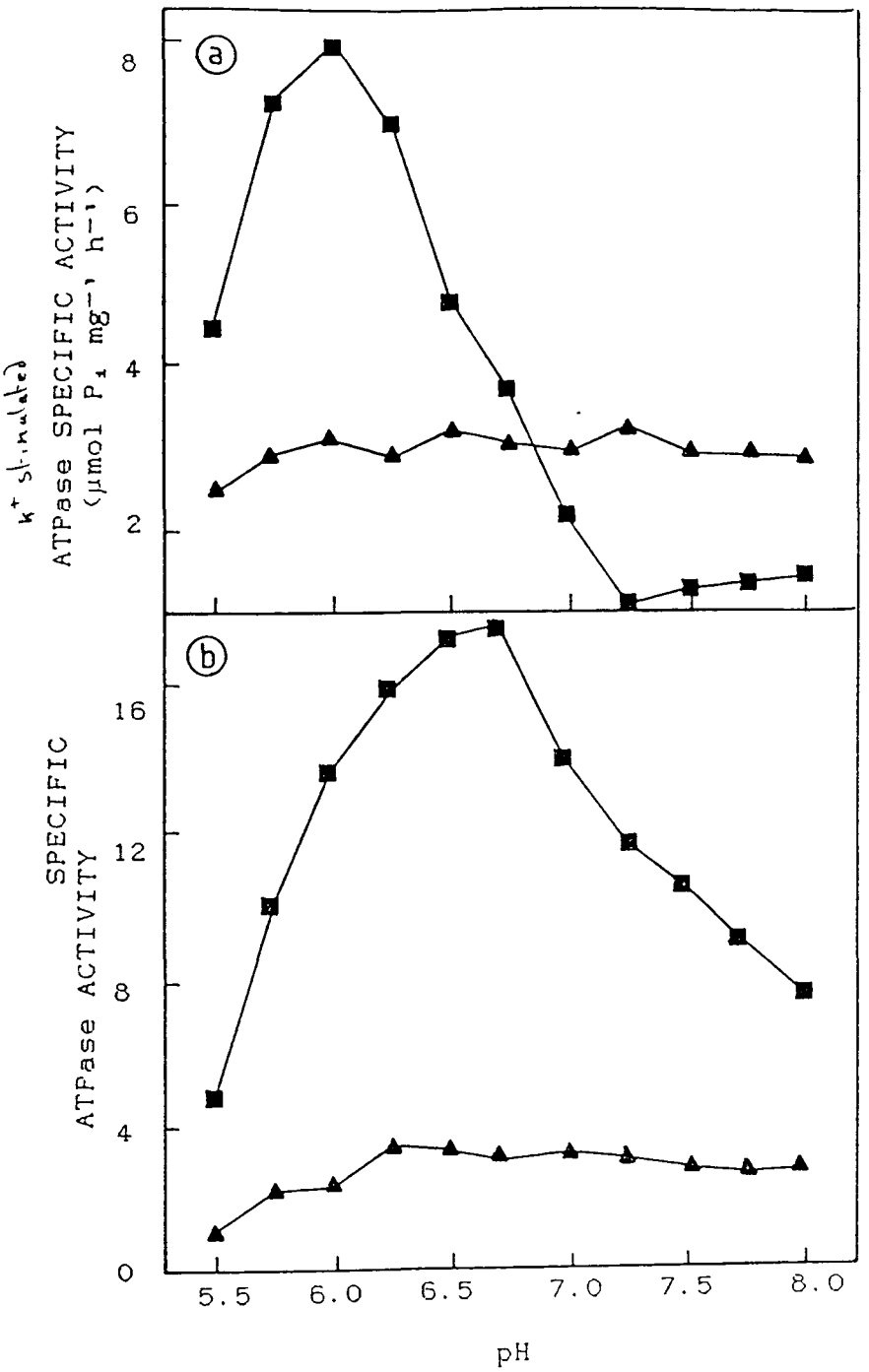


Figure 3.11a. Effect of pH on specific ATPase activity in membranes fractions from interface 4 (■), and interface 1 (▲). The standard ATPase assay was used

Figure 3.11b. ATPase specific activity in interfaces 4 (■), and 1 (▲). The standard ATPase assay was used

Result represents the mean of two experiments.

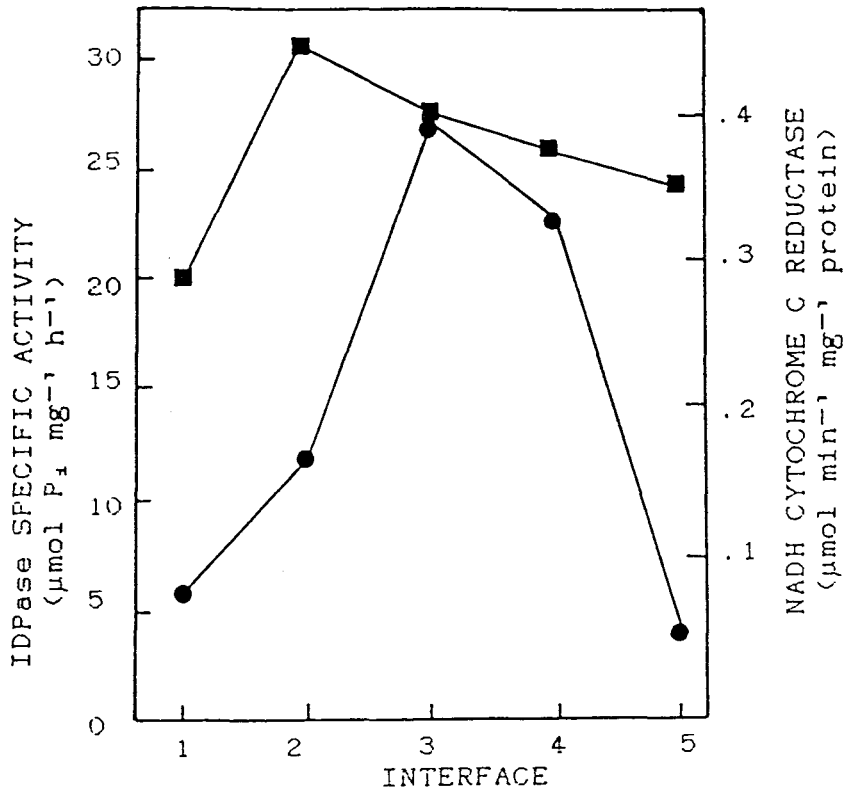


Figure 3.12. Specific IDPase activity (●) and specific NADH cytochrome C reductase (■) associated with each of the gradient interfaces. The standard assay mixture was used as described in "Materials and Methods".

3.2.3.5 Detergent and high salt washes.

A washing procedure using a weak detergent to remove loosely bound enzyme or membrane particles was investigated. The fractions were treated with Triton X-100 (0.5% v/v) for 10 min and repelleted at 100,000*g* to achieve further purification of the interfaces by removal of any extraneous membrane fragments (Blein *et al.* 1985). Inhibition by nitrate was not altered (Fig. 3.13a) but vanadate inhibition was markedly increased from 70% to nearly 90% in interface 4. However, a high inhibition of activity associated with interface 1 was still observed.

Following removal of gradient interfaces, a KI wash for 20 min on ice was given to each interface. The specific ATPase activity in all of the gradient interfaces was lowered by this procedure, but the nitrate and vanadate inhibitable components were increased (Fig. 3.13b).

The effect of Triton X-100 on ATPase activity in the interfaces was examined (Fig. 3.14a). ATPase associated with the plasma membrane fraction was markedly stimulated by Triton X-100 at 0.05% (v/v); the tonoplast ATPase showed no stimulation. In contrast 0.1% (v/v) Triton X-100 markedly inhibited activity in all interfaces.

Substrate specificity was examined in interfaces 1 and 4 (Table 3.1). ATP was the most readily hydrolyzed substrate at pH 6.75, although significant hydrolysis of IDP, ITP and UDP was also observed.

3.2.3.6 Kinetics of the ATPase in enriched membrane fractions.

The enzyme showed simple Michelis-Menten kinetics for Mg^{2+}-ATP hydrolysis (Fig. 3.15a,b). Estimation of the Lineweaver-Burk plots in both the presence and absence of K^+ at pH 6.75 gave apparent K_m values of 0. mol m^{-3} without K^+ and 0. mol m^{-3} with K^+ for interface 1. Interface 4 activity showed a K_m of 0.66 mol m^{-3} and 0.86 mol m^{-3} without and with K^+ respectively. (Fig. 3.16a,b)

The effect of gramicidin on ATPase activity was examined in each of the gradient interfaces (Fig. 3.17a).

Figure 3.13a. Percentage inhibition of ATPase activity in interfaces 1 - 5 following a Triton -100 wash (0.5%) for 10 min, by nitrate (■), and vanadate (●). The standard ATPase assay was used and with the incorporation of 50mol m^{-3} KNO_3, and $100x10^{-3}$mol m^{-3} vanadate.

Figure 3.13b. Effect of a KI wash on nitrate and vanadate inhibition in each of the gradient interfaces. Microsomal fractions were given either a resuspension buffer wash (closed symbols) or a KI wash for 15min (open symbols) as described in "Materials and Methods". The standard ATPase assay was used and percentage inhibition of activity was calculated with the incorporation of $50x10^{-3}$mol m^{-3} vanadate (◆, △) and $100x10^{-3}$mol m^{-3} (●, ○). and 50mol m^{-3} nitrate (■,□)

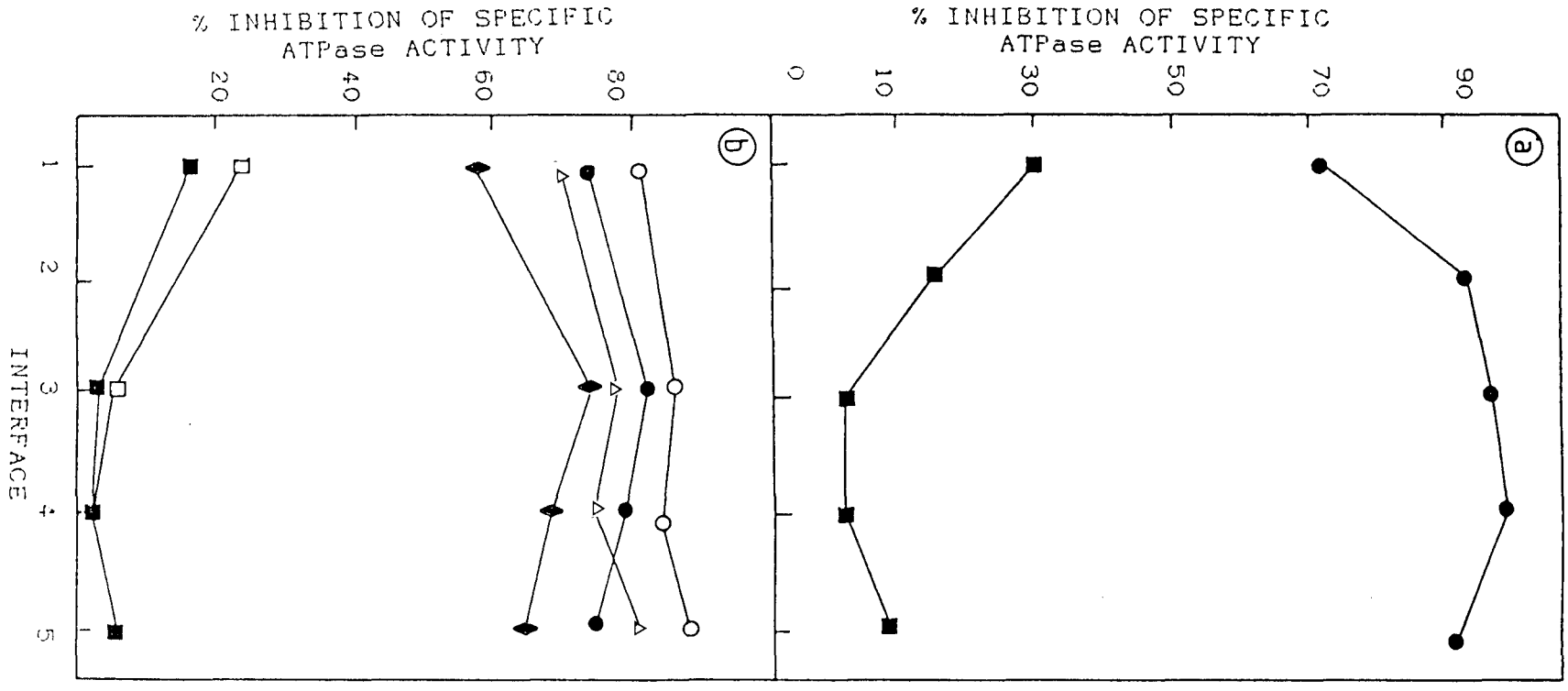
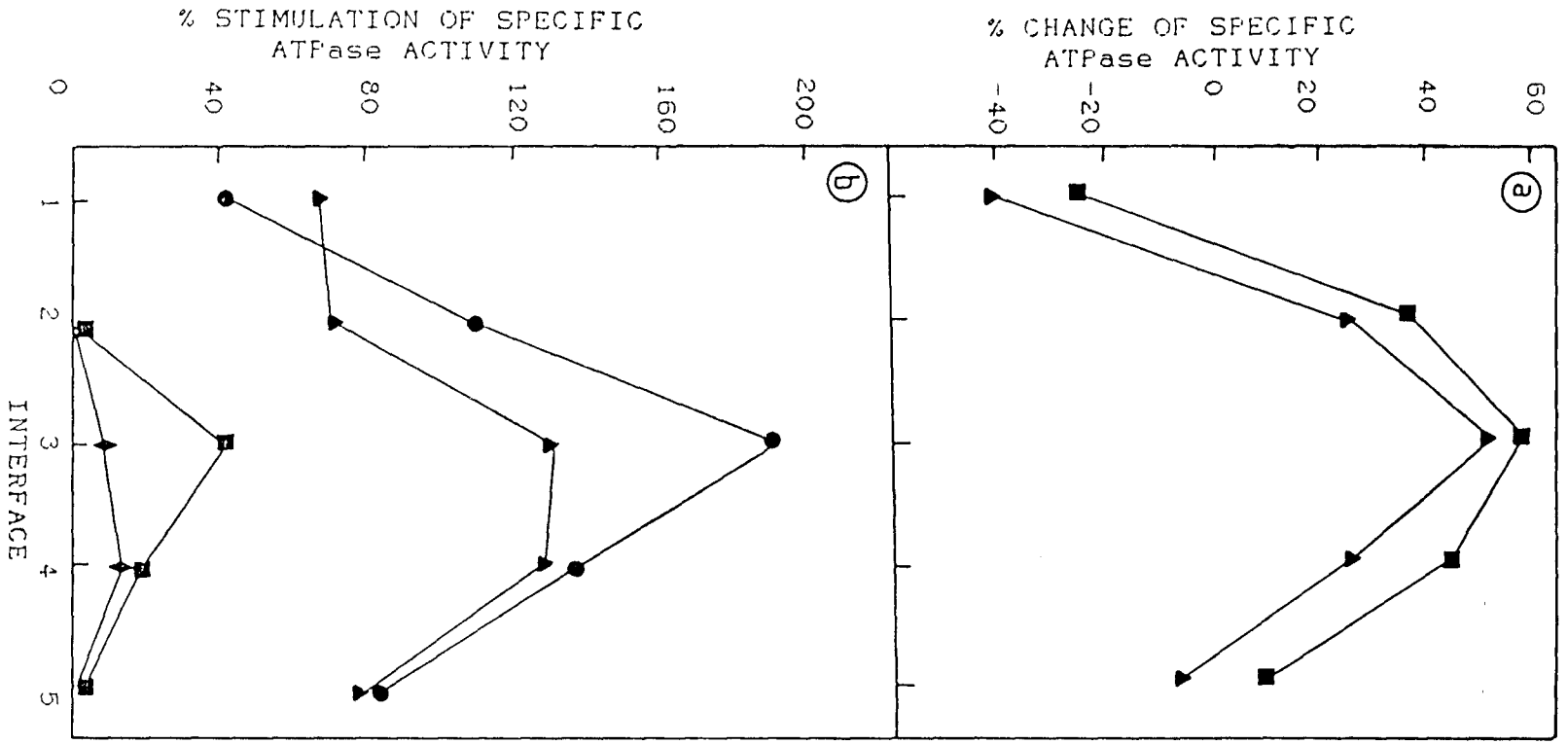

% INHIBITION OF SPECIFIC ATPase ACTIVITY
20
40
60
80
(b)
INTERFACE
1
2
3
4
5
% INHIBITION OF SPECIFIC ATPase ACTIVITY
0
10
30
50
70
90
(a)

Figure 3.14a. Percentage inhibition/stimulation of ATPase activity by Triton X-100 in each of the gradient interfaces. The standard ATPase assay was used without the incorporation of KCl (▲), and with the inclusion of 50mol m^{-3} KCl and 0.05% (v/v) Triton X-100 (■).

Figure 3.14b. Percentage stimulation of ATPase activity by a range of Triton X-100 concentrations in each of the gradient interfaces. The standard ATPase was used with the incorporation of Triton X-100 at concentrations of 0.01% (v/v) (▲), 0.02% (v/v) (●), 0.03% (v/v) (■) and 0.04% (v/v) (◆).


% CHANGE OF SPECIFIC ATPase ACTIVITY
-40
-20
0
20
40
60
(a)
% STIMULATION OF SPECIFIC ATPase ACTIVITY
0
40
80
120
160
200
(b)
INTERFACE
1
2
3
4
5

SUBSTRATE	INTERFACE 1	INTERFACE 4
ATP	100	100
ADP	19	25
AMP	2	0
ITP	47	45
IDP	71	67
UDP	78	60
GTP	31	40
Pyrophosphate	109	36
Glycerophosphate	5	1

Table 3.1. Percentage phosphohydrolase specific activity associated with interfaces 1 and 4 compared to activity with ATP as substrate. The standard ATPase assay was used with the replacement of ATP by the various substrates at a concentration of 2mol m^{-3}.

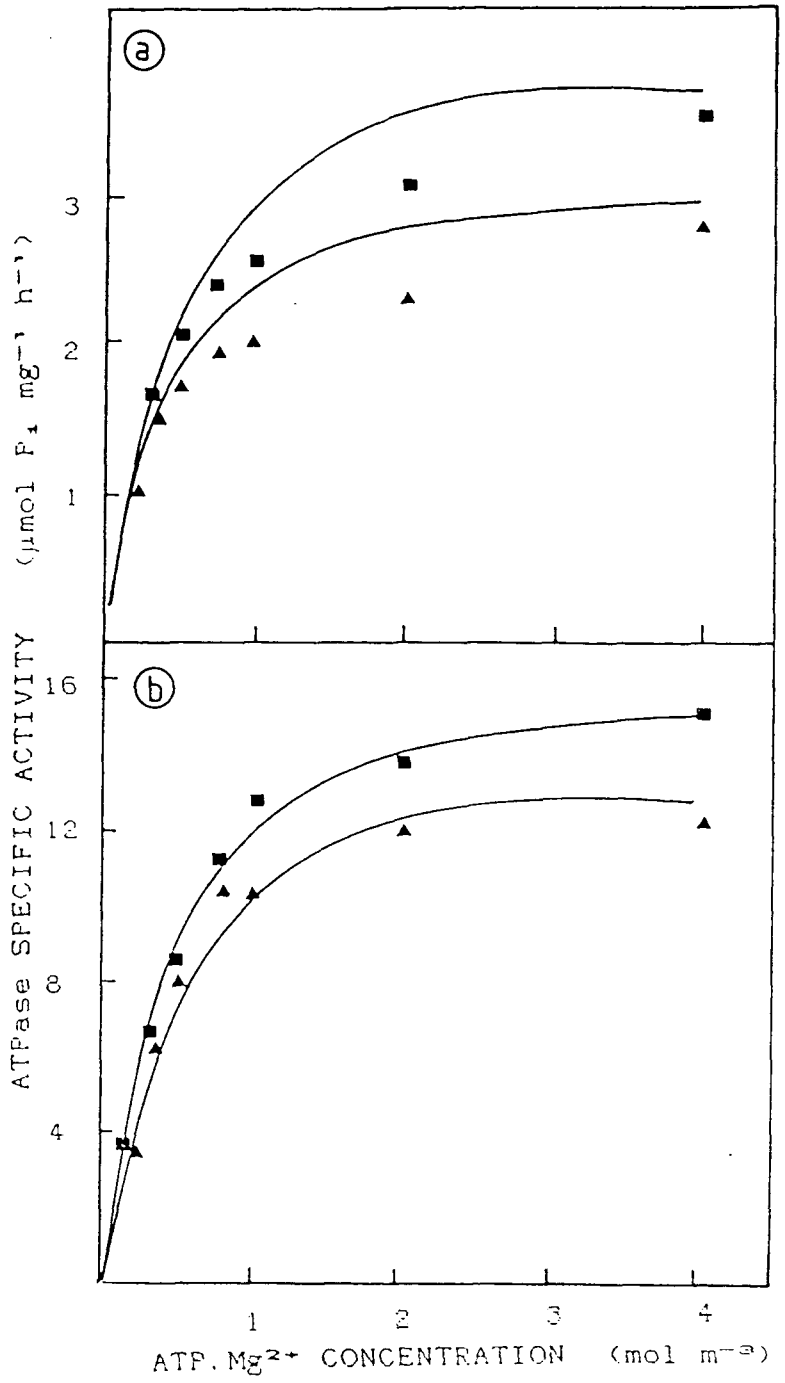


Figure 3.15a. ATPase specific activity of interface 1 as a function of increasing ATP.Mg concentrations. The standard ATPase assay was used without the inclusion of KCl (▲), and in the presence of 50mol m^{-3} (■) KCl.

Figure 3.15b. ATPase specific activity of interface 4 as a function of increasing ATP.Mg concentrations. The standard ATPase assay was used without the inclusion of KCl (▲), and in the presence of 50mol m^{-3} (■) KCl.

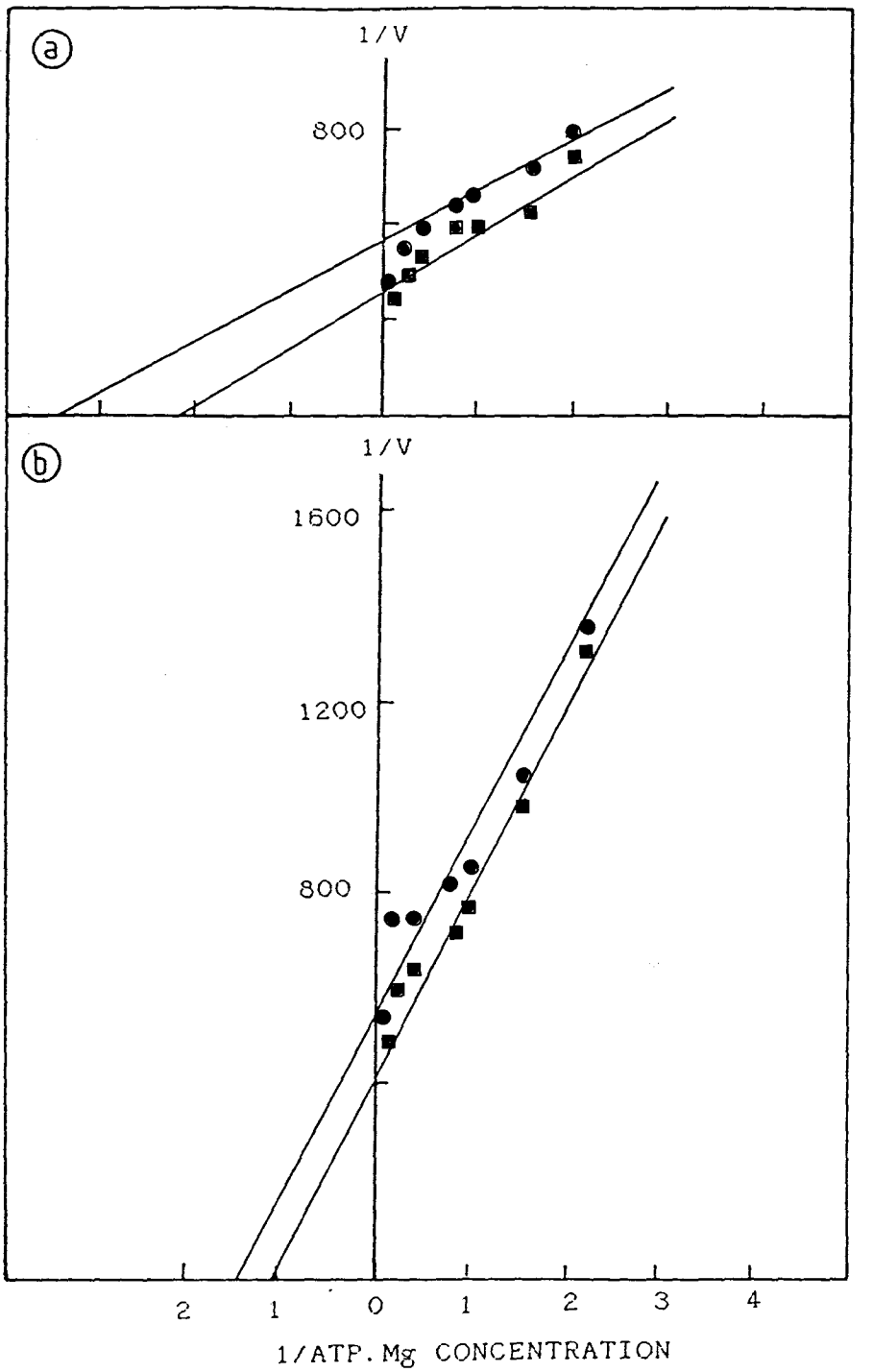
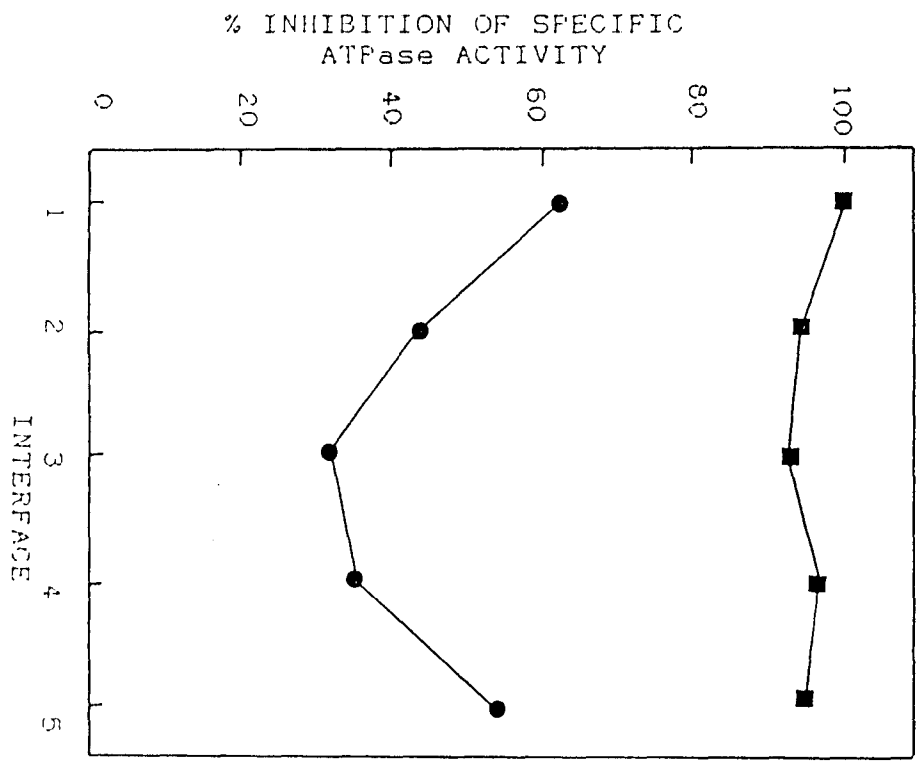


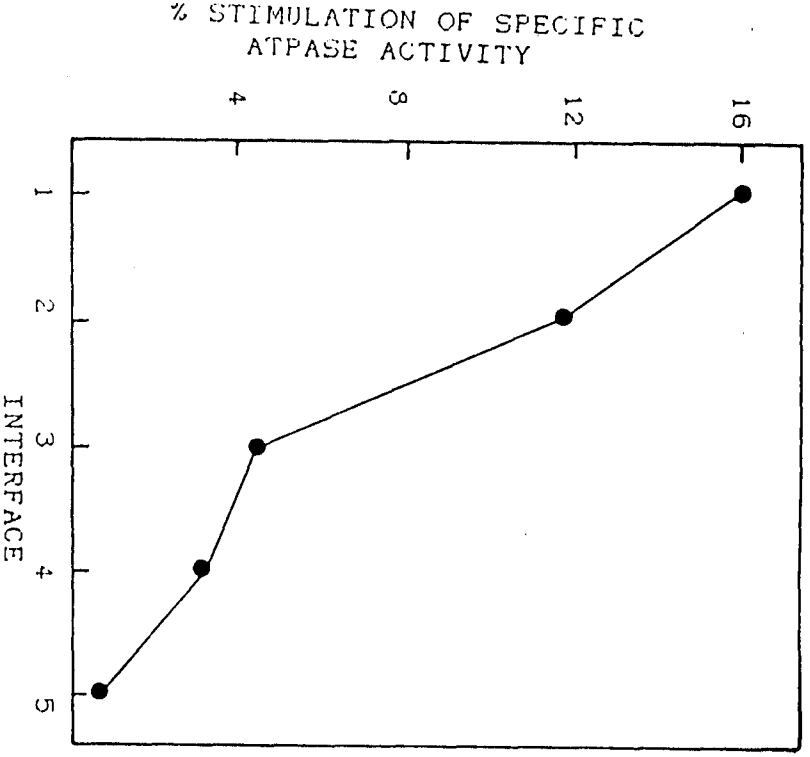
Figure 3.16a. Lineweaver-Burk plot of the data from Figure 3.14a. The standard ATPase assay was used without the inclusion of KCl (●), and in the presence of 50mol m^{-3} KCl (■).

Figure 3.16b. Lineweaver-Burk plot of the data from figure 3.14b. The standard ATPase assay was used without the inclusion of KCl (●), and in the presence of 50mol m^{-3} KCl (■).

Figure 3.17a. Percentage stimulation of ATPase specific activity by gramicidin at each of the gradient interfaces. The standard ATPase mixture was used and with the inclusion of gramicidin at a final concentration of 20x 10^{-3}mol m^{-3}. Gramicidin was made up in absolute ethanol and the final concentration of alcohol in the assay was 1% (v/v).

Figure 3.17b. Percentage inhibition of ATPase activity by glutaraldehyde in each of the gradient interfaces. The standard ATPase assay was used and with the incorporation of glutaraldehyde at final concentrations of 1% (v/v) (■) and 0.1% (v/v) (●).


% INHIBITION OF SPECIFIC
ATPase ACTIVITY
0
20
40
60
80
100
1
2
3
4
5
INTERFACE


% STIMULATION OF SPECIFIC
ATPASE ACTIVITY
4
8
12
16
1
2
3
4
5
INTERFACE

The ATPase associated with the upper region of the gradient was stimulated to a maximum of 16% and this value decreased at each of the increasing percentage sucrose steps.

3.2.3.7 Effect of inhibitors on ATPase activity.

The effect of glutaraldehyde at 1% and 0.1% (v/v) was examined on ATPase activity in each of the five gradient steps (Fig.3.17b). The upper and lowermost regions (1&5) were found to be most sensitive (about 60% with 0.1% (v/v)) and the middle steps the least (about 30% with 0.1% (v/v)). The inhibitory nature of erythrosin B was compared with that of vanadate in each of the gradient interfaces (Fig.3.18a). Inhibition by vanadate and erythrosin followed a similar trend in the lower gradient interfaces. In the upper regions of the gradient, where vanadate showed a differential effect, erythrosin B was inhibitory to similar extents in each of the gradient interfaces.

Inhibition by SW26 was found to be dependent on the protein concentration in the incubation medium whereas inhibition by vanadate and erythrosin B were relatively unaffected by the protein concentration (Fig.3.18b). The inhibition by SW26 of ATPase activity in interfaces 1 and 4 at similar protein levels was found not to vary when the fraction was prepared from different regions of the hypocotyl. Differential inhibition of ATPase from interfaces 1 & 4 (Fig.3.19a,b) was observed at similar protein levels.

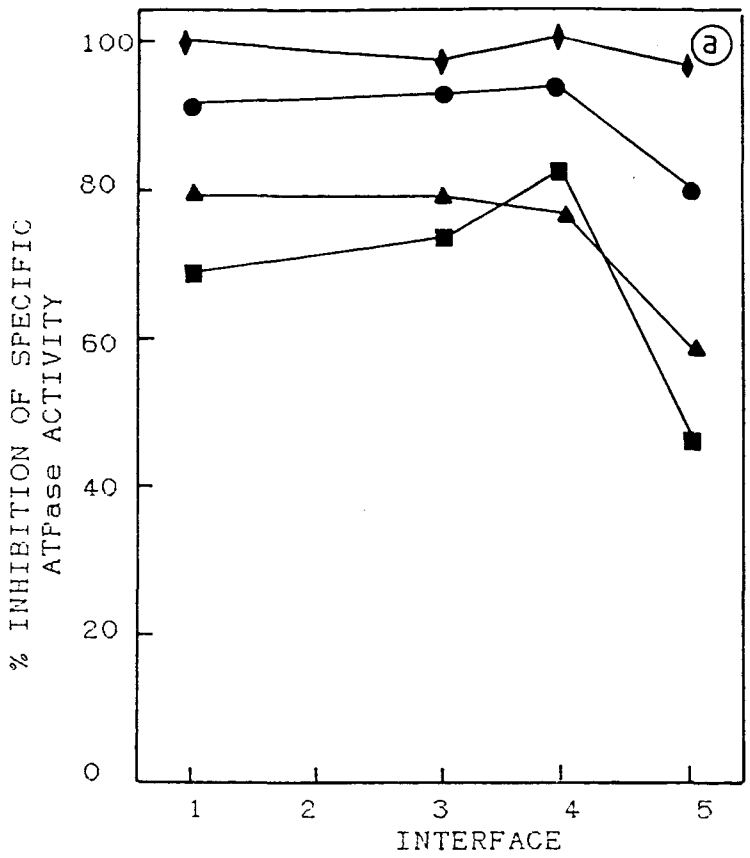
3.3.4 ATP-dependent proton transport.

3.3.4.1 General characterization.

Gramicidin-sensitive and percentage stimulation of ATPase activity by gramicidin was found notably in the tonoplast-enriched region of the gradient, and thus this fraction would be expected to show ATP-dependent H^+ transport. ATP-dependent proton transport was measured by fluorescence quenching of quinacrine and was initiated by the addition of Tris-ATP. After a lag of 2 min quenching occurred at a constant rate for 4-5 min and plateaued after 20-25 min. Fig.3.20a and b illustrates the extent of ATP-dependent H^+ transport by vesicles from interface 1

Figure 3.18a. Percentage inhibition of ATPase activity by erythrosin B and vanadate in each of the gradient interfaces. The standard ATPase assay was used with the incorporation of erythrosin B at final concentrations of $200x10^{-3}$mol m^{-3} (◆), $100x10^{-3}$mol m^{-3} (●), $50x10^{-3}$mol m^{-3} (▲), and vanadate at a final concentration of $50x10^{-3}$mol m^{-3} (■).

Figure 3.18b. Percentage inhibition of ATPase activity in a microsomal fraction by erythrosin B, SW26 and vanadate at various protein concentrations in the incubation medium. The standard ATPase assay was used with the incorporation at final concentrations of erythrosin B $50x10^{-3}$mol m^{-3} (▲), vanadate $100x10^{-3}$mol m^{-3} (■) and SW26 $50x10^{-3}$mol m^{-3} (●).


a
100
80
60
40
20
0
% INHIBITION OF SPECIFIC ATPase ACTIVITY
1
2
3
4
5
INTERFACE

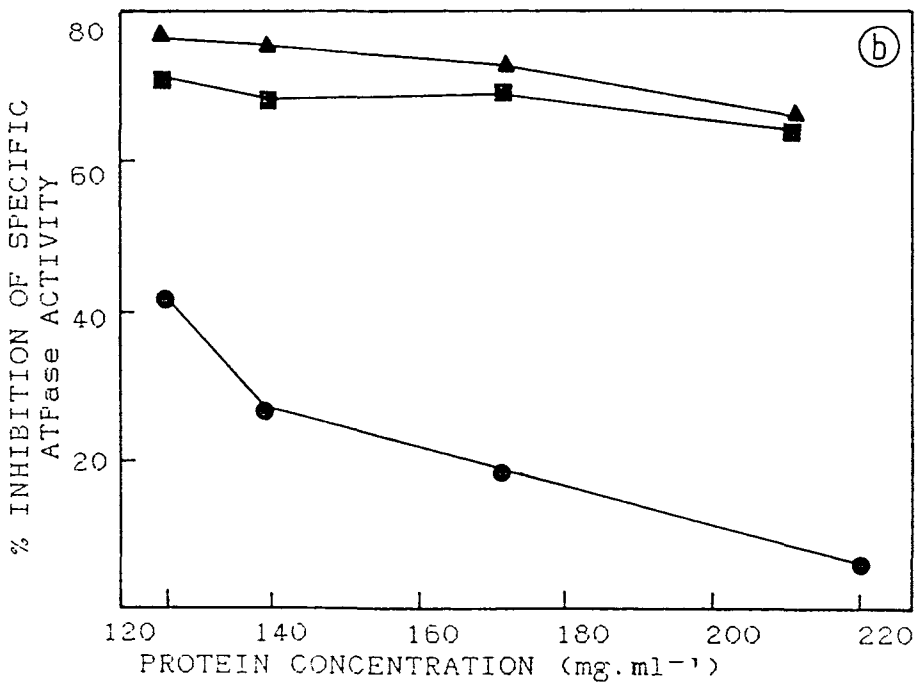
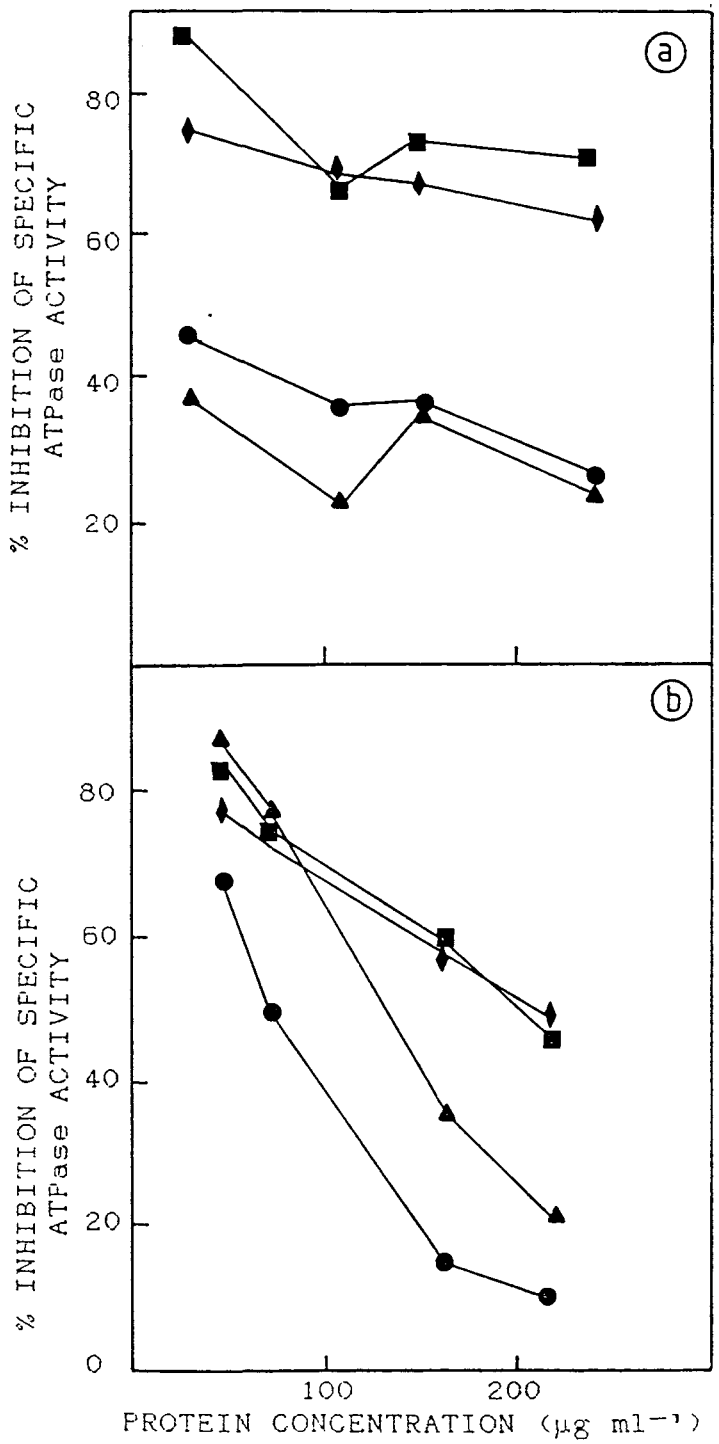

b
80
60
40
20
% INHIBITION OF SPECIFIC ATPase ACTIVITY
120
140
160
180
200
220
PROTEIN CONCENTRATION (mg.ml⁻¹)

Figure 3.19a. Percentage inhibition of ATPase activity associated with interface 1 by erythrosin B, SW26 and vanadate at varying protein concentrations. The standard ATPase assay was used with the incorporation at final concentrations of $50x10^{-3}$mol m^{-3} erythrosin B (■), $50x10^{-3}$mol m^{-3} SW26 (▲), $25x10^{-3}$mol m^{-3} SW26 (●) and $100x10^{-3}$mol m^{-3} vanadate (◆).

Figure 3.19b. Percentage inhibition of ATPase activity associated with interface 4 by erythrosin B, SW26 and vanadate at varying protein concentrations. The standard ATPase assay was used with the incorporation at final concentrations of $50x10^{-3}$mol m^{-3} erythrosin B (■), $50x10^{-3}$mol m^{-3} SW26 (▲), $25x10^{-3}$mol m^{-3} SW26 (●) and $100x10^{-3}$mol m^{-3} vanadate (◆).


a
b
% INHIBITION OF SPECIFIC ATPase ACTIVITY
% INHIBITION OF SPECIFIC ATPase ACTIVITY
80
60
40
20
0
100
200
PROTEIN CONCENTRATION (μg ml⁻¹)

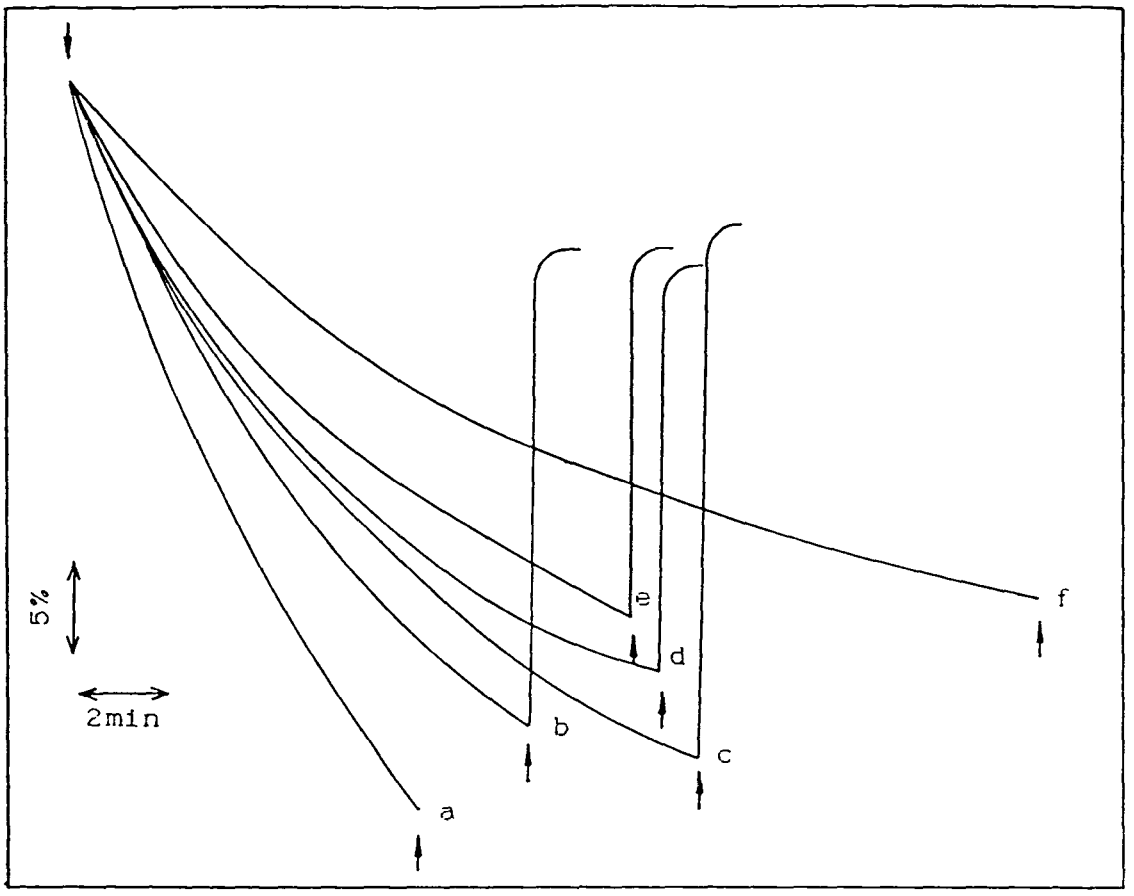


Figure 3.20. Effect of varying quinacrine concentrations on ATP-dependent quenching of fluorescence by membrane vesicles from interface 1. The assay was initiated by the addition of ATP (downward arrow) and quenching was reversed by the addition of ammonium chloride at a final concentration of 10mol m^{-3} (upward arrow). The standard fluorescence assay was used with quinacrine at final concentrations of (a) $1x10^{-3}$mol m^{-3}, (b) $1.66x10^{-3}$mol m^{-3}, (c) $3.3x10^{-3}$mol m^{-3}, (d) $3.6x10^{-3}$mol m^{-3}, (e) $5x10^{-3}$mol m^{-3} and (f) $10x10^{-3}$mol m^{-3}.

at varying quinacrine concentrations. The rate of pumping was highest at 10^{-3}mol m^{-3}, but at this level noise became a problem. Addition of gramicidin at the end of the run caused a reversal of the fluorescence quenching indicating the dissipation of the proton gradient. Calcium, when added at the end of the run was also found to dissipate the H^+ gradient, and no further reversal occurred with the addition of gramicidin at this stage (Fig.3.21a). The effect of pH on H^+ transport was examined using fraction 1 (Fig.3.22) and indicated an optimum pH of 6.75. The effect of cations on ATP-dependent H^+ transport was examined in interface 1 (Fig.3.23a). The rate of pumping was decreased in the absence of monovalent cations compared to that in the presence of 25mol m^{-3} and 50mol m^{-3} K^+. Na^+, Li^+ and Rb^+ were found to similarly stimulate ATP-dependent H^+ transport. K^+ was found to be required for H^+ transport in vesicles derived from interface 4 (Fig.3.23b).

3.3.4.2 Effect of inhibitors.

The differential response of membrane bound ATPase to vanadate and nitrate was examined in interfaces 1 and 4 from the sucrose gradient. The proton pumping ATPase associated with interface 1 was found to be markedly sensitive to nitrate, and to also contain a proportion that was sensitive to vanadate (Fig.3.24). Interface 4, on the other hand, contained a H^+-ATPase that was insensitive to nitrate relative to the control and inhibited by vanadate to a small extent at 0.15mol m^{-3} (Fig.3.25). This was examined again using interface 2 and 4. ATP-dependent H^+ transport in interface 2 was equally sensitive to 50mol m^{-3} nitrate and 0.05mol m^{-3} vanadate and interface 4 insensitive to nitrate, but sensitive to vanadate (Fig.3.26a,b). Sensitivity to vanadate in interface 4 was further examined (Fig.3.27). Inhibition increased with increasing vanadate concentration, but even at 0.5mol m^{-3} only 50% reduction was observed.

SW26 markedly affected the rate of ATP-dependent H^+ transport in vesicles from interfaces 1&4 (Fig.3.28a,b) with 6.25×10^{-3}mol m^{-3} giving nearly 50% inhibition compared with the control. Similarly erythrosin B

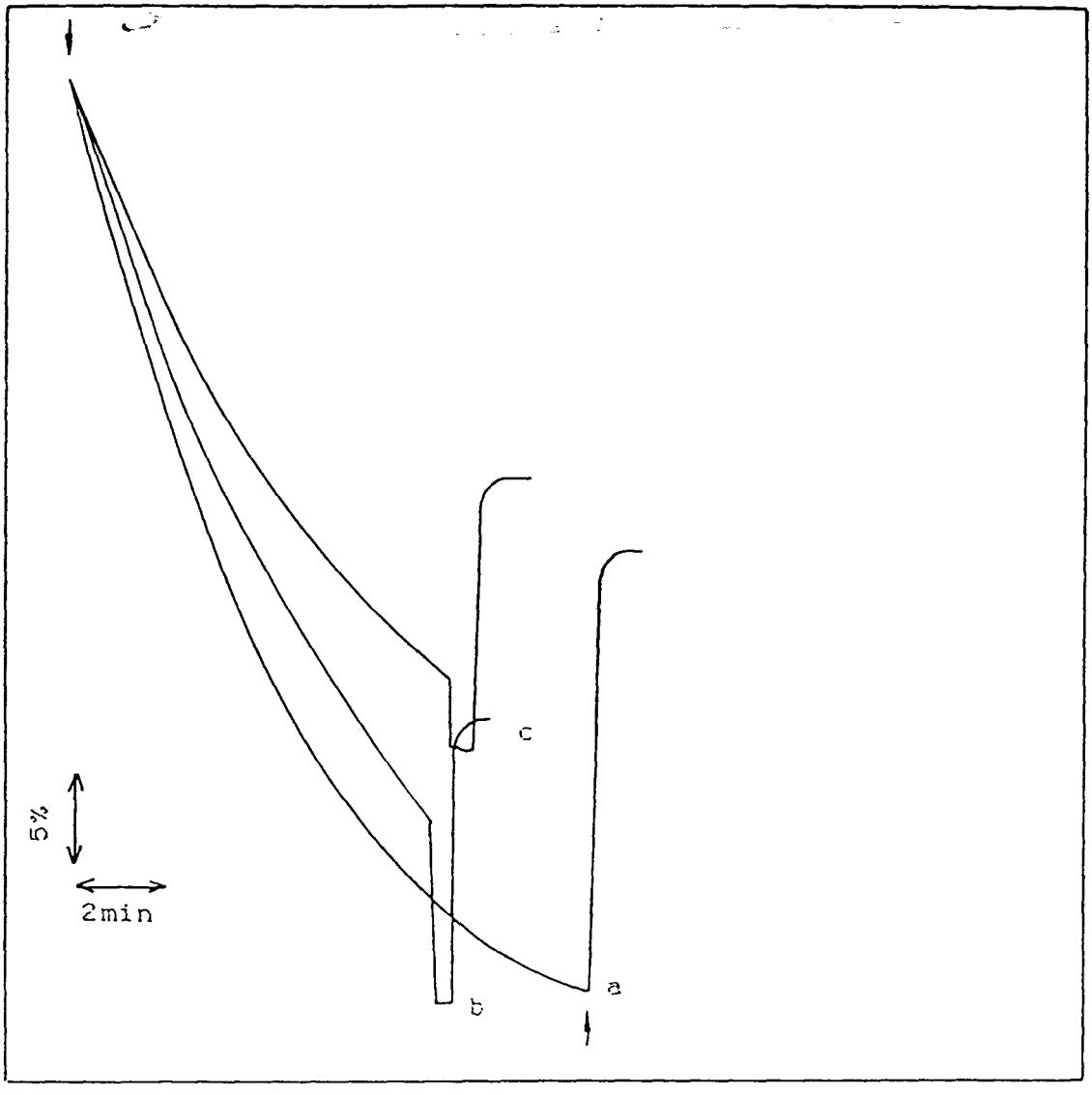


Figure 3.21. Effect of gramicidin and calcium chloride on the reversal of ATP-dependent quenching of quinacrine in membrane vesicles from interface 4. The standard fluorescence assay was used, being initiated by the addition of ATP (downward arrow). Quenching was reversed by (a) the addition of gramicidin at a final concentration of 10mol m^{-3} and calcium chloride (upward arrow) at final concentrations of (b) 0.5mol m^{-3} and (c) 0.25mol m^{-3}.

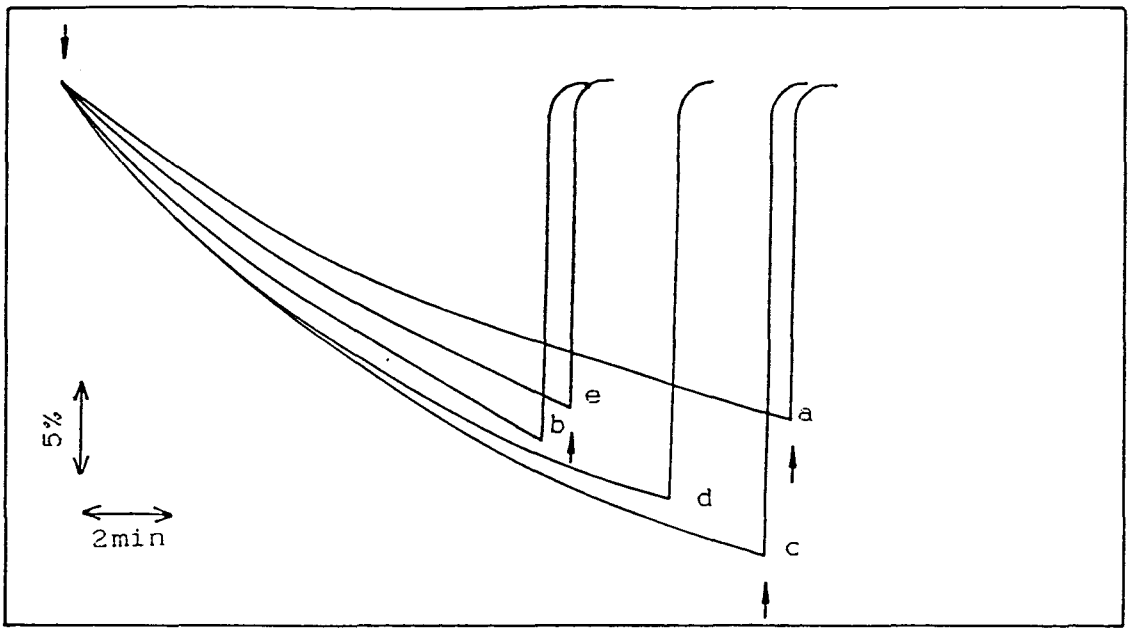
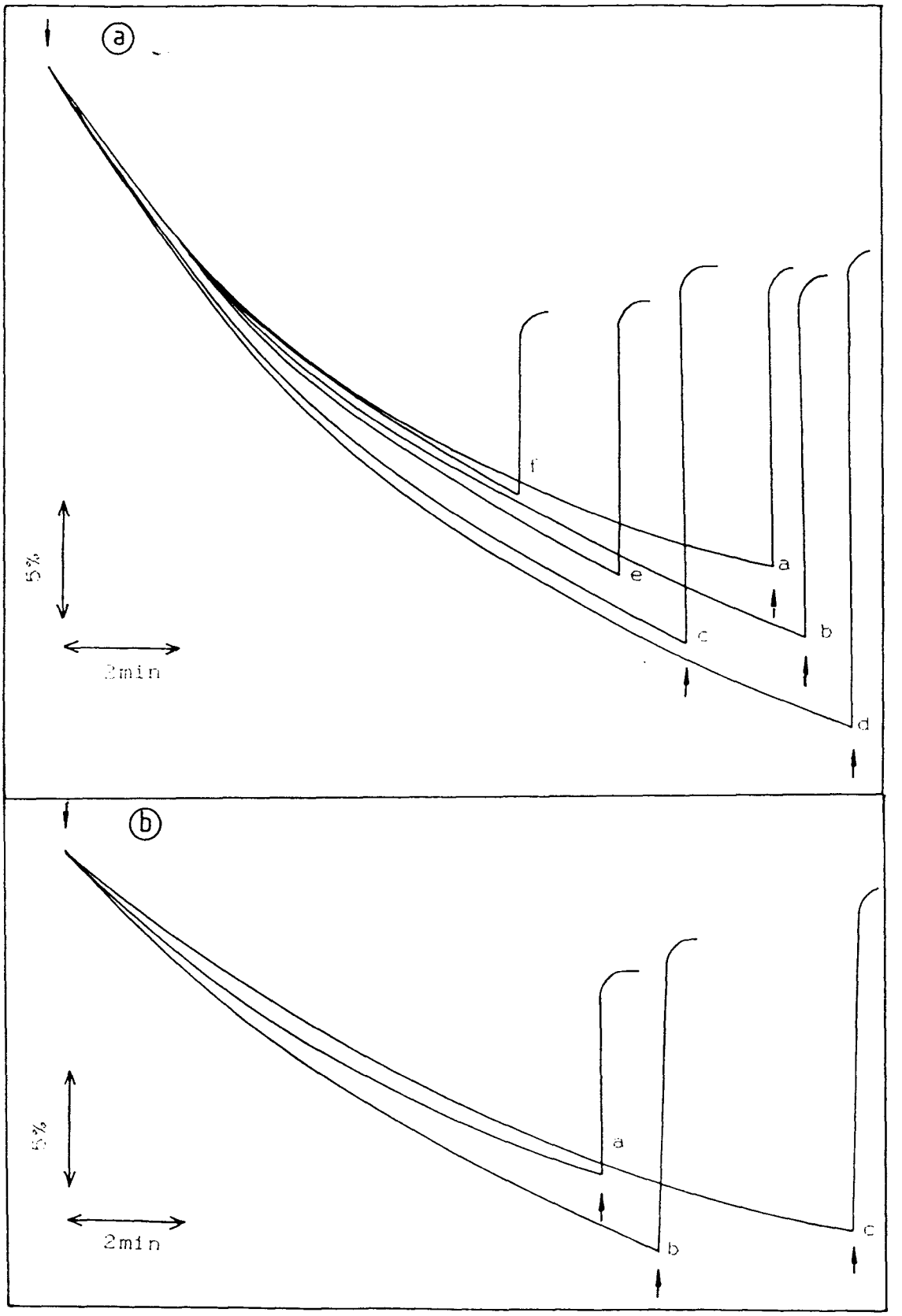


Figure 3.22. Effect of pH on ATP-dependent quenching of quinacrine in membrane vesicles derived from interface 1. The standard quinacrine assay was used with the reaction being initiated by the addition of ATP (downward arrow) and an assay pH of (a) 6.0, (b) 6.5, (c) 6.75, (d) 7.0 and (e) 7.5.

Figure 3.23a. Effect of cations on ATP-dependent H^+ transport in membrane vesicles from interface 1. The standard assay was used with quinacrine at a concentrations of $3.3x10^{-3}$mol m^{-3} and pH 7.0 and 100mol m^{-3} Cl^-. H^+ transport was examined in the presence of (a) no K^+, (b) 25mol m^{-3} K^+, (c) 50mol m^{-3} K^+, (d) 50mol m^{-3} Rb^+, (e) 50mol m^{-3} Na^+, (f) 50mol m^{-3} Li^+. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrow).

Figure 3.23b. Effect of cations on ATP-dependent H^+ transport in membrane vesicles from interface 4. The standard assay was used with quinacrine at a concentrations of $3.3x10^{-3}$mol m^{-3} and pH 7.0 and 100mol m^{-3} Cl^-. H^+ transport was examined in the presence of (a) no K^+, (b) 50mol m^{-3} K^+ and (c) 50mol m^{-3} Li^+. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrow).


a
5%
2min
f
e
c
a
b
d
b
5%
2min
a
b
c

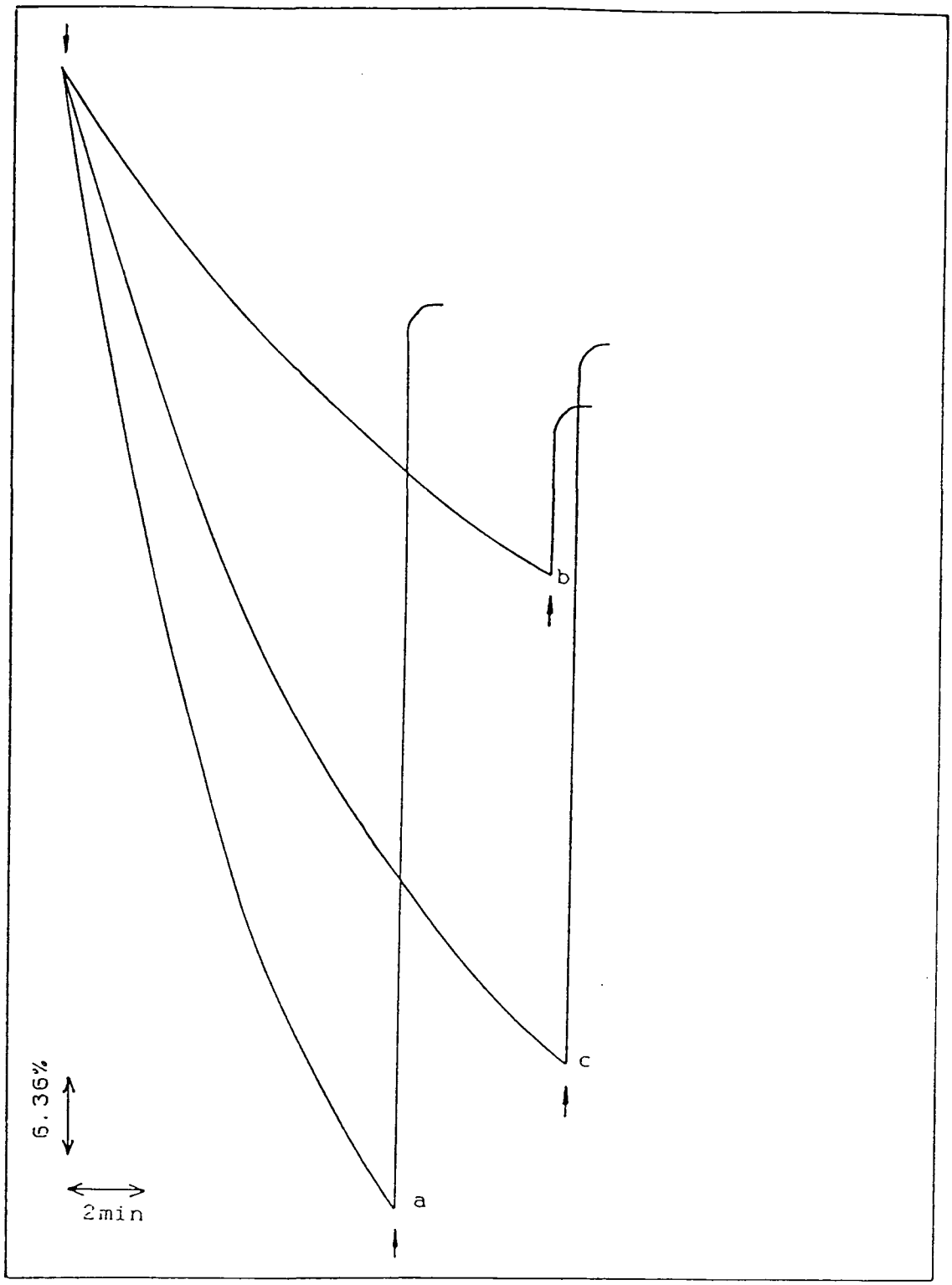


Figure 3.24. Effect of nitrate and vanadate on ATP-dependent quenching of quinacrine in membrane vesicles from interface 1. The standard quinacrine mixture was used (a) with quinacrine at final concentration of 2×10^{-3} mol m^{-3}, and with the incorporation at final concentrations of (b) 50mol m^{-3} KNO_3 and (c) 150×10^{-3}mol m^{-3} vanadate. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrow).

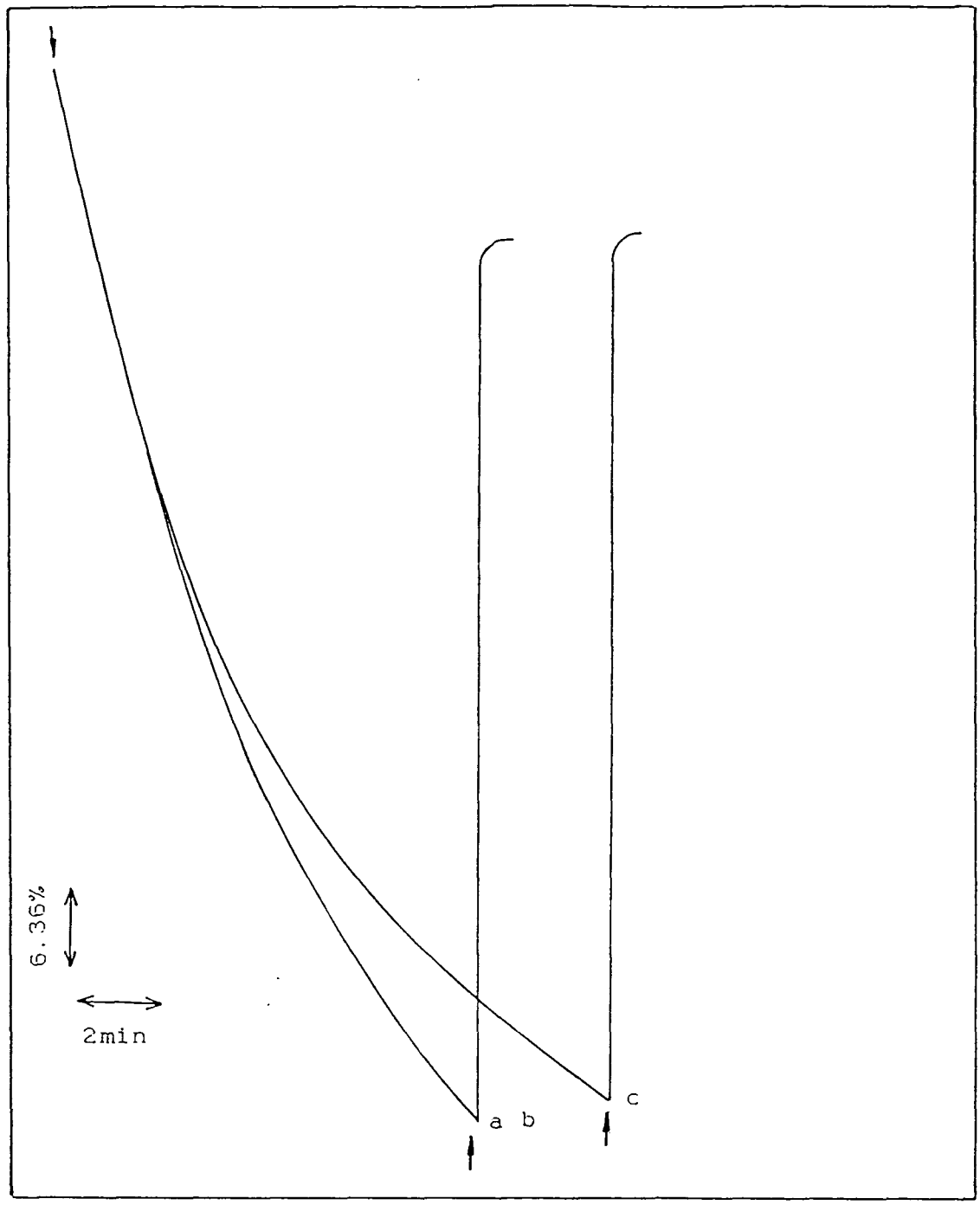
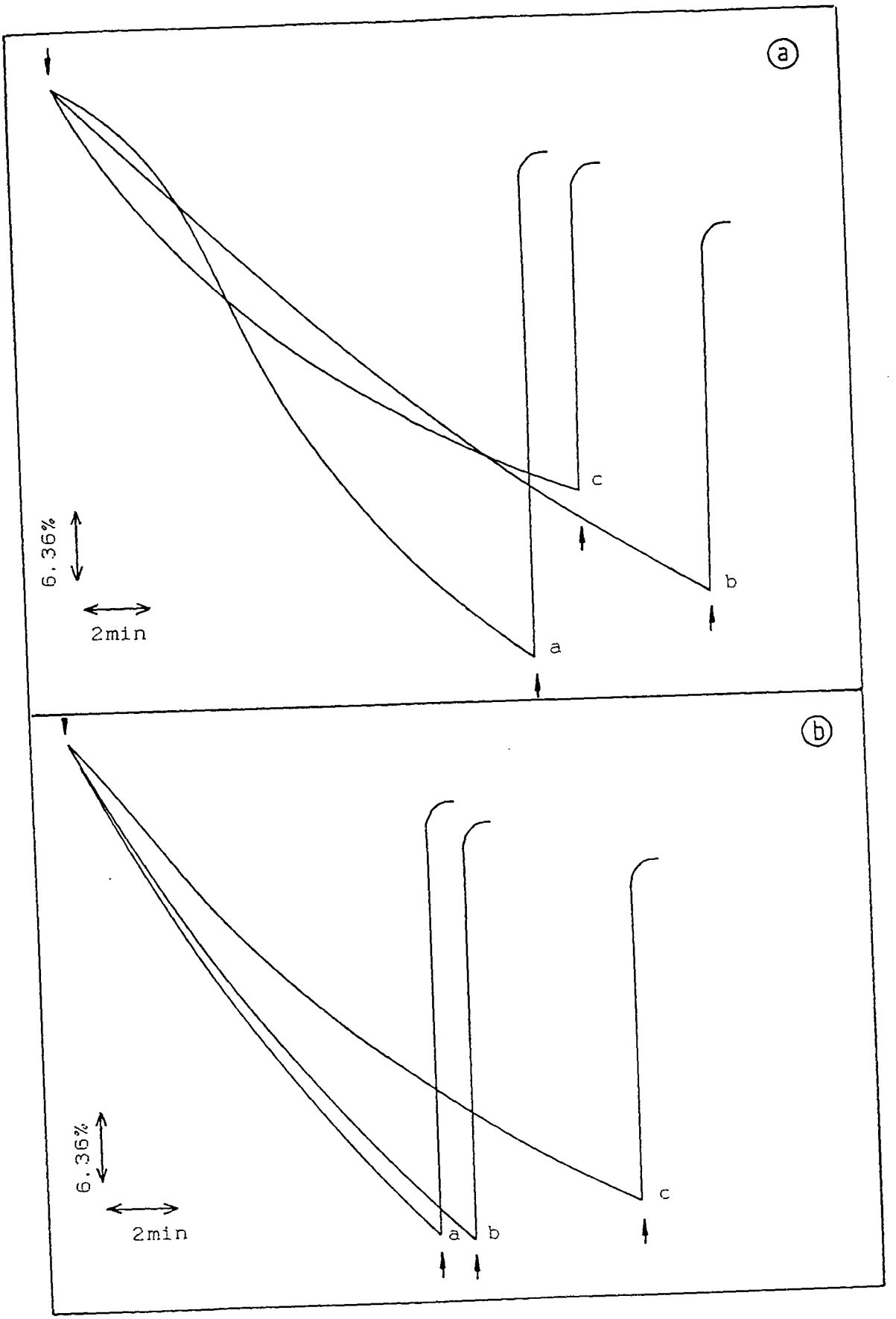


Figure 3.25. Effect of nitrate and vanadate on ATP-dependent quenching of quinacrine in membrane vesicles from interface 4. The standard quinacrine assay (a) was used with quinacrine at a final concentration of 2×10^{-3}mol m^{-3}, with the incorporation at final concentrations of (b) 50mol m^{-3} KNO_3 and (c) 150×10^{-3}mol m^{-3} vanadate. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrow).

Figure 3.26a. Effect of nitrate and vanadate on ATP-dependent quenching of quinacrine in membrane vesicles from interface 2. The standard assay was used (a) with quinacrine at a concentration of $2x10^{-3}$mol m^{-3} and pH 6.8 (a), with the incorporation at final concentrations of (b) 50mol m^{-3} KNO_3 and (c) $50x10^{-3}$mol m^{-3} vanadate. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrow).

Figure 3.26b. Effect of nitrate and vanadate on ATP-dependent quenching of quinacrine in membrane vesicles from interface 4. The standard assay was used (a) with quinacrine at a final concentration of $2x10^{-3}$mol m^{-3} and pH 6.8 and with the incorporation at final concentrations of (b) 50mol m^{-3} KNO_3 and (c) $50x10^{-3}$mol m^{-3} vanadate. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrow).


a
6.36%
2min
c
b
a
b
6.36%
2min
a
b
c

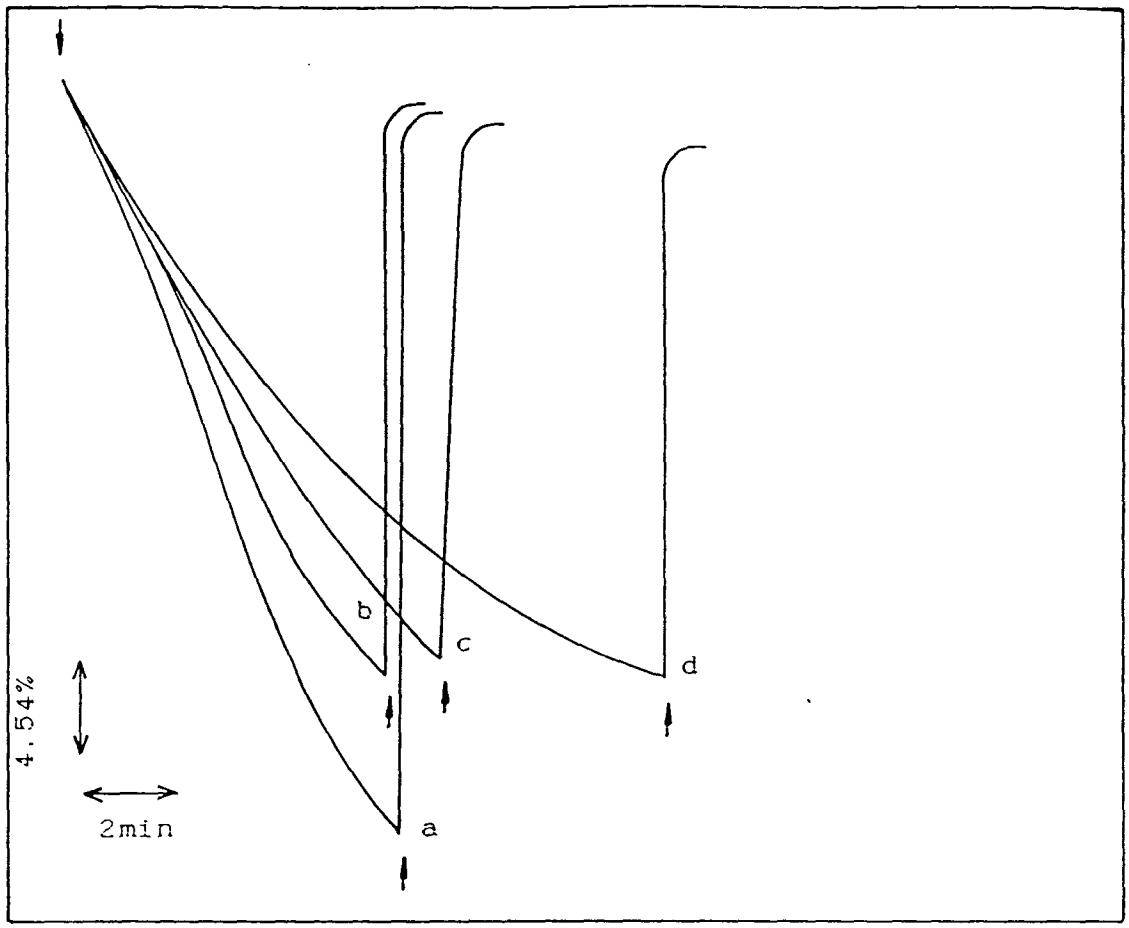
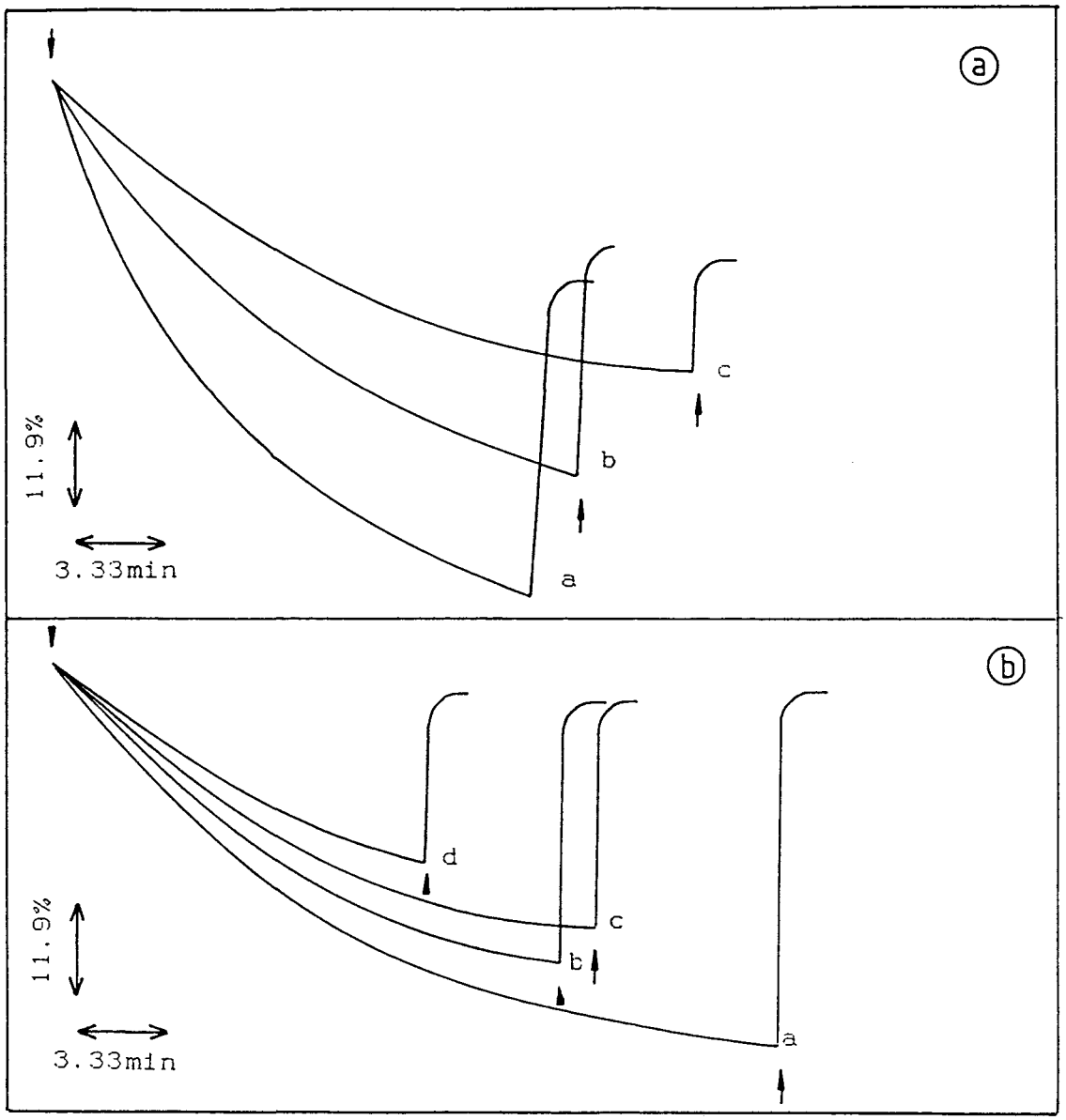


Figure 3.27. Effect of vanadate on ATP-dependent quenching of quinacrine in membrane vesicles from interface 4. The standard quinacrine assay (a) was used with vanadate at final concentrations of (b) 100×10^{-3} mol m^{-3}, (c) 200×10^{-3} mol m^{-3}, (d) 500×10^{-3} mol m^{-3}. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrow).

Figure 3.28a. Effect of SW26 on ATP-dependent quenching of quinacrine in membrane vesicles from interface 1. The standard quinacrine assay was used (a) with quinacrine at a final concentration of $2x10^{-3}$mol m^{-3} and with DMSO at a final concentration of 1% (v/v) and SW26 at final concentrations of (b) $3.12x10^{-3}$mol m^{-3}, (c) $6.25x10^{-3}$mol m^{-3}. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrow).

Figure 3.28b. Effect of SW26 on ATP-dependent quenching of quinacrine in membrane vesicles from interface 4. The standard quinacrine assay (a) was used with SW26 at final concentrations of (b) $3.1x10^{-3}$mol m^{-3}, (c) $6.25x10^{-3}$mol m^{-3}, (d) $12.5x10^{-3}$mol m^{-3}. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrow).


a
11.9%
3.33min
c
b
a
b
11.9%
3.33min
d
c
b
a

markedly affected H^+ in transport in vesicles from interfaces 1, with $5x10^{-3}$mol m^{-3} giving nearly 50% inhibition (Fig.3.29). The effect of these inhibitors on pumping in vesicles from interface 4 was not examined.

Pyrophosphate was found to be utilized as a substrate to generate a H^+ gradient within microsomal vesicles (Fig.3.30). The H^+ gradient was dissipated by the addition of gramicidin.

3.3.5 Effect of ATPase inhibitors on tissue respiration.

The effects of SW26, erythrosin B, DMSO, vanadate, cyanide and azide on hypocotyl tissue respiration were examined. Respiration over a 6 h period was reduced to a small extent by azide and cyanide compared to the control. However, erythrosin B (0.05mol m^{-3}), SW26 (0.05mol m^{-3}) and vanadate (1mol m^{-3}) had no measured effect on tissue respiration.

3.3.6 ATPase sensitivity to nitrate, vanadate and azide in a new seed batch.

A new seed batch which originated in a different country was obtained and plants were grown for a further two days before harvesting. Membrane-associated ATPase from this seed differed markedly from the previous batch in its sensitivity to nitrate and azide. Comparison of the ATPase activity in membrane enriched fractions from the two batches was carried out (Fig.3.31). In both batches, nitrate and azide stimulated tonoplast- and plasma membrane-associated ATPase.

3.3.7 ATPase localization.

ATPase localization in the hypocotyl hook region was examined using standard cytochemical techniques. In the absence of ATP (Fig.3.32a,b), slight precipitation was seen on the cell wall matrix and dense precipitation in regions of the nuclei. With the inclusion of ATP in the incubation medium, staining was seen at the plasma membrane in most of the cells (Fig.3.33a,b & 34a). No staining was seen on the tonoplast. With the inclusion of vanadate in the incubation medium, precipitation was reduced almost to that of the control Fig.34b).

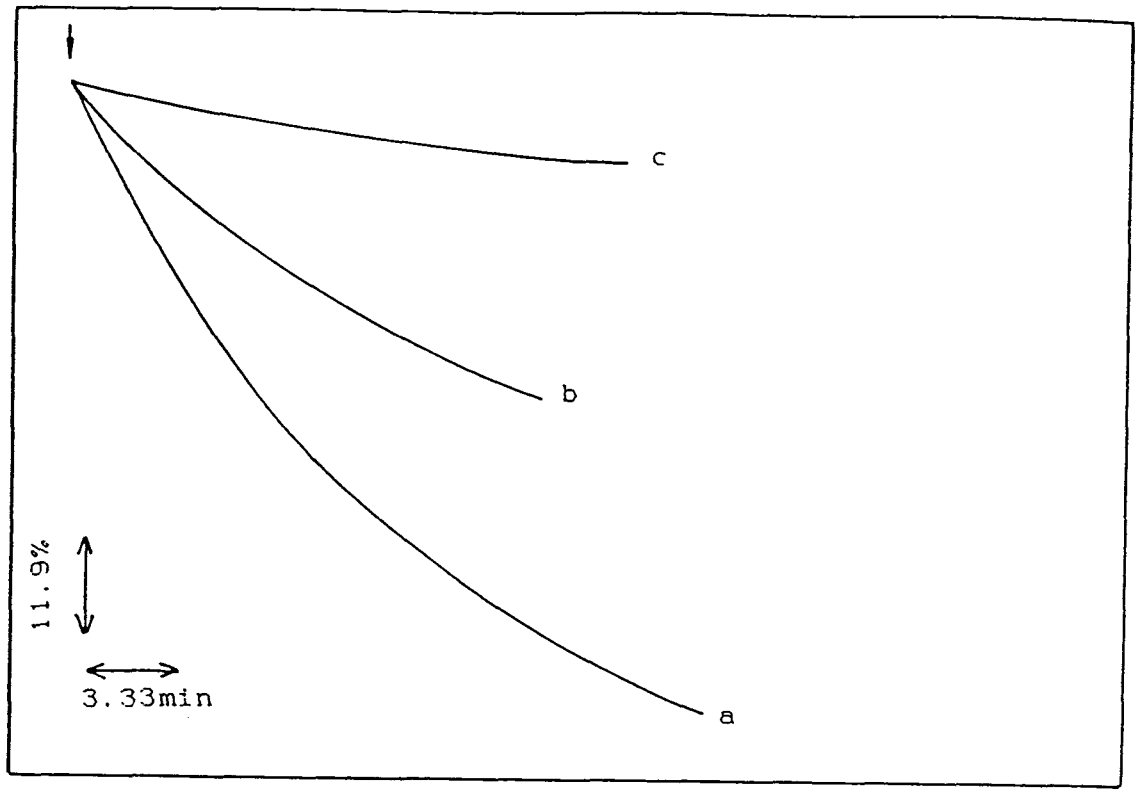


Figure 3.29. Effect of erythrosin B and calcium chloride on ATP-dependent quenching of quinacrine in vesicles from interface 1. The standard quinacrine assay was used (a) with (b) erythrosin B at a final concentration of 5x 10^{-3}mol m^{-3}, and (c) calcium chloride at a final concentration of 5mol m^{-3}. The reaction was initiated by the addition of ATP (downward arrow).

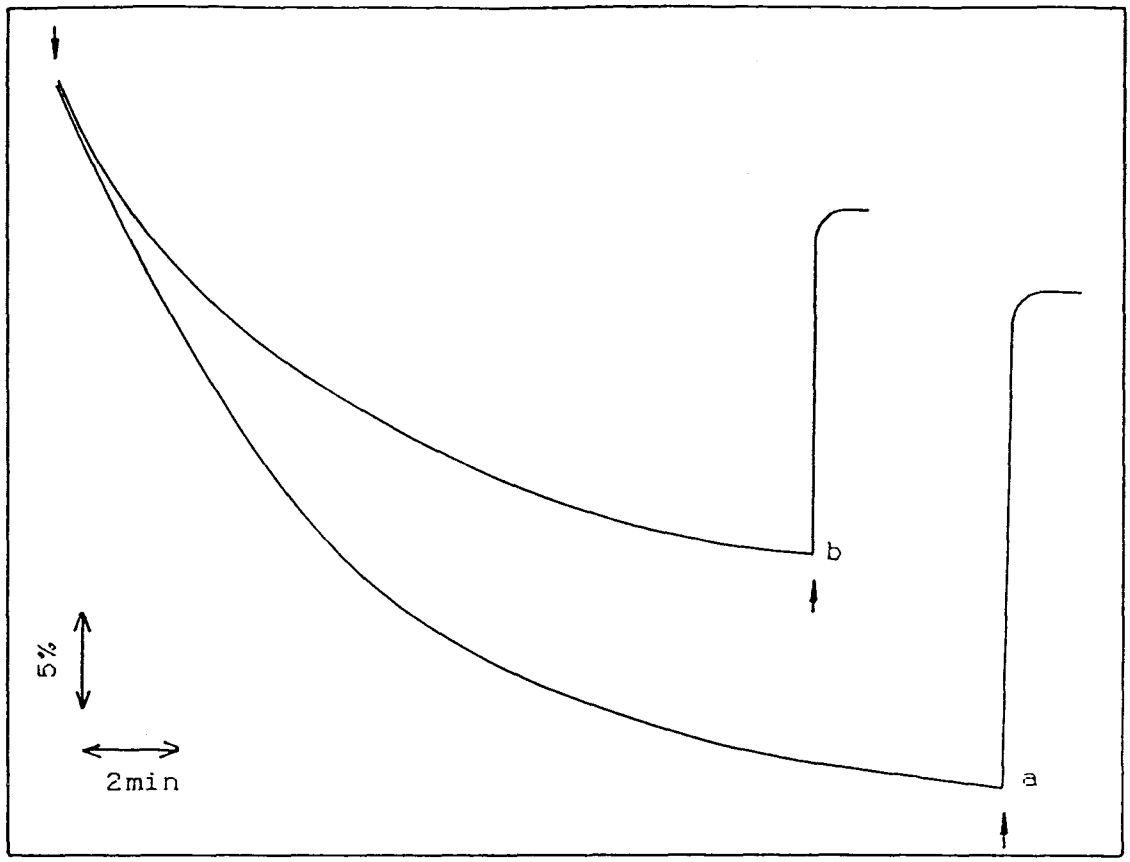


Figure 3.30. Effect of ATP and pyrophosphate as substrates for ATP-dependent quenching of quinacrine in microsomal vesicles. The standard quinacrine assay was used (a) with (b) pyrophosphate at a final concentration of 1.75mol m^{-3} replacing ATP. The reaction was initiated by the addition of ATP or PPi (downward arrow). and reversed by the addition of gramicidin (upward arrow).

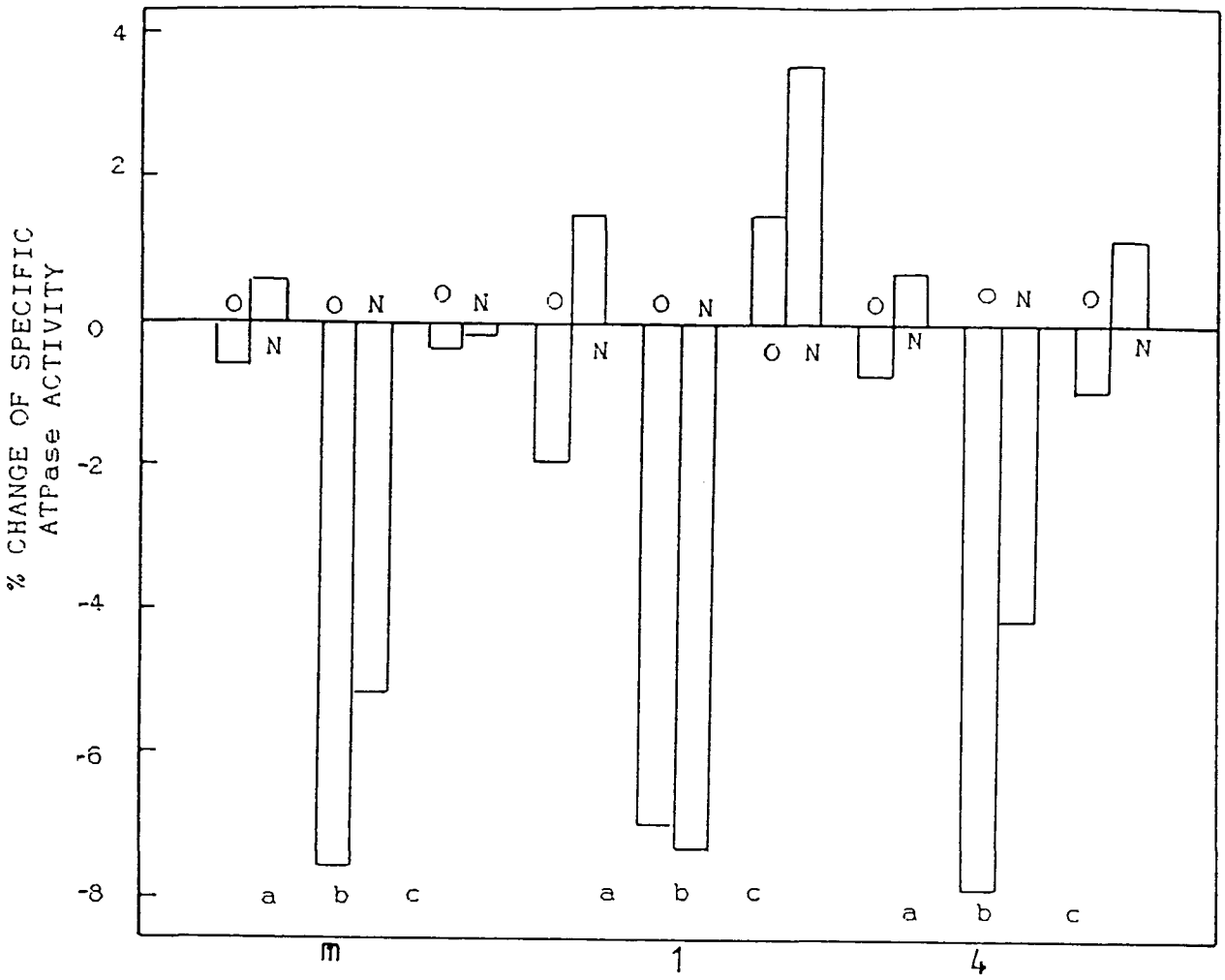
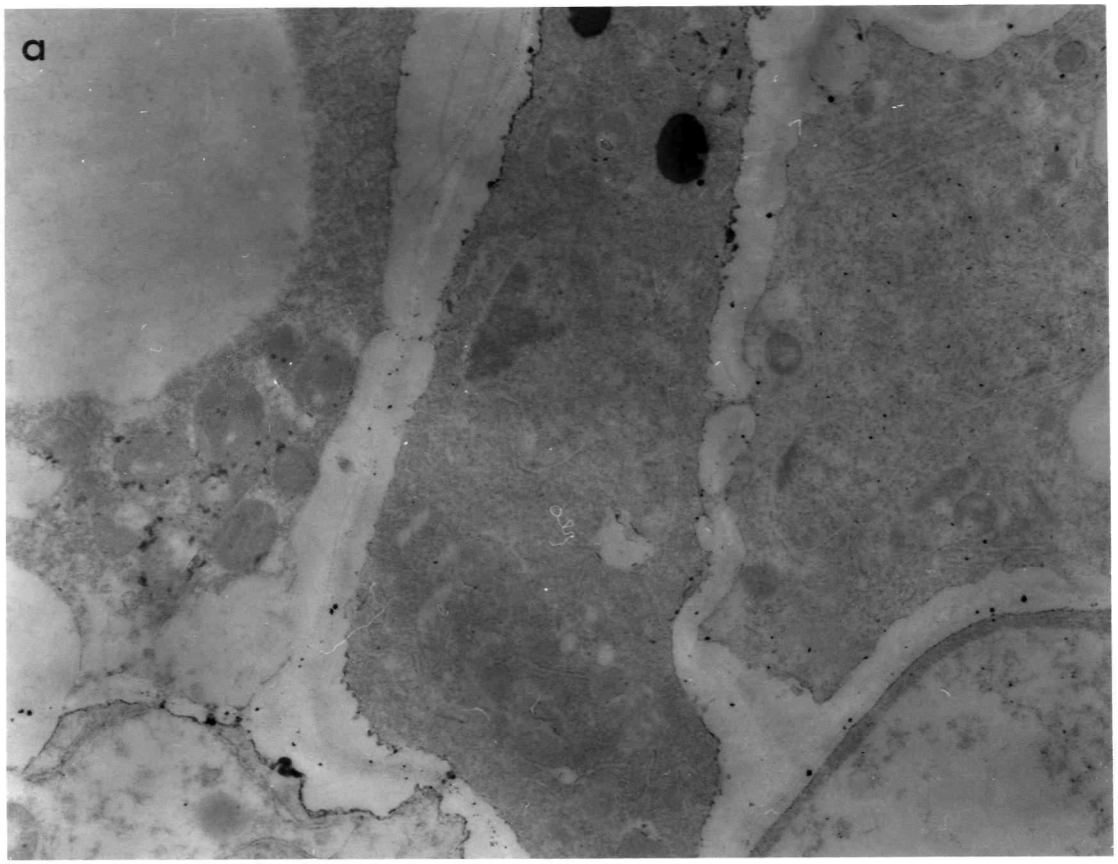


Figure 3.31. Effect of nitrate, vanadate and azide on ATPase activity prepared from a (M) microsomal fraction and interfaces 1, 4. Fractions were prepared from two different seed batches of (N) Spanish and (O) Dutch origin. The standard ATPase assay was used and activity compared as a percentage to that obtained with the addition of (a) 50mol m^{-3} KNO_3, (b) 10^{-1}mol m^{-3} vanadate and (c) 1mol m^{-3} azide.

Figure 3.32a. ATPase localization in cells of *Cucumis* hypocotyl following an incubation in the absence of ATP. Magnification 10K.

Figure 3.32b. ATPase localization in cells of *Cucumis* hypocotyl following an incubation in the absence of ATP. Magnification 2.5K.


a

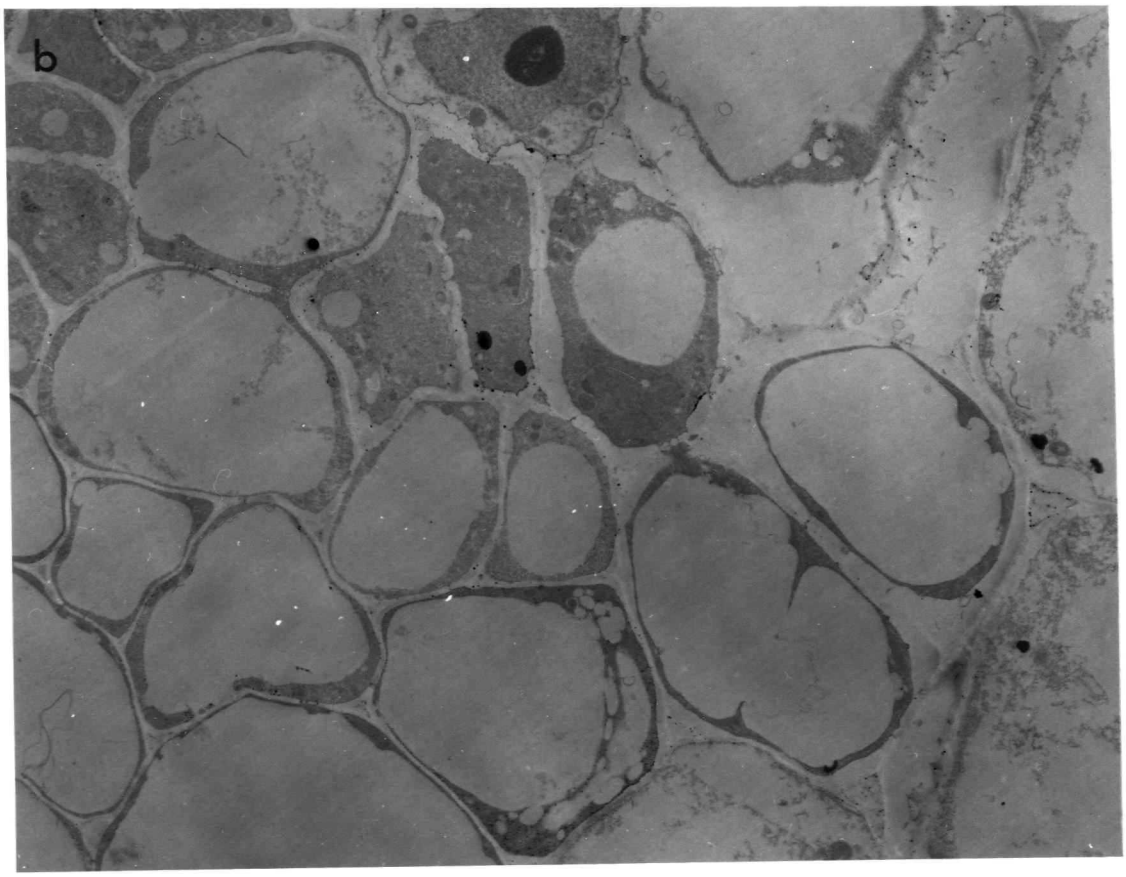
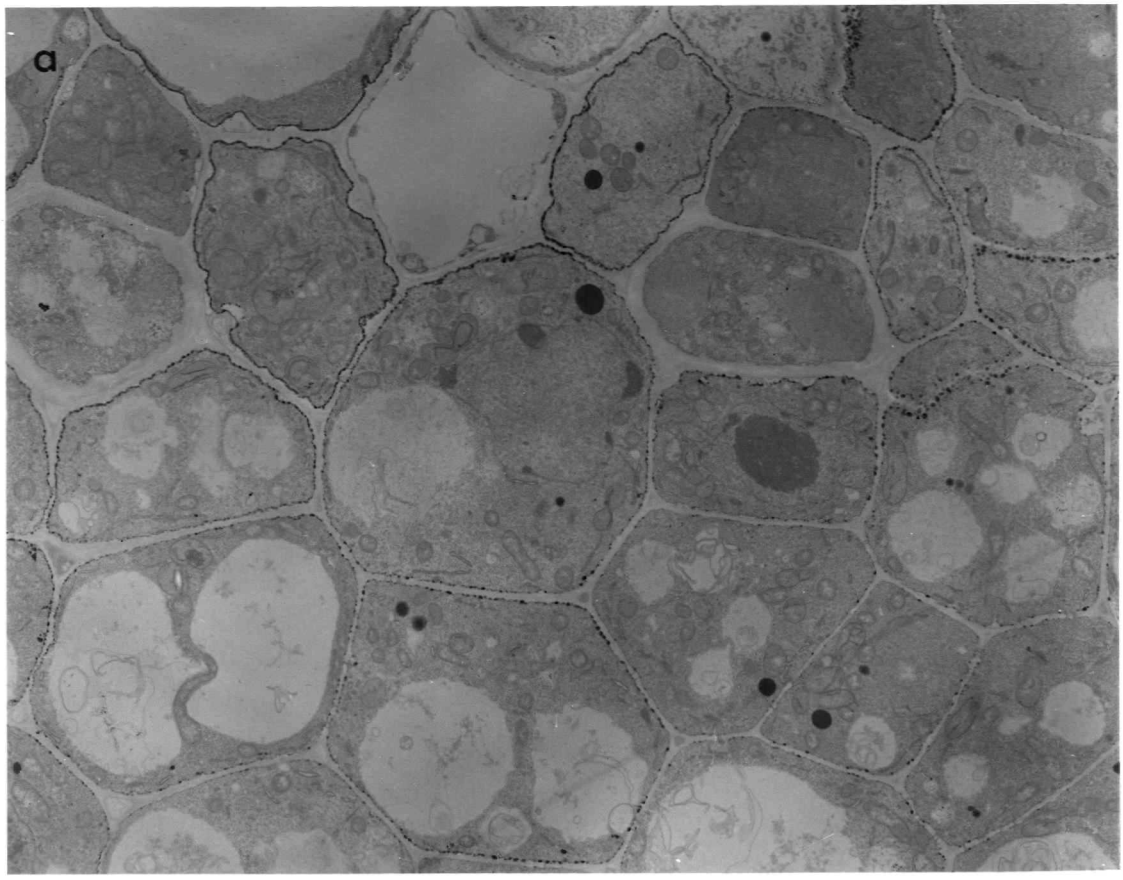

b

Figure 3.33a. ATPase localization in *Cucumis* hypocotyl cells following an incubation in the presence of ATP. Magnification 2.5K.

Figure 3.33b. ATPase localization in *Cucumis* hypocotyl cells following an incubation in the presence of ATP. Magnification 2.5K.


a

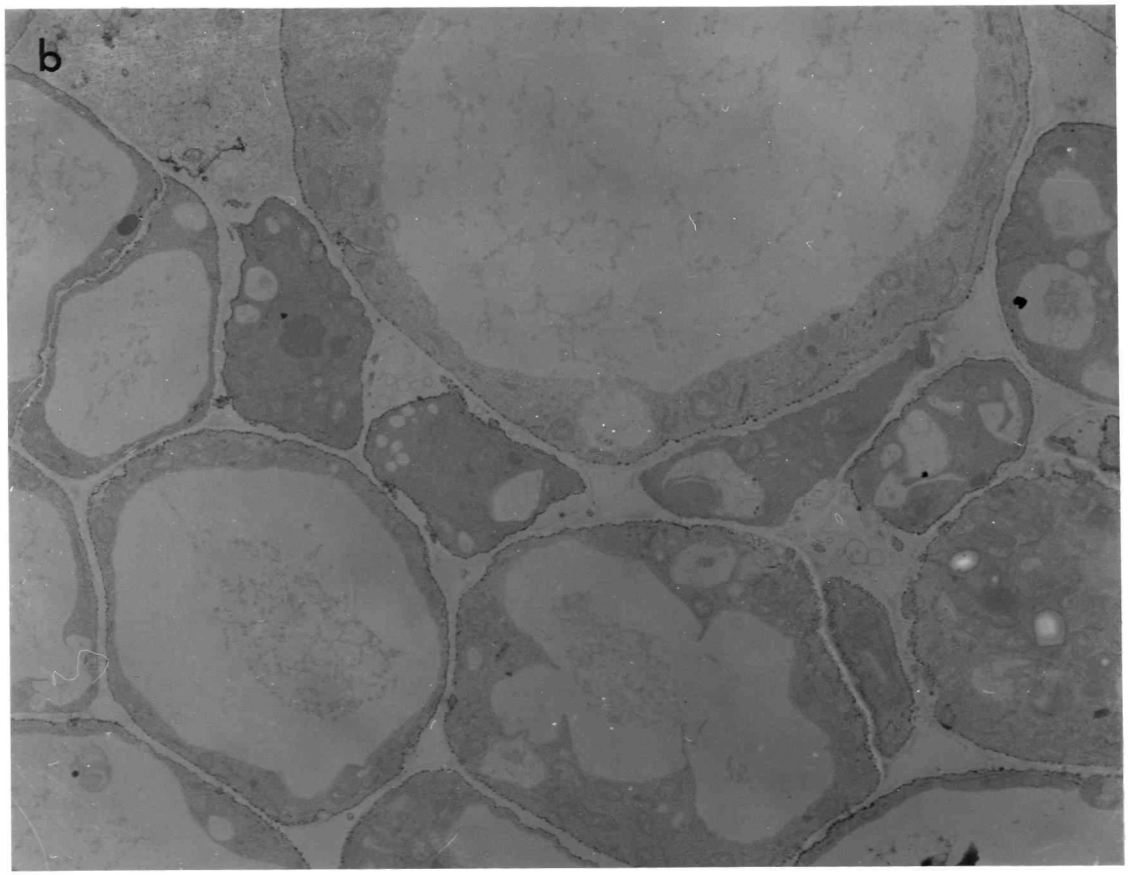
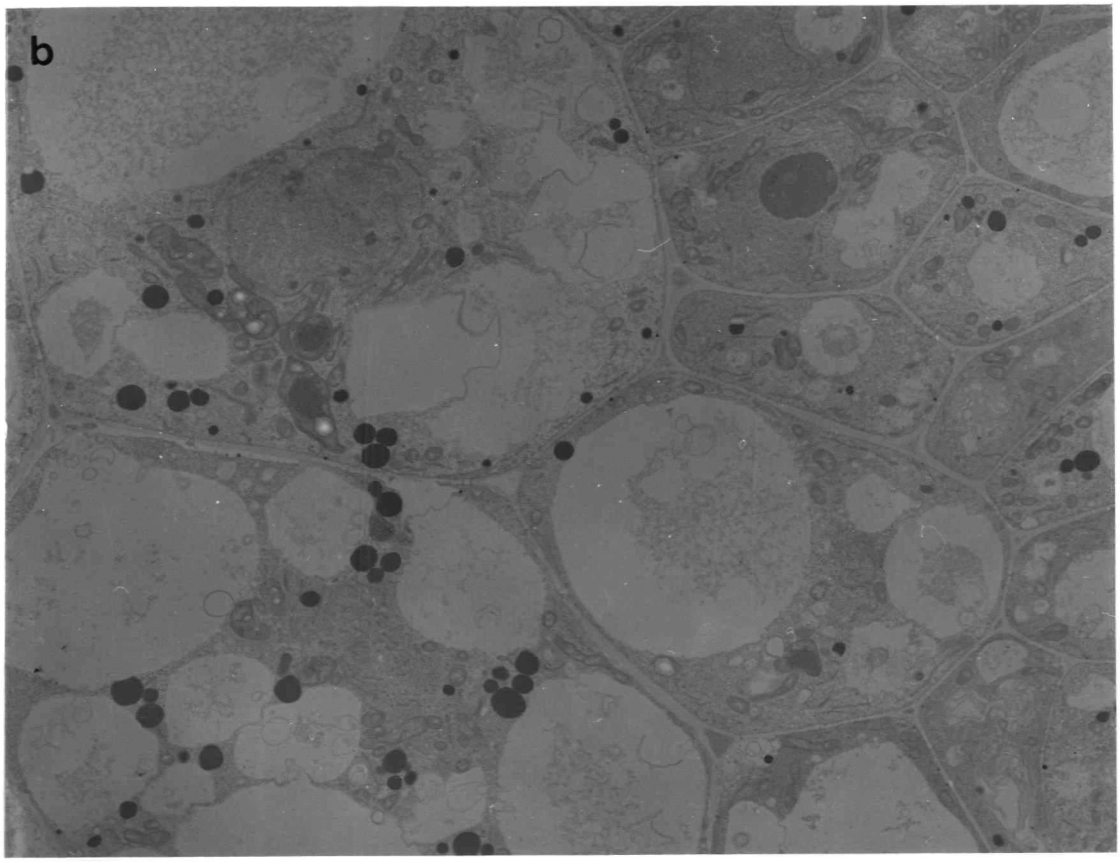

b

Figure 3.34a. ATPase localization in *Cucumis* hypocotyl cells following an incubation in the presence of ATP. Magnification 10K.

Figure 3.34b. ATPase localization in *Cucumis* hypocotyl cells following an incubation in the presence of ATP and 100×10^{-3} mol m^{-3} vanadate. Magnification 2K.


a


b

3.3.8 ATPase associated with the cotyledons.

Overall, specific ATPase activity associated with cotyledon membranes was slightly lower than that of the hypocotyls. Percentage inhibition of phosphohydrolase activity associated with the gradient interfaces indicated a similar trend to that found with the hypocotyl (Fig.3.35a) with 24% maximum inhibition by nitrate in interface 1 and 65% inhibition by vanadate in interface 3.

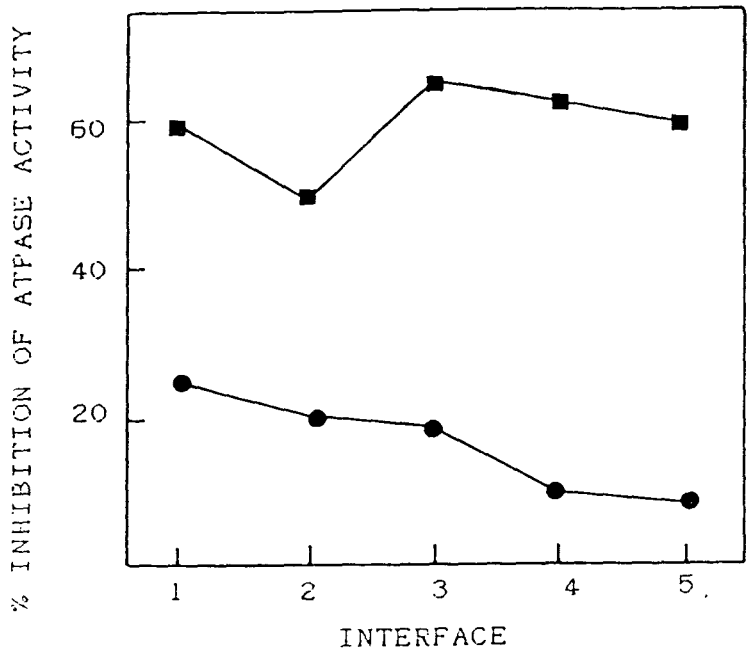
When microsomal pellets were given a KCl wash before sucrose gradient centrifugation and then assayed at pH 6.75 in the presence of Mg^{2+} or at pH 8.2 with Ca^{2+}, the wash increased the phosphohydrolase activity in most of the interfaces (Fig.3.35b). Examination of the pH profile of a microsomal fraction in the presence of Mg^{2+} or Ca^{2+} indicated the former to have an optimum at pH 7.0 and the latter one above pH 8.0 (Fig.3.36a). Triton was found to greatly stimulate the phosphohydrolase activity in the lower regions of the gradient at pH 8.0 in the presence of Ca^{2+} (Fig.3.36b). At pH 6.75 with Mg^{2+}, only phosphohydrolase activity in interface 5 was stimulated. Examination was made to determine if the Ca^{2+} stimulated phosphohydrolase activity associated with the cotyledons was anything other than due to contamination by mitochondria. At pH 8.0, ATPase activity was stimulated by Ca^{2+} but in the presence of azide additionally, activity was reduced to below the basal level in each case (Fig.3.37a). Activity at pH 6.75 was decreased by the addition of Ca^{2+} (Fig.3.37b). With the addition of azide, activity was still further reduced.

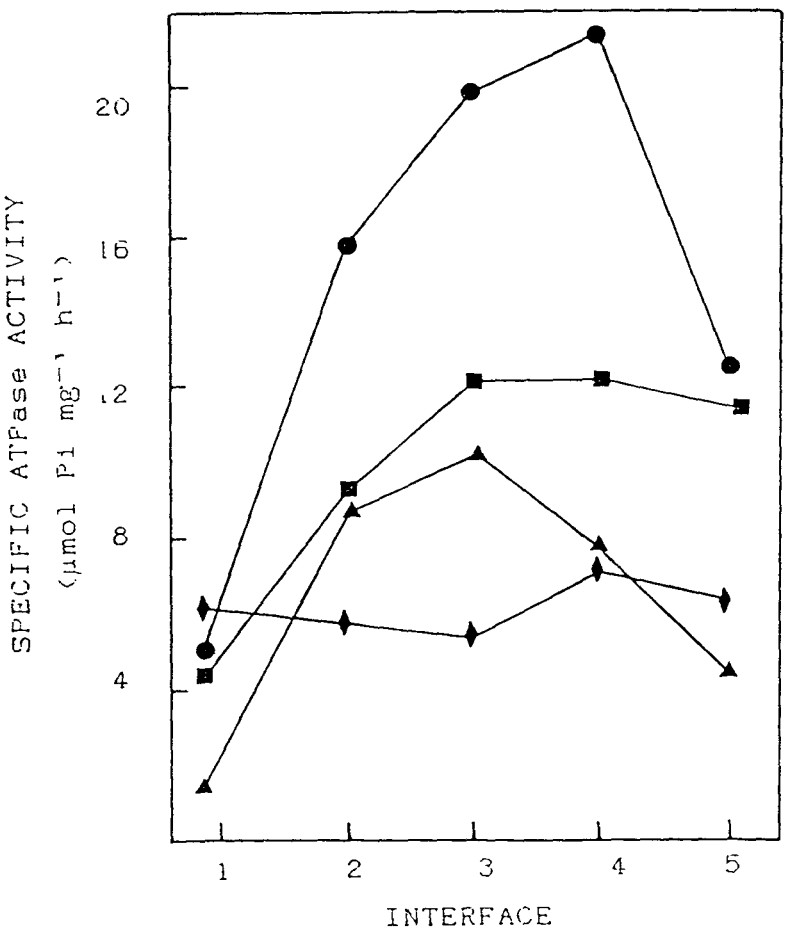
An initial attempt was made to detect ATP-driven H^+-pumping in cotyledon vesicles (Fig.3.38). Pumping differed from that of the hypocotyl in that with the addition of gramicidin quenching was completely reversed to 100%. The fraction was found to be fairly insensitive to 10^{-1}mol m^{-3} vanadate, but possibly [illegible] by 8×10^{-3}mol m^{-3} IAA.

An electron microscopical examination was made of the cotyledon tissue (Fig.3.39, 3.40). A large number of lipid vesicles is very evident, and it was noted that a thick layer of lipid formed on the surface of the supernatant during the low speed spin. Again, it is a

Figure 3.35a. Percentage inhibition of ATPase activity by nitrate and vanadate in the five gradient fractions prepared from the cotyledons of *Cucumis*. The standard ATPase assay was used and activity compared as a percentage with that obtained in the presence of 50mol m^{-3} KNO_3 (●) and $100x10^{-3}$mol m^{-3} vanadate (■).

Figure 3.35b. ATPase specific activity associated with each of the gradient interfaces prepared from cotyledons of *Cucumis*. The microsomal fraction was given a high concentration potassium chloride wash as described in "Materials and Methods" (●, ▲) and a control was used (■, ◆). The standard ATPase assay was used at pH 6.75 (■, ●), and at a pH of 8.0 (◆, ▲) and 2mol m^{-3} Ca^{2+}


% INHIBITION OF ATPASE ACTIVITY
60
40
20
1
2
3
4
5
INTERFACE


SPECIFIC ATPase ACTIVITY
(μmol Pi mg⁻¹ h⁻¹)
20
16
12
8
4
1
2
3
4
5
INTERFACE

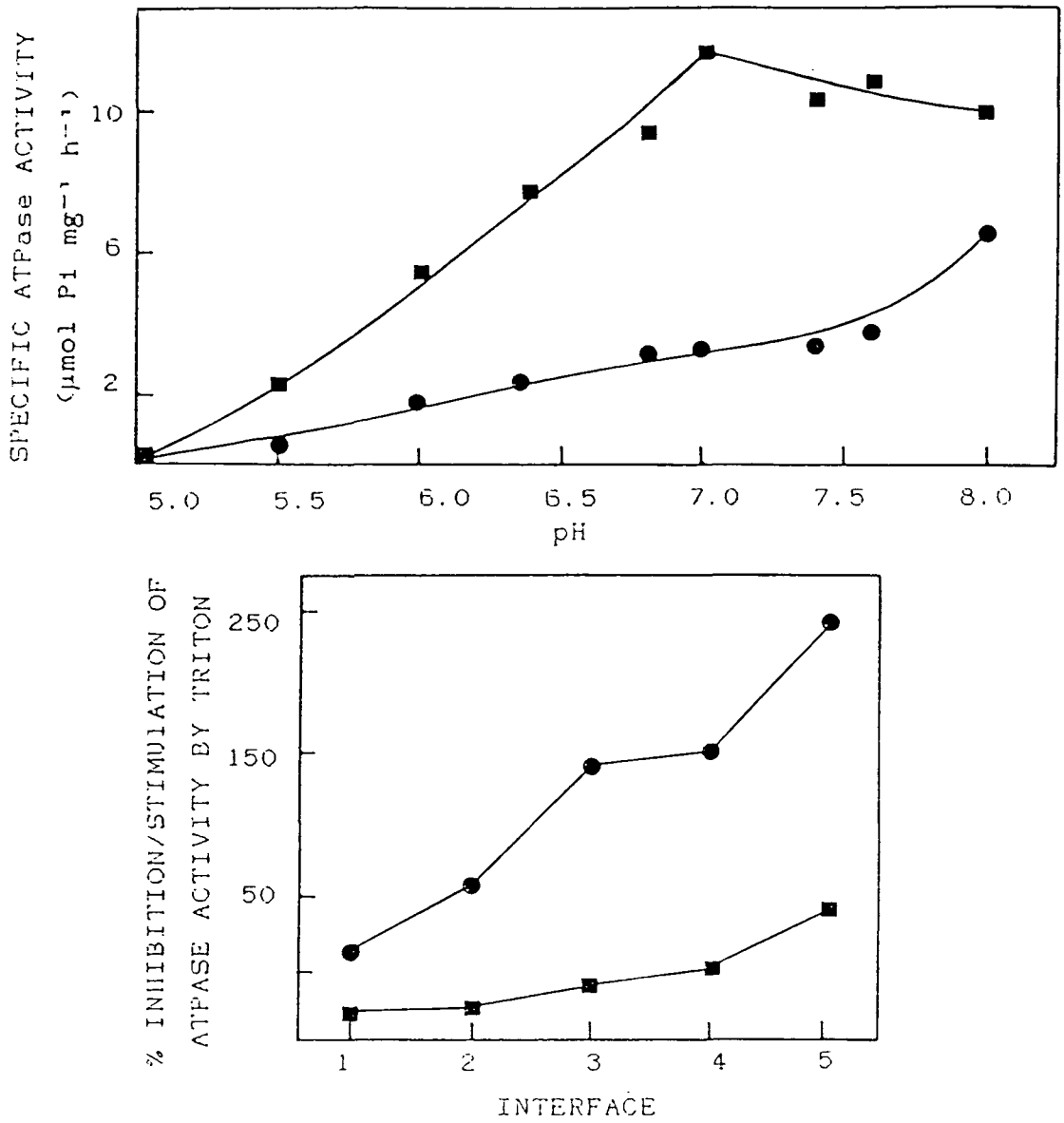


Figure 3.36a. Effect of pH on ATPase specific activity of a microsomal fraction prepared from the cotyledons. The standard ATPase assay was used (■), and with the replacement of magnesium by 2mol m^{-3} $CaCl_2$ (●).

Figure 3.36b. Percentage inhibition/stimulation of ATPase activity by Triton X-100 in a microsomal fraction prepared from cotyledons. The standard ATPase assay was used with the incorporation of Triton X-100 at a final concentration of 0.03% (v/v) and pH 8.0 (●), and pH 6.75 (■).

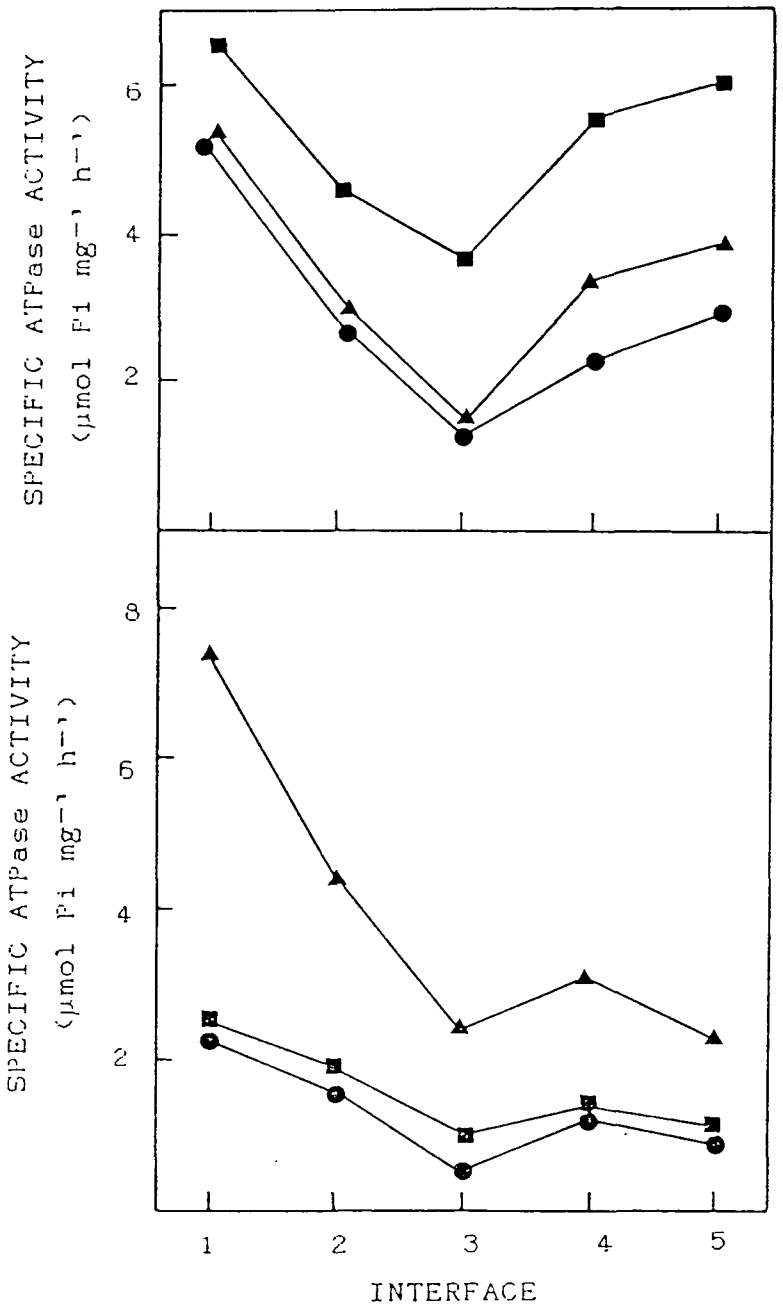


Figure 3.37a. Specific ATPase activity in the five interfaces of the gradient prepared from cotyledons. The standard ATPase assay was used at pH 8.0 in the absence of magnesium (▲), with the replacement of magnesium with 2mol m^{-3} $CaCl_2$ (■) and with 2mol m^{-3} $CaCl_2$ replacing magnesium and with the addition of 1mol m^{-3} azide (●).

Figure 3.37b. Specific ATPase activity in the five interfaces of the gradient prepared from cotyledons. The standard ATPase assay was used at pH 6.75 in the absence of magnesium (▲), with the replacement of magnesium with 2mol m^{-3} $CaCl_2$ (■) and with 2mol m^{-3} $CaCl_2$ replacing magnesium and with the addition of 1mol m^{-3} azide (●).

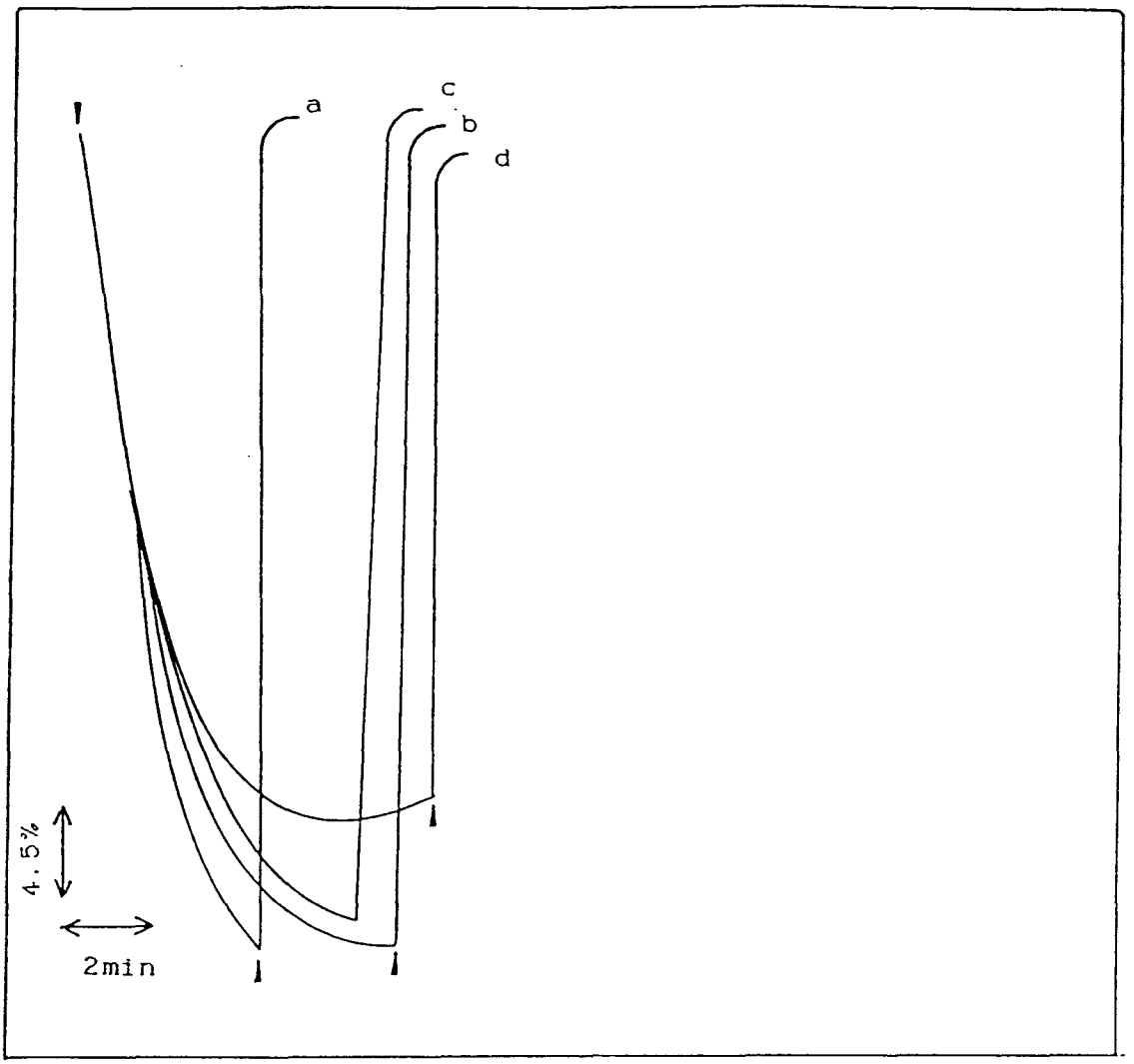
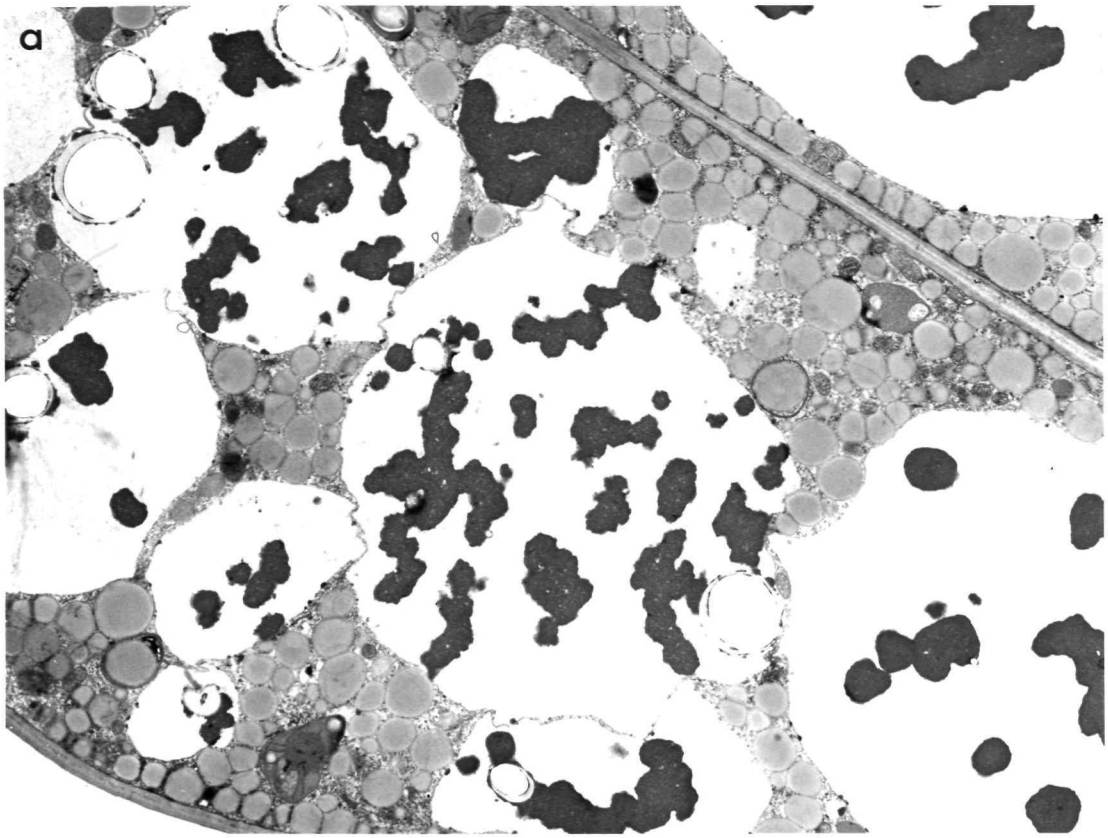


Figure 3.38. ATP-dependent quenching of quinacrine in vesicles prepared from cotyledons. The standard (a) fluorescent mixture was used, with the addition of (b) 50×10^{-3}mol, (c) 250×10^{-3}mol m^{-3} vanadate and (d) $8 \times 10^{-}$ mol m^{-3} IAA. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrows).

Figure 3.39a. Electron micrographs of tissue from the cotyledons of *Cucumis*. Magnification 2.5K.
Figure 3.39b. Electron micrographs of tissue from the cotyledons of *Cucumis*. Magnification 2.5K.



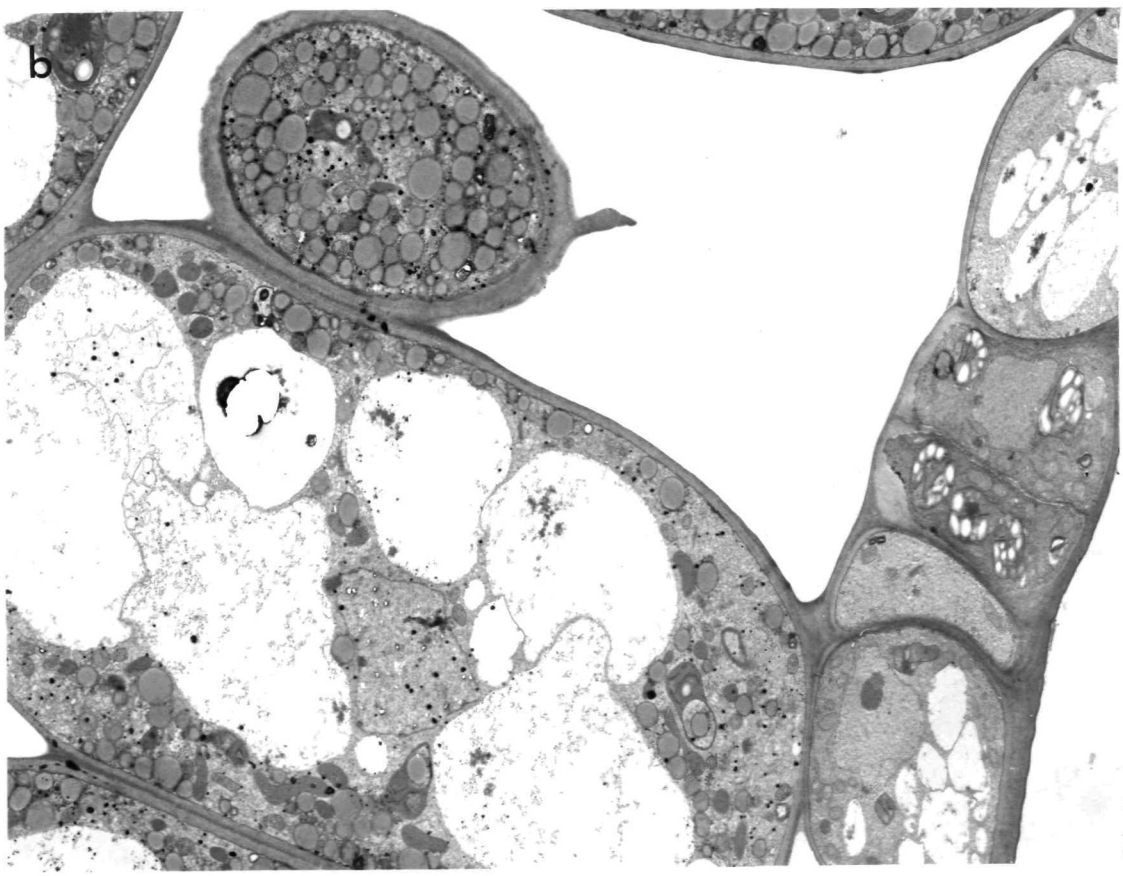
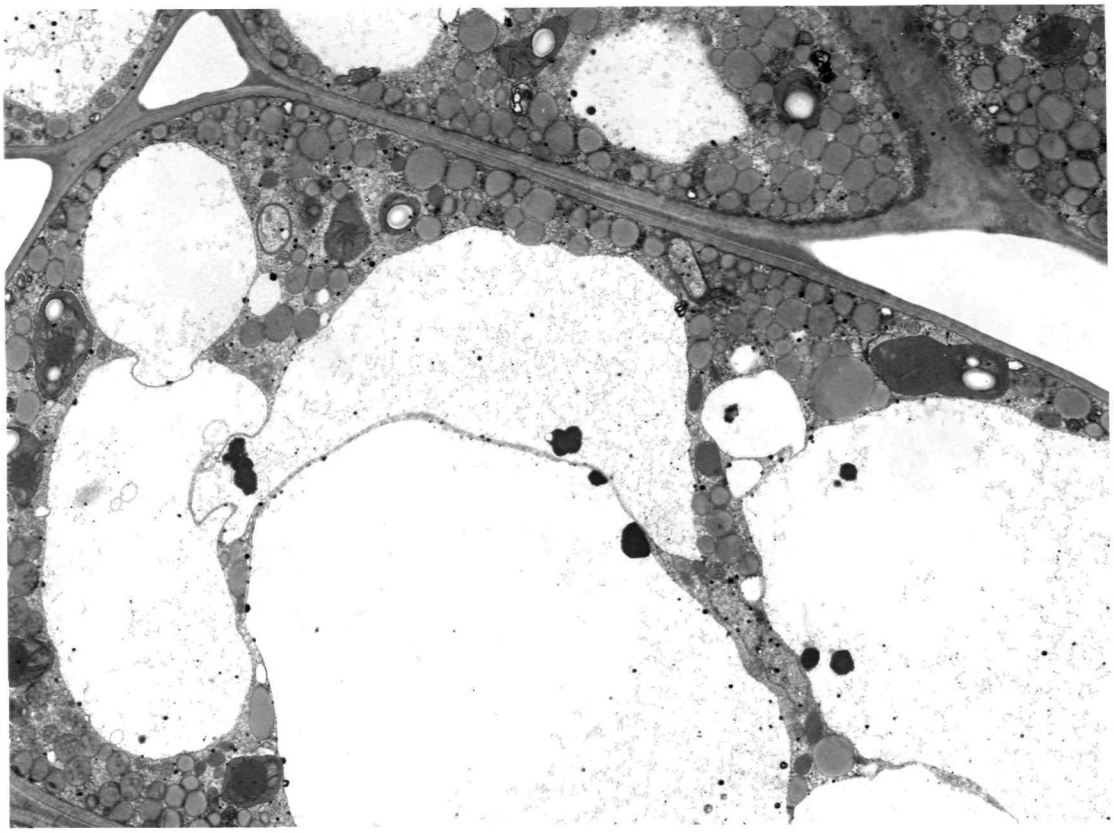


Figure 3.40. Electron micrograph of tissue from the cotyledons of *Cucumis*. Magnification 2.5K.



very vacuolate tissue with the cytoplasm being pushed to the periphery of the cells.

3.4 Discussion.

The preparation of plasma membrane sheets by sedimentation with charged silica microbeads resulted in contamination by mitochondrial membranes, as detected with biochemical assays, and was additionally shown by the electron micrographs. In addition the micrographs showed that many membranes were beaded while others were not. Whether there is a differential beading or whether the beads were not fully neutralised and became detached from the plasma membrane sheets and attached to other intracellular membranes cannot be assessed. The biochemical and morphological data presented by Polenko and Maclachlan (1984) reports that highly purified sheets of plasma membrane may be prepared by this method without mitochondrial contamination. The discrepency may be due to the tissue difference used in this work.

Enzyme inhibitor sensitivity has been expressed by different workers as a percentage inhibition of the total ATPase activity, the inhibitor-sensitive specific activity and as inhibitor sensitivity on a per fraction basis. The latter method is typically employed when a continuous gradient is used and equal aliquots are removed. The results in this chapter however were expressed using the first two methods. The percentage sensitivity of a preparation to an inhibitor gives information on the purity of a preparation but gives no indication of the specific activity that is sensitive to the inhibitor. This information is gained by expressing data on the basis of inhibitor sensitive activity.

Several gradient types were tested and the one chosen for routine use gave the clearest separation of vanadate-sensitive ATPase activity from nitrate- and azide-sensitive ATPase. Reports from other tissues indicate the wide variation in ATPase sensitivity to inhibitors that is obtained in membrane enriched fractions. For example, plasma membrane and tonoplast fractions of high purity are typically obtained from *Beta vulgaris*. A 92% and 20%

inhibition of the ATPase associated with a 16/26% (w/w) and 34/40% (w/w) fraction by 50X10^{-3}mol m^{-3} vanadate was obtained respectively and 26% and 89% inhibition by 50mol m^{-3} nitrate (Bennett *et al.* 1984). Similarly, a tonoplast enriched fraction from *Zea mays* was found to be 90% inhibited by 50mol m^{-3} nitrate and 10% inhibited by 100x10^{-3}mol m^{-3} vanadate (Mandala & Taiz 1985). However, separation of plasma membrane, and particularly tonoplast fractions from dicotyledonous stems using sucrose gradient centrifugation or phase partitioning has not been achieved to such a purity. For example, in a plasma membrane enriched fraction from *Cucumis sativus* roots, the associated ATPase was 39% inhibited by 100x10^{-3}mol m^{-3} vanadate at pH 6.0 (Memon *et al.* 1987). In a plasma membrane fraction prepared from hypocotyls of *Vigna radiata* (mungbean), the ATPase was inhibited by 84% at a vanadate concentration of 100x10^{-3}mol m^{-3}. Nitrate caused no inhibition of this fraction. In a purified tonoplast fraction from the same tissue, 50x10^{-3}mol m^{-3} vanadate caused 9% inhibition of the ATPase and 50mol m^{-3} KNO_3 inhibited activity by 39% (Yoshida *et al.* 1986a,b). These results illustrate the difficulties encountered in the purification of tonoplast and plasma membrane fractions from dicotyledonous stem tissue. However, the ATPase sensitivity data in this work suggests that a plasma membrane enriched fraction was achieved by sucrose density centrifugation that was sensitive to vanadate and insensitive to azide. A tonoplast enriched fraction was also obtained that showed increased sensitivity to nitrate but also had a vanadate-sensitive component.

Although nitrate and vanadate are conventionally used as ATPase inhibitors, variable vanadate inhibition of the plasma membrane ATPase in the intact system has been reported. Also vanadate is thought to penetrate intact cells very slowly (Saftner *et al.* 1983, Brummell *et al.* 1986) and consequently higher concentrations may be required to achieve inhibition of physiological processes. Recently, two other potential inhibitors of the plasma membrane ATPase have been reported: SW26, a herbicide derivative and weak photosynthetic inhibitor has been demonstrated to inhibit the plasma membrane

phosphohydrolase activity, ATP-dependent H^+ transport and growth of sycamore cell cultures (Blein *et al.* 1986). SW26 appeared to be specific for the plasma membrane in this tissue but inhibition was found to depend on the concentration of membrane protein in the assay. At low protein concentration, nearly 100% inhibition was achieved and this declined to 10% inhibition at higher protein concentrations. Erythrosin B, a derivative of fluorescein, has been shown to inhibit plasma membrane and tonoplast ATPases *in vitro* (Cocucci 1986). Erythrosin B at 25mol m^{-3} caused a similar differential inhibition of the ATPase activity as $100x10^{-3}$ vanadate in each of the gradient interfaces indicating that its effect is similarly specific for the plasma membrane, but more potent. Erythrosin B and vanadate showed no variation of inhibition with protein concentration per assay. Erythrosin B may therefore be useful as a plasma membrane associated ATPase inhibitor, but SW26 appears to show variable inhibition.

The differential pH optimum of the ATPases reported in the two membrane types from this tissue has similarly been found in plasma membrane and tonoplast ATPases from other tissues. In red beet, for example the pH optimum for the 16/26% associated ATPase was 7.0, while that for the 34/40% interface was 6.5 (Bennett *et al.* 1984). Similar pH optima were reported for the ATPase associated with the tonoplast and plasma membrane from *Vigna radiata* (Yoshida *et al.* 1986a,b). The pH optimum typically reported for the ATPases associated with the plasma membrane appears to be a little lower than the reported cytoplasmic pH (Brummell & Hall 1987). This may indicate that a pH-stat mechanism may be operative with maximal ATPase activity being achieved as the cytoplasm acidifies or that local variation occurs within the cytoplasm; alternatively it may indicate that enzyme activity has been affected by membrane isolation procedures.

Substrate specificity is routinely examined in membrane fractions to ascertain membrane purity. ATP is typically hydrolyzed to a greater extent than any other nucleotide phosphate in plasma membrane and tonoplast fractions, but may be followed closely by other

substrates depending on the tissue and membrane type. In a plasma membrane-enriched fraction from *Vigna radiata*, ATP hydrolysis was followed closely by UDP ≥ IDP ≥ UTP (Yoshida *et al.* 1986a) while in a tonoplast-enriched fraction from the same tissue, ATP was hydrolyzed to a greater extent than any other substrate (Yoshida *et al.* 1986b). In a tonoplast fraction from tobacco, ADP was hydrolyzed to a greater extent than ATP, and UDP was hydrolyzed nearly to the same extent as ATP. It is not known whether hydrolysis of these other substrates represents membrane contamination or the ability of the ATPase to hydrolyze these substrates.

Mitochondrial associated ATPase has been reported to hydrolyze GTP and ITP as well as or better than ATP (Grubmeyer & Spencer 1979). The high rate of hydrolysis of UDP, ITP, and IDP in both the tonoplast and plasma membrane enriched fractions from this tissue suggests contamination by other membrane types. IDPase activity, a marker for the Golgi, is negligible in most plasma membrane preparations (Lundborg *et al.* 1981); however, some activity has been detected in plasma membrane fractions from orchard grass and wheat (Yoshida *et al.* 1983, Sommarin *et al.* 1985). Whether these activities are due to the K^+, Mg^{2+}-ATPase activity or are due to Golgi contamination are uncertain. In this tissue, the distribution of IDPase within the gradient indicated the presence of Golgi membranes, particularly in interface 3.

Pyrophosphatase activity is used as a tonoplast marker (e.g. Wagner & Mulready 1983). Although the plasma membrane-enriched fraction contained a lower activity than the tonoplast-enriched fraction, substantial hydrolysis of this substrate occurred, suggesting that pyrophosphate may be unspecifically broken down.

Simple Michelis-Menten kinetics were observed with increasing ATP-Mg concentrations for both the tonoplast and plasma membrane enriched fractions from *Cucumis* hypocotyls; both V_{max} and the K_m were altered in the presence of K^+. The K_m for Mg.ATP was higher in the tonoplast-enriched fraction than the plasma membrane fraction. A relatively high K_m for ATP.Mg was obtained for the plasma membrane ranging from 0.5 - 2mol m^{-3} (Marrè

& Ballarin-Denti 1985) and similarly in a plasma membrane fraction prepared from *Zea mays* root, a K_m of 0.93mol m^{-3} and 0.65mol m^{-3} for $-K^+$ and $+K^+$ respectively was reported (Leonard & Hotchkiss 1976), and a plasma membrane preparation prepared from oat roots a K_m was obtained for ATP.Mg of 0.38mol m^{-3}. KCl was shown to have no effect on the K_m, but altered V_{max} (Leonard & Hodges 1973).

ATPase specific activity associated with the plasma membrane from different plants has been shown to lie within the range of 10 - 30µmol Pi mg^{-1} h^{-1}. No distinct range of activity has emerged for dicotyledons, monocotyledons, shoots or roots. The ATPase specific activity associated with the tonoplast enriched fraction in this tissue was significantly lower than that associated with the plasma membrane. This difference has been shown by other workers. For example, the plasma membrane fraction from *Vigna radiata* was reported to have a specific ATPase activity of 25.2µmol Pi mg^{-1} h^{-1} and from a tonoplast fraction a value of 5.19µmol Pi mg^{-1} h^{-1} was obtained (Yoshida *et al.* 1986a,b). Similarly red beet ATPase values of 12.06 and 32.96µmol Pi mg^{-1} h^{-1} were obtained for the 16/26% and 34/40% membrane interfaces (Bennett *et al.* 1984). If the protein per unit area of the tonoplast and plasma membrane could be determined, a physiological role might be ascribed to these differences in ATPase levels.

Potassium stimulation of membrane-bound ATPase activity has previously been used as a marker for the plasma membrane, but recent reports suggest it to be more of a marker for the tonoplast. For example, the ATPase associated with a plasma membrane fraction from *Vigna radiata* showed no stimulation by KCl (Yoshida *et al.* 1986a). In a tonoplast-enriched fraction from the same tissue, significant stimulation of the ATPase was found (Yoshida *et al.* 1986b). Similarly, in this tissue, stimulation by KCl of the plasma membrane associated ATPase was found to be variable or non-existent, while in the tonoplast-enriched fraction, significant stimulation was always observed particularly at lower pH values. In both of the enriched fractions from this tissue, KCl was found not to alter the pH optimum. In other tissues a

change of the pH optimum for the ATPase has sometimes been reported on addition of KCl to the incubation medium. For example, in a red beet plasma membrane-enriched fraction, KCl reduced the pH optimum from 7.5 to 6.5 (Leonard & Hodges 1973). On the other hand, in preparations made from *Vigna radiata*, no alteration in the ATPase optimum was found in either a plasma membrane or tonoplast fraction by KCl (Yoshida *et al.* 1986a,b).

More generally, ATPase sensitivity to a range of cations has been used as a marker to distinguish between the plasma membrane and tonoplast. It is reported that sensitivity to cations occurs with plasma membrane associated ATPase, while tonoplast associated ATPase is unaffected (Sze 1985). For example, red beet tonoplast ATPase was shown to have little sensitivity to K^+, Na^+, Li^+ and Rb^+ (Bennett *et al.* 1984) while a tonoplast fraction prepared from *Vigna radiata* showed little differential response to K^+, Na^+, or Li^+ (Yoshida *et al.* 1986b). Similar results are reported in this tissue from the gradient fractions. In this tissue calcium was found to be a potent inhibitor of both ATPases and may inhibit activity by interfering with the ATP.Mg.enzyme complex (Balke *et al.* 1974, Dahl & Hokin 1974, Robinson 1974, Leonard & Hotchkiss 1976). However, when Ca^{2+} was removed prior to the assay, stimulation of ATPase activity by Ca^{2+} was observed, which may indicate the presence of a Ca^{2+}-ATPase in this tissue. With a plasma membrane fraction from roots of *Cucumis*, no evidence for a Ca^{2+}-ATPase was found (Memon *et al.* 1987).

Anion sensitivity is considered to be a characteristic of the tonoplast associated ATPase. In this tissue Cl^- and Br^- were both very inhibitory of the tonoplast associated ATPase and I^- inhibited the ATPase associated with both membrane fractions, particularly the tonoplast associated ATPase. In a tonoplast-enriched fraction from *Vigna radiata*, KI was found to be stimulatory to the ATPase (Yoshida *et al.* 1986b); however, with red beet tonoplast fractions the ATPase was found to be very sensitive to KI. KI has been used as a specific inhibitor of the tonoplast associated ATPase. This method has been employed in the preparation of vanadate-sensitive

ATPase from corn root vesicles by incubating membrane fractions in 250mol m^{-3} KI for 15 min on ice. Tonoplast associated ATPase was found to be inhibited (de Michelis & Spanswick 1986). In this tissue, a KI wash decreased ATPase activity associated with both the tonoplast and plasma membrane.

Enzyme latency has been reported in many membrane ATPase systems (e.g. Memon *et al.* 1987) and is overcome by the use of detergents such as Triton X-100. ATPase latency was found to be associated with all the membrane fractions from this tissue and stimulation was achieved with the addition of low concentrations of Triton. Higher concentrations were found to be inhibitory, presumably causing denaturation of the enzyme. Enhanced ATPase activity by Triton indicates the presence of sealed membrane vesicles, being either inside-out in the case of tonoplast or right way round for plasma membrane. The lower activation of the ATPase associated with the tonoplast fraction by Triton compared to that of the plasma membrane enriched fraction indicated that tonoplast vesicles may have reannealed the right way round in the majority of cases.

For a H^+ symport or antiport to be operative, initially a proton gradient must be established at the tonoplast or plasma membrane using metabolic energy. When in operation, this process works to maintain a H^+ gradient and will also contribute to the regulation of the pH of the free space, cytoplasm and vacuole. ATPases associated with the tonoplast and plasma membrane have now been well established as present, but it has been only relatively recently that ATP-dependent H^+ transport been demonstrated in isolated membrane vesicles (Sze 1980). With the use of fluorescent dyes, H^+ transport is now routinely measureable and is being biochemically characterized to establish that the ATPases associated with the tonoplast and plasma membrane are the same. ATP dependent H^+ transport was demonstrated in vesicle fractions from hypocotyls from this tissue and were found to be sensitive to both nitrate and vanadate. ATP-dependent H^+ transport was subsequently demonstrated in membrane fractions from a sucrose gradient. ATP-dependent H^+ transport in the

tonoplast enriched fraction had a greater sensitivity to nitrate than the plasma membrane enriched fraction. The nitrate-sensitive ATPase may therefore similarly transport H^+. Vanadate on the other hand caused similar inhibition in both fractions, and at relatively high concentrations only minor inhibition occurred. This may indicate that plasma membrane vesicles from the hypocotyl of *Cucumis* do not form tightly sealed vesicles which are capable of carrying out ATP-dependent H^+ transport. However, there are other reports of plasma membrane fractions capable of carrying out ATP-dependent H^+ transport that have similarly shown reduced inhibition by vanadate when compared to ATPase activity; for example, with a plasma membrane fraction from *Ricinus* cotyledons (Williams & Hall 1987). Following reconstitution of ATPases into liposomes, ATP-dependent transport has been reported to have an increased sensitivity to vanadate (Williams 1987), indicating that vandate may not be readily penetrating regions of the enzyme in the native vesicle.

Markers for other membranes have been used to determine their location within the gradient in relation to the plasma membrane and tonoplast. Cytochrome c reductase has been used as a specific marker for the endoplasmic reticulum and on this basis all the gradient fractions from *Cucumis* contained membranes of endoplasmic reticulum origin. Cytochrome c reductase activity has been reported to be associated with plasma membrane fractions and recent reports have indicated that the plasma membrane also contains P-450/420 as judged from CO-difference spectra (Schelin *et al.* unpublished, Larsson 1985).

ATPase localization examined in hypocotyl sections indicated intense ATPase activity at the plasma membrane, but consistently no activity at the tonoplast. Glutaraldehyde was used as a fixative in the localization experiments and it was found that this chemical (at 1%) strongly inhibited ATPase activity in all of the gradient interfaces. At 0.1%, differential inhibition was observed, with the tonoplast being the most sensitive and the plasma membrane fraction the least. The use of this fixative for localization studies needs to be re-examined

since a similar lack of tonoplast associated ATPase activity has been observed when glutaraldehyde was used (Williams & Hall 1987).

3.5 Conclusions.

ATPase characteristics, inhibitor data and marker enzymes indicate that fractions enriched in plasma membrane and tonoplast membranes were obtained from a sucrose gradient. These fractions were found to be capable of carrying out ATP-dependent H^+ transport detected by the fluorescence quenching of the probe quinacrine in which the H^+ gradient established was quenched by gramicidin.

CHAPTER FOUR.

Distribution of ATPase activity in the cucumber hypocotyl and the effect of auxin and other plant regulators on ATPase activity, growth and acidification.

4.1 Introduction.

Growing plants exhibit a range of cell developmental stages including dividing, elongating, differentiating and mature zones. Although these developmental stages may be isolated from developing organs and are present in different regions of many tissues, little work has been done on the associated ATPase activity and ATP-dependent H^+-transport in tissues from these different stages. There have been reports of varying levels of ATPase activity associated with the plasma membrane and tonoplast from fruits and tubers at various developmental stages (Ishikawa & Yoshida 1985, Chedhomme & Rona 1986). ATPase, and NADH cytochrome C reductase activity has been reported to vary in microsomal vesicles prepared from tissues of different developmental stages from corn roots (Dupont *et al.* 1982b), while ATP-dependent H^+ transport was also examined in vesicles prepared from these zones. Activity in the dividing zone was markedly higher than in the elongating and differentiating zones.

4.1.1 H^+ excretion and correlation with auxin action.

The detection of proton extrusion by intact tissue in response to auxins has been reported in monocotyledonous and dicotyledonous tissues provided the cuticle is made permeable to H^+ (Cleland 1980). It is believed that this acidification of the wall solution is part of the mechanism of the rapid auxin-induced growth (Hager *et al.* 1971, Rayle & Cleland 1977). A plasma membrane associated ATPase is thought to be responsible for this wall acidification by the electrogenic export of protons from the cytoplasm into the wall space (Hager *et al.* 1971) and an ATP-dependent H^+ transport has unequivocally been demonstrated in plasma membrane vesicles (e.g. Sze 1984).

There is much evidence in the literature that the release of H^+ by plant tissue due to a plasma membrane located ATPase is stimulated by K^+ (Marrè 1979, Travis & Booz 1974). Lin (1981) assumes that the H^+ extrusion from maize root tips is brought about by a H^+/K^+ antiport driven by the membrane ATPase rather than a direct effect mediated by an ATPase. However, active extrusion of protons by roots of intact maize plants has been shown to occur without the concomitant uptake of cations and thus H^+ extrusion was shown to be an active process driven by a plasma membrane located ATPase and not occurring *via* a K^+/H^+ antiport (Mengel & Schubert 1985). Supportive evidence is further provided by the report that auxin-stimulated growth is inhibited by vanadate (Brummell *et al.* 1986), suggesting that IAA may mediate a control *via* the plasma membrane ATPase. This is confirmed by reports that auxin-induced proton excretion from intact tissue is inhibited by vanadate (e.g.Jacobs & Taiz 1980). Auxin has been reported to directly increase membrane associated ATPase activity; for example, IAA (10^{-1}mol m^{-3}) enhanced specific ATPase activity by 20-30% in a plasma membrane-enriched fraction from *Cucurbita maxima* hypocotyls (Scherer 1981). The hormone specificity was further examined by comparing the effect of the weak auxin analogue 2,3-D with that of 2,4-D, an active auxin on ATPase activity. Activity was stimulated by 29% by 2,4-D at a concentration of 10^{-3}mol m^{-3}, and by one-fifth of this value using 2,3-D at the same concentration (Scherer 1984b).

Subsequent studies using *Cucurbita pepo* have shown that the K_m of the plasma membrane ATPase for ATP is 122×10^{-3}mol m^{-3}, whereas IAA *in vitro* decreased this value to 72×10^{-3}mol m^{-3} (Scherer 1984a). On average the K_m was decreased by a factor of 2.2 in the presence of the hormone at a concentration of 10^{-3}mol m^{-3}. V_{max} was also increased slightly in the presence of IAA, but only at very low ATP concentrations, of the order of 3.8×10^{-3}mol m^{-3}. ATP concentrations of 14 and 54×10^{-3}mol m^{-3} were inhibitory. The physiological significance of these observations is questionable, as the ATP concentration *in vivo* is well above those used in these *in vitro* assays.

The alteration of K_m rather than V_{max} by IAA suggests that the affinity of the enzyme for its substrate is being altered rather than a change occurring in the membrane.

However, this effect of IAA on ATPase activity and the decrease in K_m for ATP caused by auxin has proved to be difficult to reproduce. Some workers have found V_{max} to be changed by plant hormones (Kasamo & Yamaki 1974) whereas others have found ATPase *in vitro* to be unaffected. IAA at a concentration of 10^{-3}mol m^{-3} was reported to have a slight affect only on ATPase activity in pea root plasma membrane vesicles, at low ATP concentrations (Gabathuler & Cleland 1985).

4.1.2 Effect of phenyl acetic acid and other plant regulators on growth and ATPase activity.

3-Indoleacetic acid has been regarded for many years to be the only naturally occurring auxin to play any role in plant physiological processes. A number of related chemicals have been isolated: for example, 4-chloro-3-indoleacetic acid is present in immature seeds of *Pisum* and *Lathyrus* (Gander & Nitsch 1967, Marumo *et al.* 1968, Engvild *et al.* 1978 1980); 3-indolepropionic acid has been found in tobacco leaves and squash hypocotyls by Bayer (1969) Wightman & Lighty (19[illegible]). IAA and PAA have both been isolated from sunflower, pea, barley, *Zea mays*, tobacco and tomato and both cause stimulation of growth (Wightman 19[illegible]). If it is assumed that 75% of the shoot fresh weight is water, the calculated PAA concentration is of the order $1-3x10^{-3}$mol m^{-3} (Wightman 19[illegible]). These values are somewhat lower than those giving optimal growth-promoting activity in standard auxin tests.

The effect of PAA on ATPase activity *in vitro* in the presence and absence of IAA has been examined (Scherer 1981). PAA alone caused very little stimulation of the ATPase at 10^{-1}mol m^{-3}, but in the presence of IAA ATPase activity was stimulated by 35-50%, with maximum effect at 10^{-3}mol m^{-3} IAA. Similarly, PAA and 2,4-D stimulated ATPase activity when added together, but 2,3-D had no effect.

4.1.3 Regulatory role of auxins on ATP-dependent H^+ transport.

With the isolation of sealed inside-out membrane vesicles capable of carrying out ATP-dependent proton transport which is sensitive to vanadate (Stout & Cleland 1982, Serrano 1983, Sze 1984), the direct effect of IAA on ATP-driven H^+ transport has been examined. Auxin was reported to influence ATP-dependent proton transport into vesicles from pea root and had no effect on V_{max} (Gabathuler & Cleland 1985). At ATP concentrations of 10^{-1}mol m^{-3}, activity was greatly increased, with the K_m for ATP being reduced by nearly 50% from 0.75 to 0.35mol m^{-3} in the presence of 10^{-2}mol m^{-3} IAA. The significance of enhanced H^+ efflux observed at low ATP concentrations *in vitro* is questionable as cytoplasmic ATP levels *in vivo* are in the region of 1-2mol m^{-3} (Bieleski 1973).

4.1.4 Auxin movement in relation to H^+ gradient.

As well as the possibility that auxin has a direct effect on ATPase activity, the H^+ gradient itself may influence auxin distribution and thus physiological processes. The negatively-charged and slightly alkaline pH of the symplast compared with pH 5-6 for the wall space (Roberts et al. 1981) favours the accumulation of IAA^- within the cytoplasm. The plasma membrane is relatively impermeable to IAA, but some transport may occur by transmembrane diffusion of undissociated IAA (IAAH). The major route of entry of IAA is *via* a H^+/IAA^- symport, which is proposed to be evenly distributed throughout the plasma membrane (Rubery 1977, 1978, Sussman & Goldsmith 1981a,b). The chemiosmotic theory of Rubery and Sheldrake (1974) envisaged that the driving force for active auxin transport is *via* a pH gradient across the plasma membrane, with the higher pH on the cytoplasmic face driving the uptake of undissociated IAAH and/or IAA uptake *via* a saturable H^+/IAA^- symport. Efflux of IAA occurs *via* saturable anion carriers located in the plasma membrane and are thought to be located predominantly in the basal region of the cell (Hertel *et al.* 1969, Goldsmith & Ray 1973, Hertel *et al.* 1983). This site is non-competitively inhibited by TIBA and NPA (Thomson *et al.* 1973, Katekar

et al. 1981, Goldsmith 1982) and accumulation of IAA occurs in the presence of these anion carrier inhibitors in plasma membrane vesicles prepared from *Cucurbita pepo* (Hertel *et al.* 1983).

Although these mechanisms have been proposed to explain the polar movement of IAA, it has not yet been conclusively demonstrated that stimulation of ATPase activity by IAA is not mediated *via* the dissipation of the H^+ gradient generated within vesicles. If this were so, it would imply that IAA efflux also takes place *in vivo* by a IAA^-/H^+ symport.

For auxin to affect the initial fast phase of growth in the intact system it must enter the cytoplasm *via* the symport, and either mediate an effect directly, or else amplify itself by stimulating the plasma membrane ATPase which further acidifies the apoplast. While the plasma membrane ATPase consists of a single catalytic peptide (Scarborough & Addison 1984), it may well require other peptides for efficient proton transport in a manner analogous to the F_1F_o-ATPase (McCarty 1985). The protein that binds auxin is not the catalytic subunit (Cross *et al.* 1978) but might be a proton channel peptide (Chastain & Hanson 1982) or a regulating peptide.

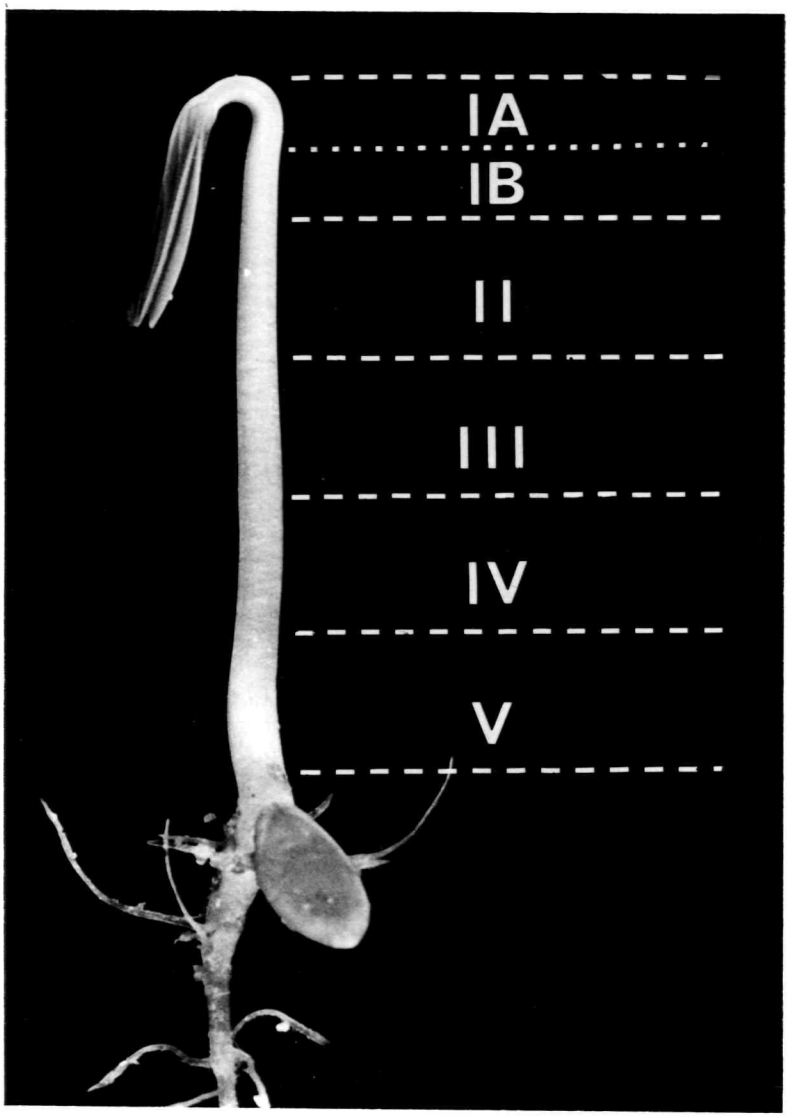
4.2 RESULTS.

4.2.1 Levels of ATPase activity associated with different regions of the hypocotyl.

The six regions of the *Cucumis* hypocotyl (1A, 1B, 11, 111, 1V, V) examined are illustrated in Figure 4.1. ATPase specific activity associated with different regions of the hypocotyl varied in separate experiments; however, comparison of the relative specific activities in the regions within each experiment showed the same trend.

There was a marked difference between the ATPase specific activity associated with membrane enriched fractions. Four experiments were carried out, and results from two are averaged and illustrated. Plasma membrane-associated ATPase activity was typically four times higher

Figure 4.1. Hypocotyls 50 mm in height were used which were divided into 10 mm segments I - V, region I being equally sub-divided in some experiments into regions IA and IB.
In experiments using cotyledons, excision was made so that no hypocotyl tissue was included, but this tissue did contain the apical axis.


IA
IB
II
III
IV
V

than tonoplast-associated activity, in each of the five regions from the hypocotyl (Fig.4.2a). ATPase specific activity associated with these two fractions remained relatively constant in regions 1 and 11 of the hypocotyl, but increased basipetally to region 111 and plateaued or declined slightly in region 1V.

Potassium chloride had little effect on ATPase activity associated with the plasma membrane-enriched fraction, and little variation was found in different regions of the hypocotyl (Fig.4.2b). However, the tonoplast-associated ATPase was stimulated to a greater extent. Potassium chloride stimulated ATPase activity from region 1A of the hypocotyl by up to 20% and this increased basipetally to region 111 plateauing at 75%.

4.2.2 Vanadate and nitrate sensitivity of ATPase in different regions of the hypocotyl.

Thirteen per cent of the total ATPase activity was found to be sensitive to nitrate (50mol m^{-3}) in region 1A, which increased to 60% in region 11 (Fig.4.3a). Nitrate-sensitive specific ATPase activity followed the same trend as did the nitrate-sensitive component associated with the plasma membrane-enriched fraction (Fig.4.3b).

Sixty per cent of the ATPase activity associated with the plasma membrane-enriched fraction was sensitive to vanadate at $100x10^{-3}$mol m^{-3} and remained constant in regions 1 to 111 (Fig.4.4). In region 1V this sensitivity increased to 90%. Vanadate-sensitive ATPase (Fig.4.5a) steadily increased basipetally to region 111 and increased further in region 1V. The level of vanadate-resistant ATPase associated with the same regions (Fig.4.5b) rose similarly to region 111, declining markedly in region 1V. Nitrate and vanadate sensitivity was examined in three further experiments, and the same trend was found in each.

4.2.3 Pyrophosphatase activity associated with different regions of the hypocotyl.

Specific pyrophosphatase activity associated with the regions of the hypocotyl was measured in the tonoplast-enriched fractions 1 and 2 in one experiment (Fig.4.6). PPase specific activity was highest in the region

Figure 4.2a. ATPase specific activity in interfaces 1 (▲) and 4 (■) from the sucrose gradient, with tissue taken from the five regions of the hypocotyl 1A, 1B, 11, 111, 1V. The standard ATPase assay was used and carried out as outlined in "Materials and Methods". Each assay was performed in triplicate and a mean taken. Experiments were repeated at least twice. Result represented is the mean of two experiments.

Figure 4.2b. Percentage stimulation of Mg^{2+}-dependent ATPase activity by 50mol m^{-3} KCl in interfaces 1 (▲) and 4 (■) of a sucrose gradient, with tissue taken from regions 1A, 1B, 11, 111, 1V of the hypocotyl. The standard ATPase mixture was used in the absence and presence of 50mol m^{-3} KCl, and the change in ATPase activity expressed as a percentage. Results represented are the mean of two experiments.

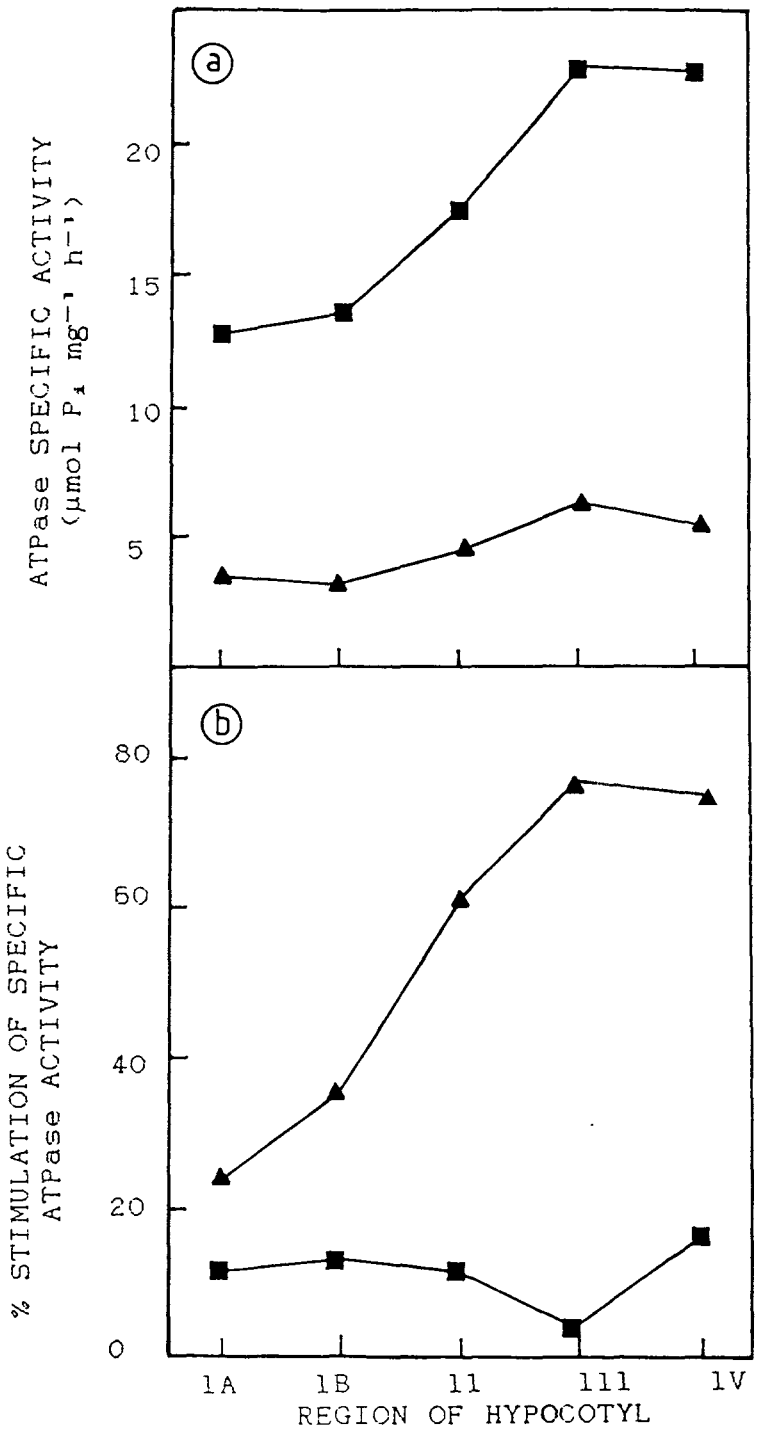
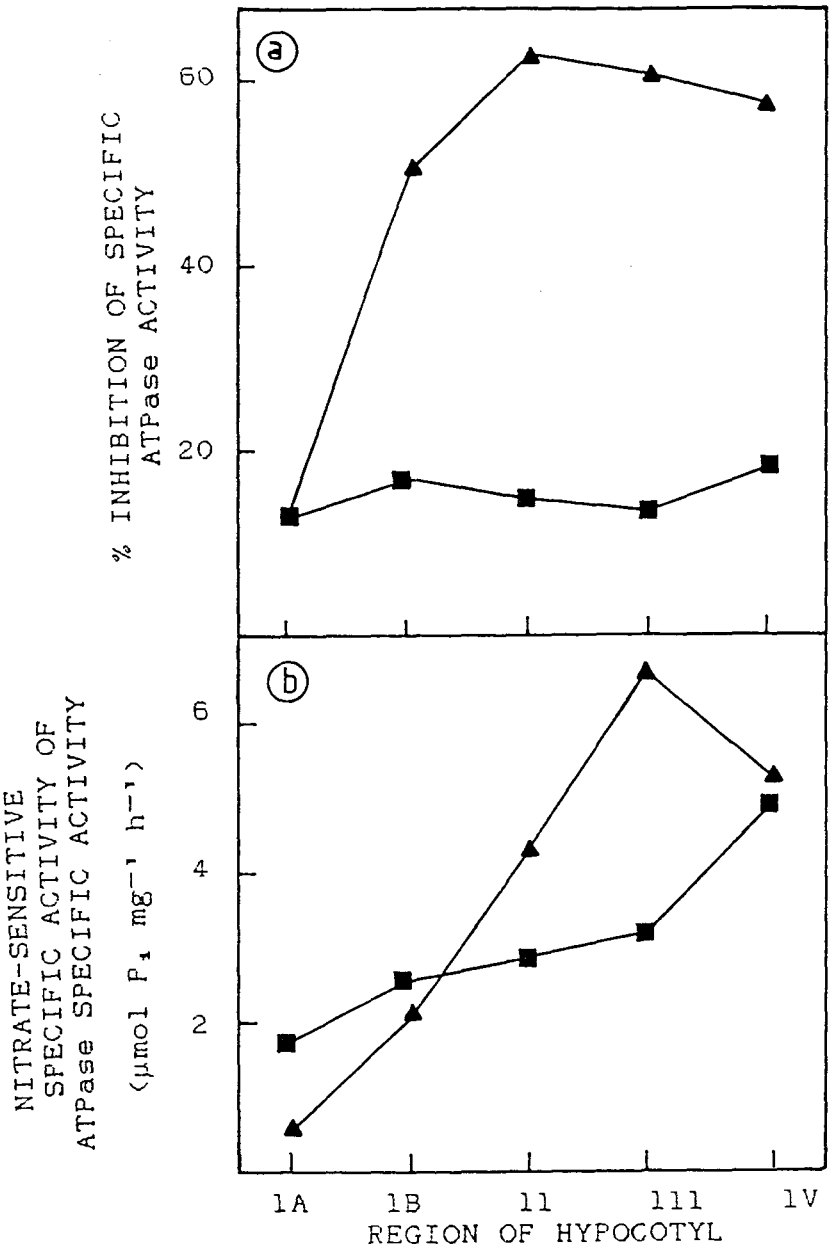

a
b
ATPase SPECIFIC ACTIVITY
(µmol P_i mg^{-1} h^{-1})
% STIMULATION OF SPECIFIC
ATPase ACTIVITY
20
15
10
5
80
60
40
20
0
1A
1B
11
111
1V
REGION OF HYPOCOTYL

Figure 4.3a. Percentage inhibition of ATPase activity by nitrate in interfaces 1 (▲) and 4 (■) with tissue taken from regions 1A, 1B, 111, 1V of the hypocotyl. In all experiments where KNO_3 was added, the standard ATPase assay was used and activity was compared with that obtained with the replacement of KCl with 50molm^{-3} KNO_3. Result is the mean of two experiments.

Figure 4.3b. Nitrate-sensitive ATPase specific activity associated with interfaces 1 (▲) and 4 (■) from regions 1A, 1B, 11, 111, 1V of the hypocotyl. Nitrate-sensitive ATPase specific activity was calculated by subtracting ATPase activity when assayed in the presence of 50mol m^{-3} KNO_3 from ATPase activity assayed in the presence of 50mol m^{-3} KCl. Result represents the mean of two experiments.


a
60
40
20
% INHIBITION OF SPECIFIC
ATPase ACTIVITY
b
6
4
2
NITRATE-SENSITIVE
SPECIFIC ACTIVITY OF
ATPase SPECIFIC ACTIVITY
(μmol Pi mg⁻¹ h⁻¹)
1A
1B
11
111
1V
REGION OF HYPOCOTYL

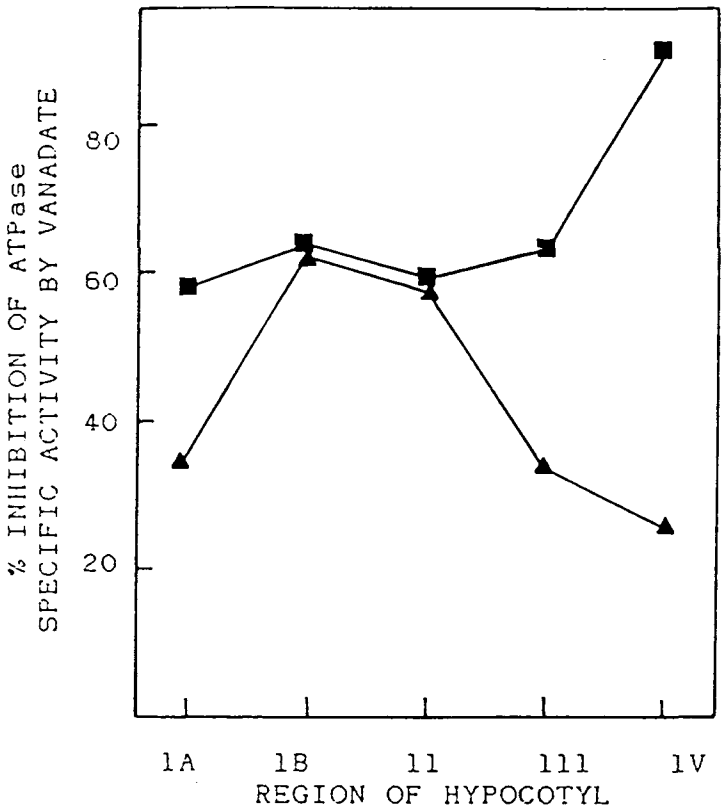


Figure 4.4. Percentage inhibition of ATPase activity by vanadate in interfaces 1 (▲) and 4 (■) from regions 1A, 1B, 111, 1V of the hypocotyl. In this and subsequent experiments, the standard ATPase assay was used and activity compared with that obtained when assayed with the addition of $50 \times 10^{-3} mol m^{-3}$ vanadate to the assay. Result is the mean of two experiments.

Figure 4.5a. Vanadate-sensitive ATPase specific activity associated with interfaces 1 (▲) and 4 (■) from regions 1A, 1B, 11, 111, 1V of the hypocotyl. Vanadate sensitive ATPase activity was calculated by subtracting ATPase activity when assayed in the presence of $50x10^{-3}$mol m^{-3} vanadate from ATPase activity assayed with the standard ATPase assay as outlined in "Materials and Methods". Data represents the mean of two experiments.

Figure 4.5b. Vanadate insensitive ATPase specific activity of interface 4 (■) from regions 1A, 1B, 11, 111, 1V of the hypocotyl. Vanadate insensitive ATPase activity is the activity remaining after the subtraction of the vanadate sensitive component. Vanadate was included at a concentration of $50x10^{-3}$mol m^{-3} in the standard ATPase assay as outlined in "Materials and Methods". Data represents the mean of two experiments.

SPECIFIC ACTIVITY OF VANADATE-INSENSITIVE
ATPase SPECIFIC ACTIVITY (µmol Pi mg-1 h-1)
1
2
3
4
b
SPECIFIC ACTIVITY OF VANADATE-SENSITIVE
ATPase SPECIFIC ACTIVITY (µmol Pi mg-1 h-1)
5
15
25
a
1A
1B
11
111
1V
REGION OF HYPOCOTYL

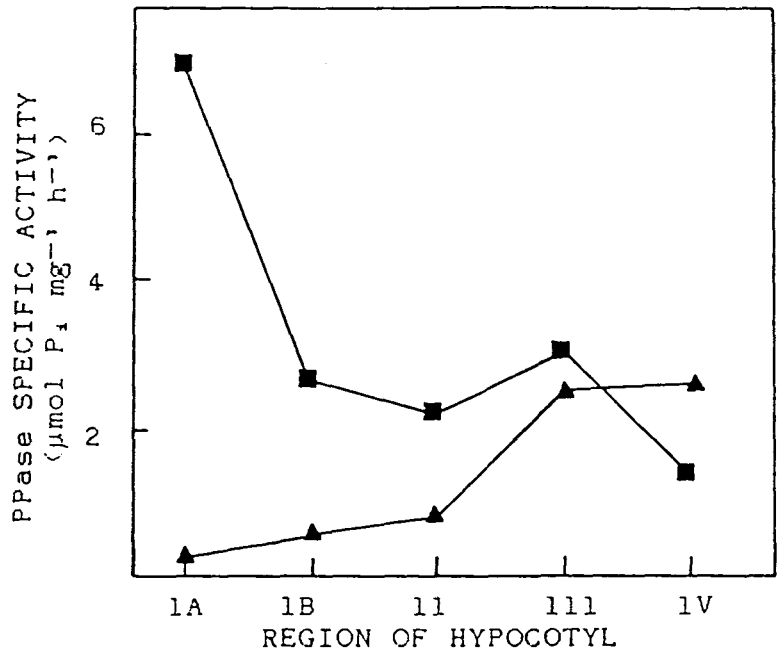


Figure 4.6. Pyrophosphatase specific activity associated with interfaces 1 (▲) and 2 (■) and prepared from regions 1A, 1B, 11, 111, 1V of the hypocotyl. The standard assay was used as outlined in "Materials and Methods".

adjacent to the cotyledons with activity decreasing basipetally.

4.2.4 pH profile of ATPase activity associated with different regions of the hypocotyl.

ATPase activity was examined over a range of pH values in microsomal fractions prepared from regions 1A and 1V in two experiments (Fig.4.7a,b). The optima for both regions were rather broad, but a small difference was apparent. The optimum for region 1A was pH 6.5, while that for region 1V was pH 6.75. Potassium-stimulated ATPase activity in region 1A and 1l was examined over the similar pH range (Fig.4.8). Stimulation of the ATPase was similar in the two regions at pH 6.25 and above, but below this pH, region 1l of the hypocotyl was stimulated by KCl to a greater extent than region 1A.

Levels of protein associated with interfaces 1 and 4 varied from the different regions 1A, 1 , 1l, 1ll, 1V (Fig.4.9a). Protein associated with interface 1 was higher when the tissue was taken from the upper regions of the hypocotyl. The protein concentration associated with interface 4 was lower than that associated with interface 1, and a protein peak was exhibited in the fraction prepared from the hypocotyl region 1B. ATPase activity associated with interfaces 1 and 4 from regions 1A, 1B, 2, 3, 4 of the hypocotyl (Fig.4.9b) was found to differ from the activity when expressed on a protein basis. Total activity was higher in region 1B than the others when membranes were taken from interface 1. The total ATPase activity associated with membranes taken from interface 4 was highest in region 1l of the hypocotyl.

An investigation was made to relate ATPase activity to the surface area of the plasma membrane. Total cell area was calculated per 10 mm segments from regions 1 and 1l using thick longitudinal and transverse sections (Figs.4.10a, 4.10b, 4.10c & 4.10d). Epidermal and stelar tissues were excluded from the calculations. The individual cell surface area increased four-fold from region 1 to region 1l, but the total cell surface area in a 10 mm segment showed only a small increase in region 1l between these regions (Table 4.1).

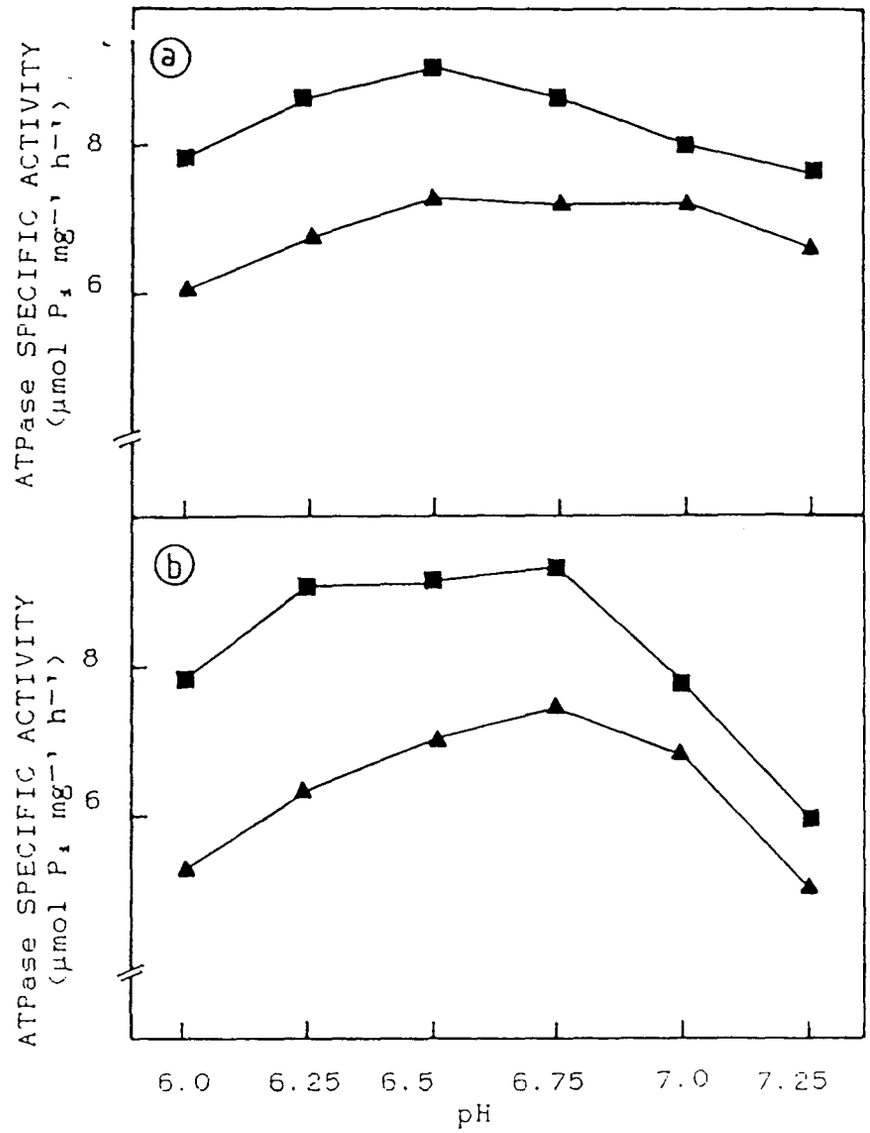


Figure 4.7a. pH profile of ATPase specific activity associated with a microsomal fraction prepared from region 1A of the hypocotyl. The standard ATPase assay was used without the inclusion of KCl (▲) and with 50mol m^{-3} KCl (■). Result represents the mean of two experiments.

Figure 4.7b. pH profile of ATPase specific activity associated with a microsomal fraction prepared from region 1V of the hypocotyl. The standard ATPase assay was used without (▲), and with the inclusion of 50mol m^{-3} KCl (■). Result represents the mean of two experiments.

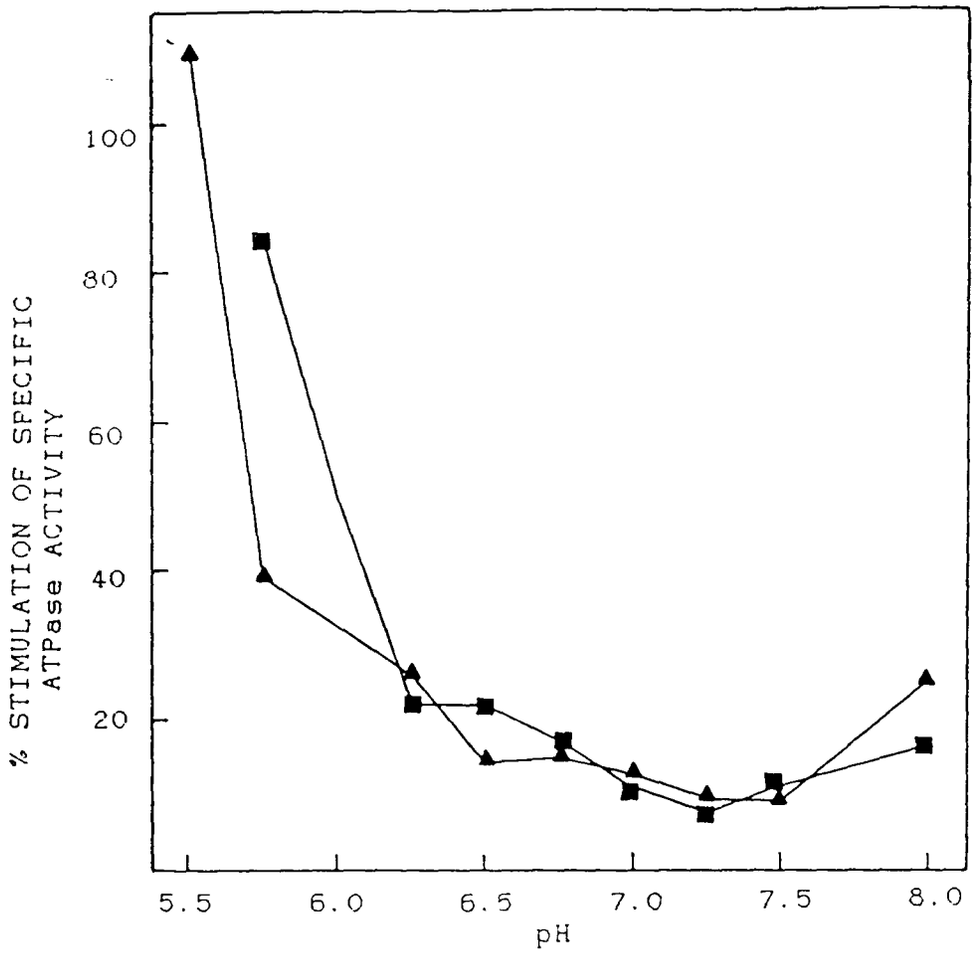
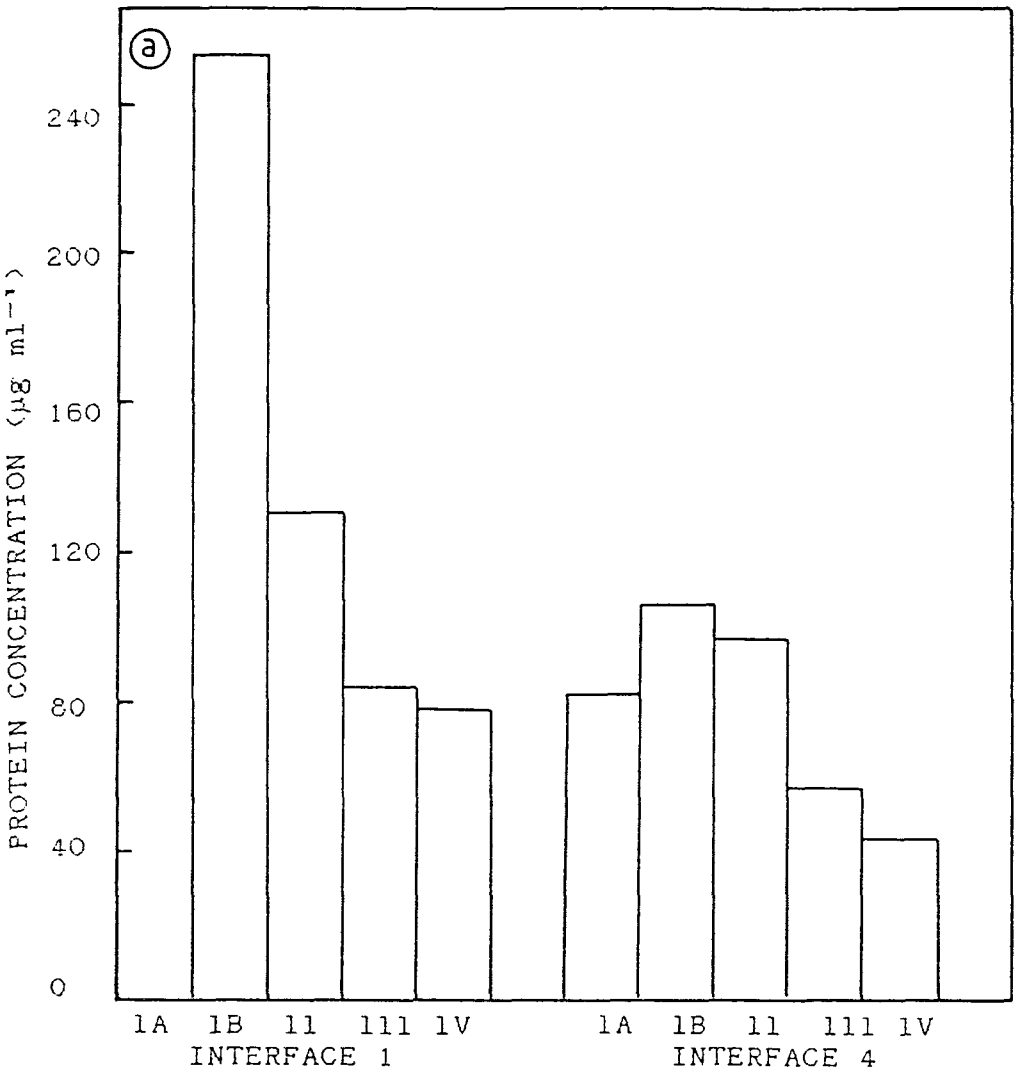


Figure 4.8. Percentage stimulation of ATPase activity associated with a microsomal fraction by KCl at various pH values in regions 1A (▲) and 11 (■) of the hypocotyl. The standard assay medium was used without and with 50mol m^{-3} KCl and potassium stimulated ATPase activity represented as a percentage. Result represents the mean of two experiments.

Figure 4.9a. Protein concentration associated with interfaces 1 and 4 prepared from regions 1A, 1B, 11, 111, 1V of the hypocotyl. 160 segments were cut from each region of the hypocotyl.

Figure 4.9b. Total ATPase specific activity associated with interfaces 1 and 4 prepared from regions 1A, 1B, 11, 111, 1V of the hypocotyl. The standard ATPase assay was used.


a
PROTEIN CONCENTRATION (μg ml⁻¹)
240
200
160
120
80
40
0
1A 1B 11 111 1V
INTERFACE 1
1A 1B 11 111 1V
INTERFACE 4

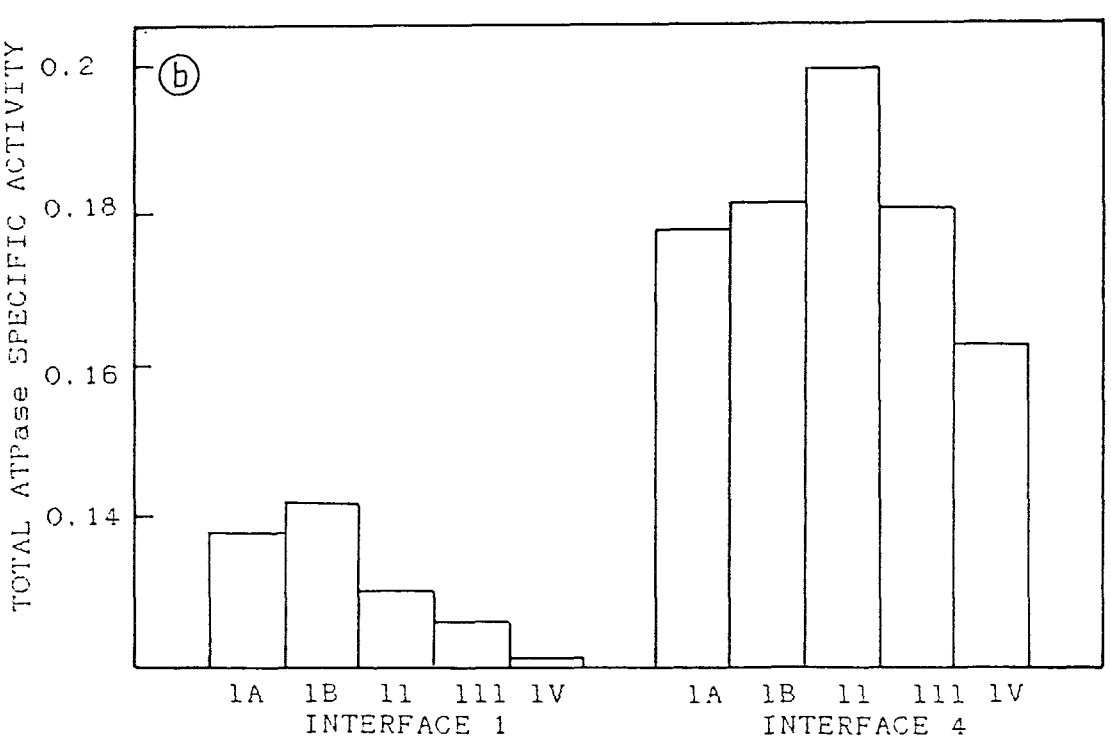

b
TOTAL ATPase SPECIFIC ACTIVITY
0.2
0.18
0.16
0.14
1A 1B 11 111 1V
INTERFACE 1
1A 1B 11 111 1V
INTERFACE 4

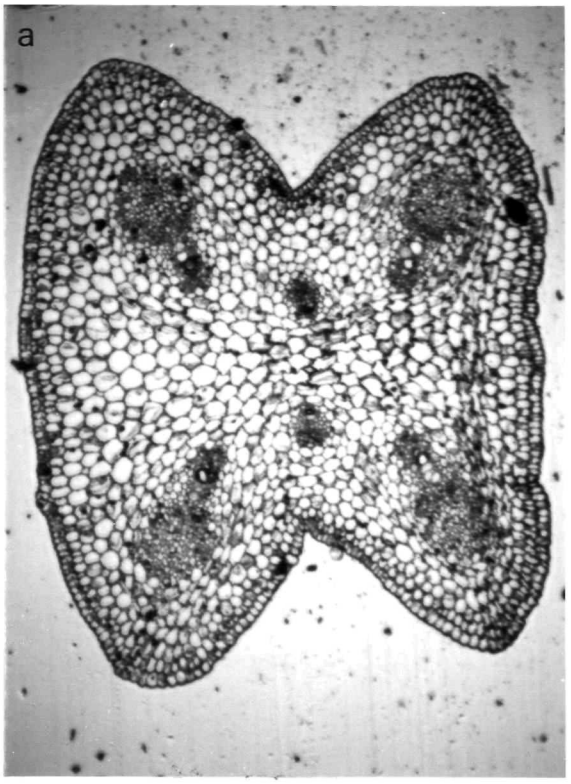
Figure 4.10a. Transverse thick section from region I of the hypocotyl.

Figure 4.10b. Longitudinal thick section from region I of the hypocotyl.

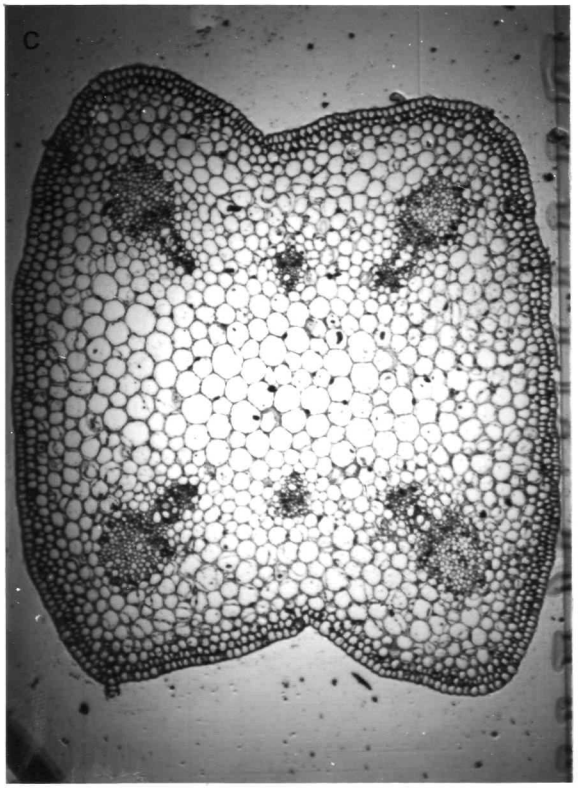
Figure 4.10c. Transverse thick section from region II of the hypocotyl.

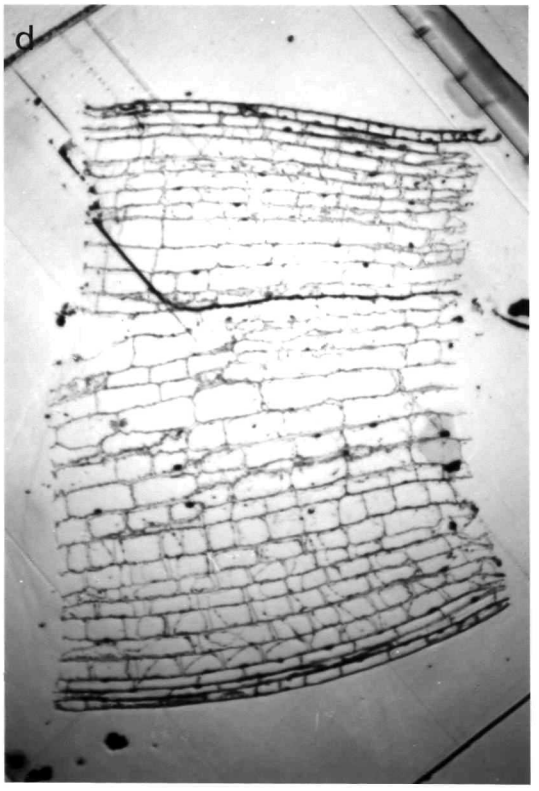
Figure 4.10d. Longitudinal thick section from region II of the hypocotyl.

(Approximate magnification of all sections X36)


a


b


c


d

	REGION	
	1	11
Average cell diameter (mm)	0.02	0.04
Average cell length (mm)	0.02	0.07
Total surface area of cell (mm^2)	0.0021	0.0096
Total surface area of one layer of cells (mm^2)	2.32	9.20
Number of cell layers per 10mm	492.6	141.5
Total surface area of cells in 10mm hypocotyl (mm^2)	1143.1	1302.6

Table 4.1. Total surface area of cells in a 10 mm hypocotyl section excluding epidermis and stelar tissue taken from regions 1 and 11.

4.2.5 ATPase activity associated with cortical and epidermal tissues.

ATPase specific activity associated with the cortical and epidermal tissues of the hypocotyl in tonoplast- and plasma membrane-enriched fractions was examined in one experiment (Fig.4.11a,b,c). The enzyme activity in the plasma membrane-enriched fraction prepared from the cortical tissue was five times higher than that in the same fraction prepared from epidermal tissue. Stimulation of this ATPase by KCl was greater than the stimulation by the epidermal associated ATPase (Table 4.2). Stimulation of both tissues by KCl was higher in the tonoplast-enriched fractions. Inhibition of the cortical ATPase by nitrate was twice the inhibition achieved with the epidermal ATPase. Vanadate (10^{-1}mol m^{-3}) totally inhibited the epidermal ATPase activity, but only inhibited the cortical and vascular associated enzyme by 66%.

4.2.6 Effect of IAA *in vivo* and *in vitro* on ATPase activity in enriched membrane fractions.

In six experiments, application of IAA *in vitro* was found to have no effect on ATPase activity in the concentration range 1mol m^{-3} - 10^{-3}mol m^{-3} when the fractions were prepared from different regions of the hypocotyl that had been given no auxin or water pretreatment. However, when tissue was used from the apical region of the hypocotyl and incubated in distilled water for 3 h, IAA *in vitro* was found to be stimulatory to ATPase activity. The effect of IAA on ATP-driven H^+-transport in isolated vesicles was also examined by monitoring the fluorescence change of quinacrine. At low ATP concentrations (0.5mol m^{-3}), IAA (8×10^{-3}mol m^{-3}) stimulated pumping in two experiments (Fig.4.12). On the third occasion, IAA had no effect on H^+ transport.

Excised hypocotyls (regions I-V) when incubated *in vivo* in a range of IAA concentrations for 2½ h and enriched membrane fractions prepared. In these fractions it was found that IAA altered ATPase activity in each of the gradient interfaces in two experiments (Fig.4.13). IAA (10^{-3}mol m^{-3}) caused up to 26% stimulation of ATPase activity in interfaces 3 and 4.

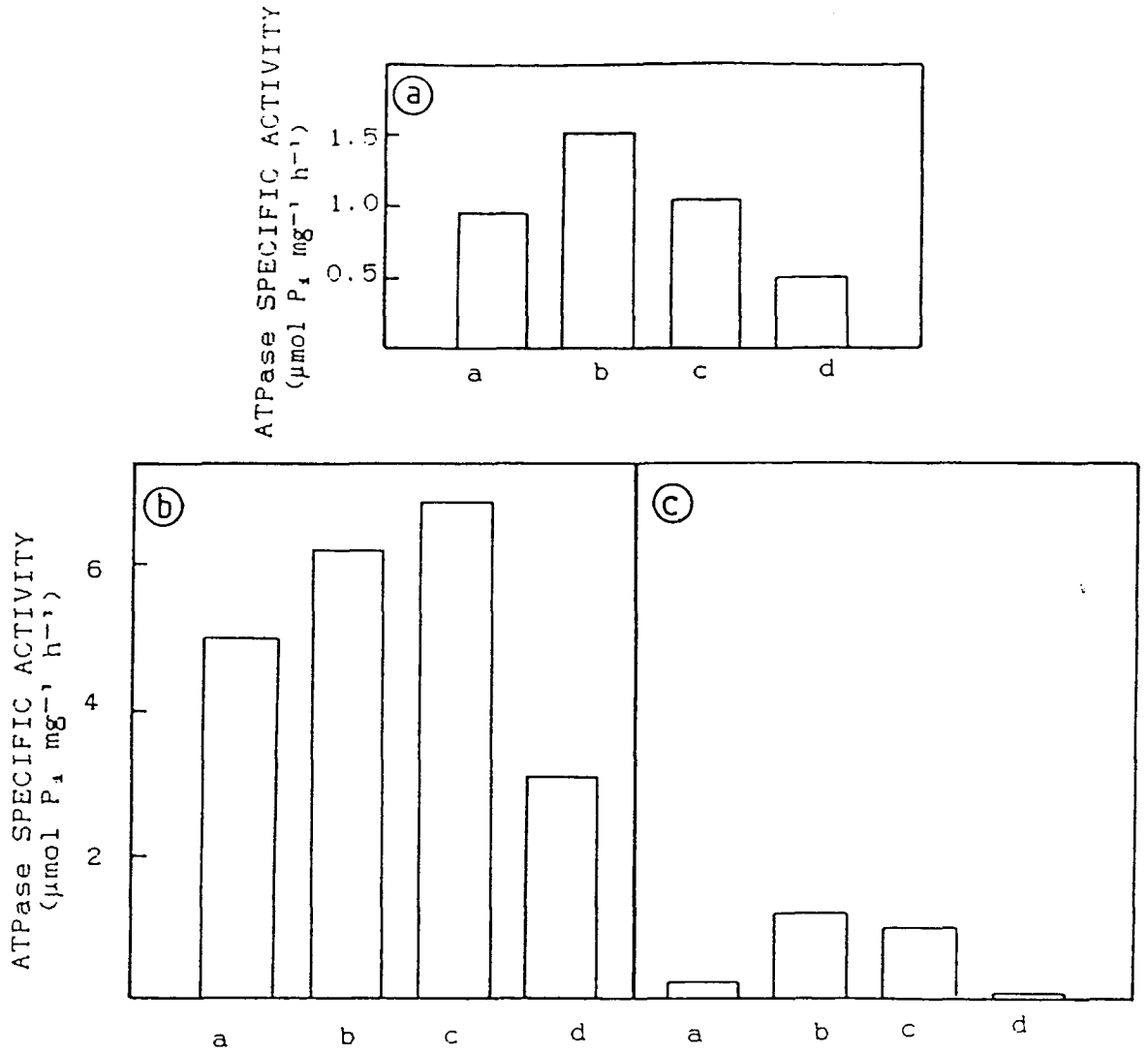


Figure 4.11a. ATPase specific activity of cortical hypocotyl tissue associated with in interface 1. The standard ATPase assay was used (a) without the inclusion of KCl, (b) 50mol m^{-3} KCl, (c) 50 mol m^{-3} KNO_3 replacing KCl, (d) $50x10^{-3}$mol m^{-3} vanadate.

Figure 4.11b. ATPase specific activity of cortical hypocotyl tissue associated with interface 4. The standard ATPase assay was used (a) without the inclusion of KCl, (b) 50mol m^{-3} KCl, (c) 50mol m^{-3} KNO_3 replacing KCl, (d) $50x10^{-3}$mol m^{-3} vanadate. Result represents the mean of two experiments.

Figure 4.11c. ATPase specific activity from epidermal hypocotyl tissue associated with interface 4. The standard ATPase assay was used (a) without the inclusion of KCl, (b) 50mol m^{-3} KCl, (c) 50mol m^{-3} KNO_3 replacing KCl, (d) $50x10^{-3}$mol m^{-3} vanadate.

Interface	Cortex 1	Cortex 1V	Epidermis 1V
KCl (50mol m^{-3})	+52.6	+13.7	+401
Nitrate (50mol m^{-3})	-31.5	+12.0	-17.1
Vanadate ($50x10^{-3}$mol m^{-3})	-65.9	-66.0	-100

Table 4.2. Percentage inhibition/stimulation of ATPase activity of cortical and epidermal tissue by nitrate and vanadate. The standard ATPase assay was used with and without the incorporation of KCl and with 50mol m^{-3} KNO_3 and $50x10^{-3}$mol m^{-3} vanadate.

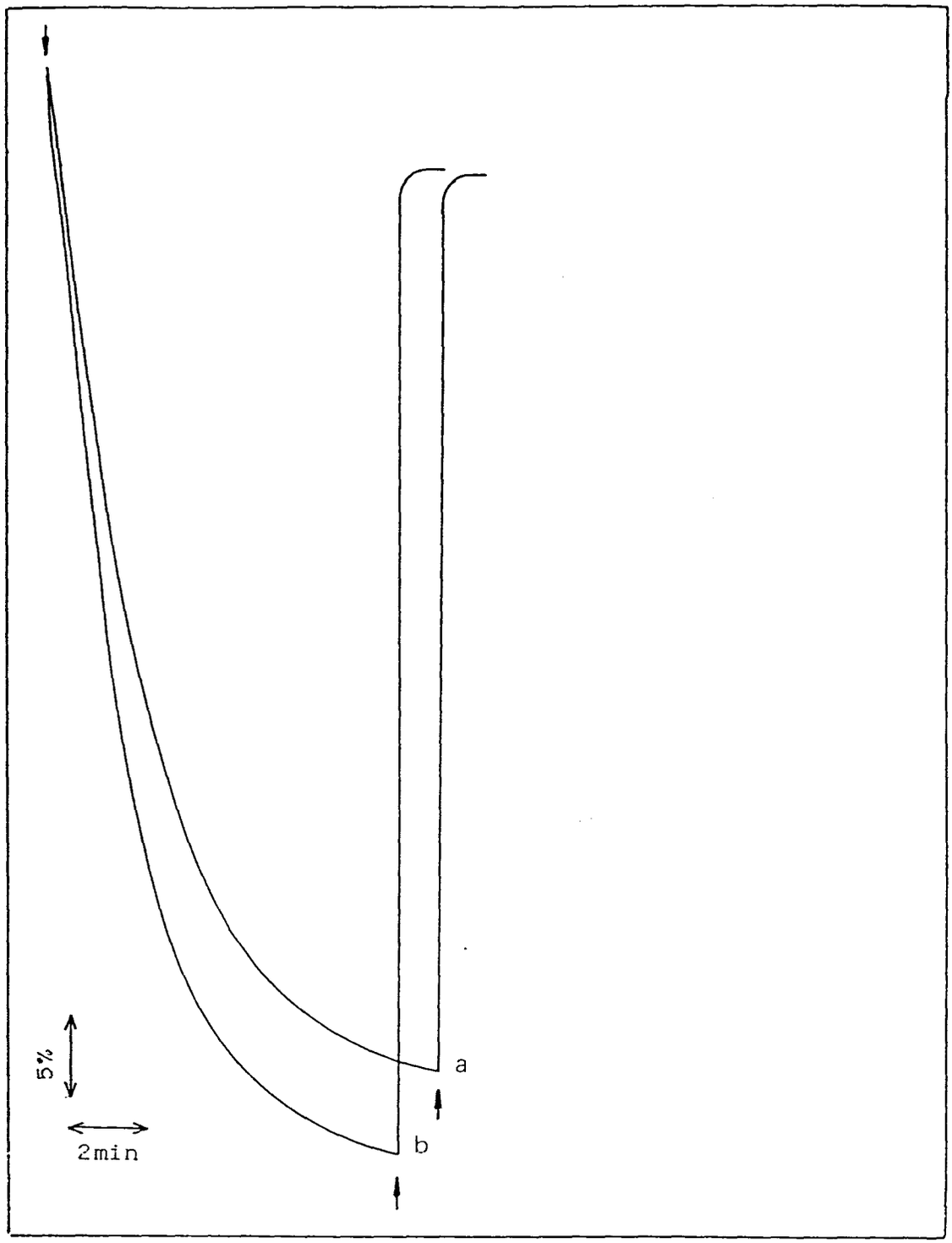


Figure 4.12. Effect of IAA on ATP-dependent H^+ transport in membrane vesicles from interface 4 of the sucrose gradient. The standard (a) quinacrine assay was used with ATP-BTP at a concentration of 0.5mol m^{-3}, with (b) the inclusion of $8x10^{-3}$mol m^{-3} IAA. The reaction was initiated by the addition of ATP (downward arrow) and reversed by the addition of gramicidin (upward arrow).

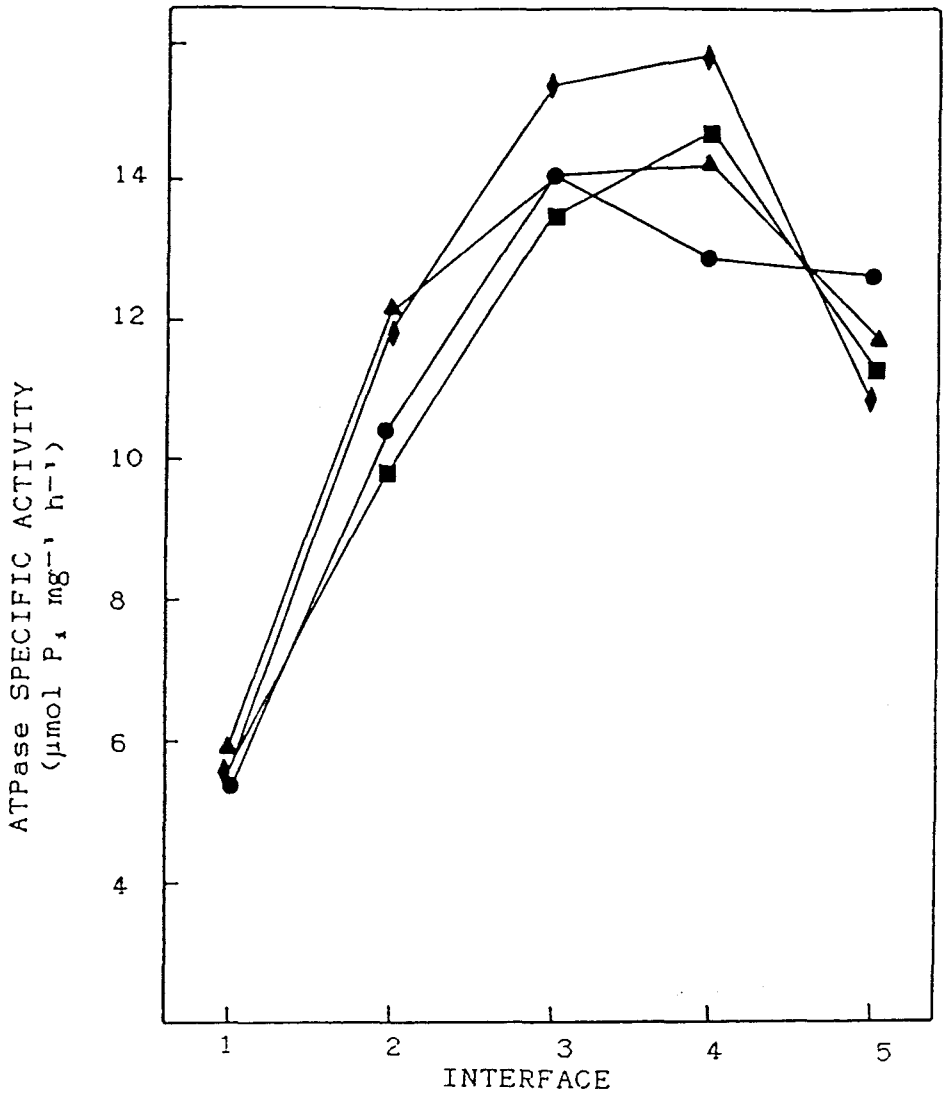


Figure 4.13. Effect of incubating tissue *in vivo* in IAA on ATPase specific activity in interfaces 1 - 5. Tissue was incubated for 2½ h in distilled water (●), 10^{-1}mol m^{-3} IAA (▲), 10^{-3}mol m^{-3} IAA (◆) and 10^{-5}mol m^{-3} IAA (■). ATPase assay was measured with the standard assay. Result represents the mean of two experiments.

In two further experiments, tissue was preincubated for 3½ h in IAA (10^{-1}mol m^{-3}) and little further change in ATPase activity occurred. In three further experiments, Mg and K^+-ATPase activity was examined following an *in vivo* incubation in IAA (10^{-1} mol m^{-3}) for 11 h (Fig.4.14a). Increased ATPase activity was observed in interfaces 3, 4 and 5.

KCl stimulation of ATPase activity was enhanced in interfaces 4 and 5 following an IAA pre-treatment *in vivo* (Fig.4.14b). Following pre-treatment of the tissue *in vivo* in IAA, ATPase activity was examined at 37°C and 25°C (Fig.4.15a), the latter being nearer the optimum for growth in *Cucumis*. At 37°C ATPase activity was stimulated by 36% and 35% in interfaces 3 & 4 respectively and 32% and 30% respectively at 25°C. KCl stimulation of the ATPase *in vitro* was enhanced by IAA pretreatment in fractions from interfaces 4 & 5, with this effect being greater at 25°C (Fig.15b). The experiment was repeated twice and on each occasion ATPase activity associated with interface 4 was stimulated to the greatest extent. The lower incubation temperature did not stimulate activity further, and in subsequent experiments only interface 4 was examined, and incubations were made at 37°C.

Following an *in vivo* incubation in 10^{-3}mol m^{-3} IAA the effect of IAA *in vitro* on ATPase activity was examined in three experiments (Figure 4.16a). In auxin-depleted tissue, IAA *in vitro* was found to be stimulatory at 10^{-6} to 10^{-7}mol m^{-3} concentration. ATPase activity in tissue that had been incubated in IAA *in vivo* for 2½ h was greater than that of the control. Addition of IAA *in vitro* at 10^{-1} to 10^{-7}mol m^{-3} further stimulated ATPase activity.

4.2.7 Effect of phenyl acetic acid on ATPase activity.

ATPase activity in interface 4 was examined from a tissue preparation that had been incubated *in vivo* with PAA (10^{-1}mol m^{-3}) for 2½ h (Fig.4.16b). ATPase activity in membrane fraction 4 following this incubation showed an increase in ATPase activity compared with that of the control. In tissue that had been given a water pretreatment *in vivo* addition of PAA (10^{-1} to 10^{-7}mol m^{-3})

Figure 4.14a. Effect of incubating tissue *in vivo* in IAA on ATPase specific activity in interfaces 1 - 5. Tissue was incubated for 11 h in distilled water (▲■), 10^{-1}mol m^{-3} IAA (●◆). ATPase assay was carried out with the standard assay in the absence of KCl (▲●) and in its presence (■◆). Result represents the mean of two experiments.

Figure 4.14b. Effect of a 11 h incubation of tissue in IAA on potassium stimulated ATPase specific activity in interfaces 1 - 5. Tissue was incubated in distilled water (▲), 10^{-1}mol m^{-3} IAA (●). ATPase assay was carried out with the standard assay as described in "Materials and Methods" but in the absence of KCl and with 50mol m^{-3} KCl. The percentage increase in ATPase activity was calculated.

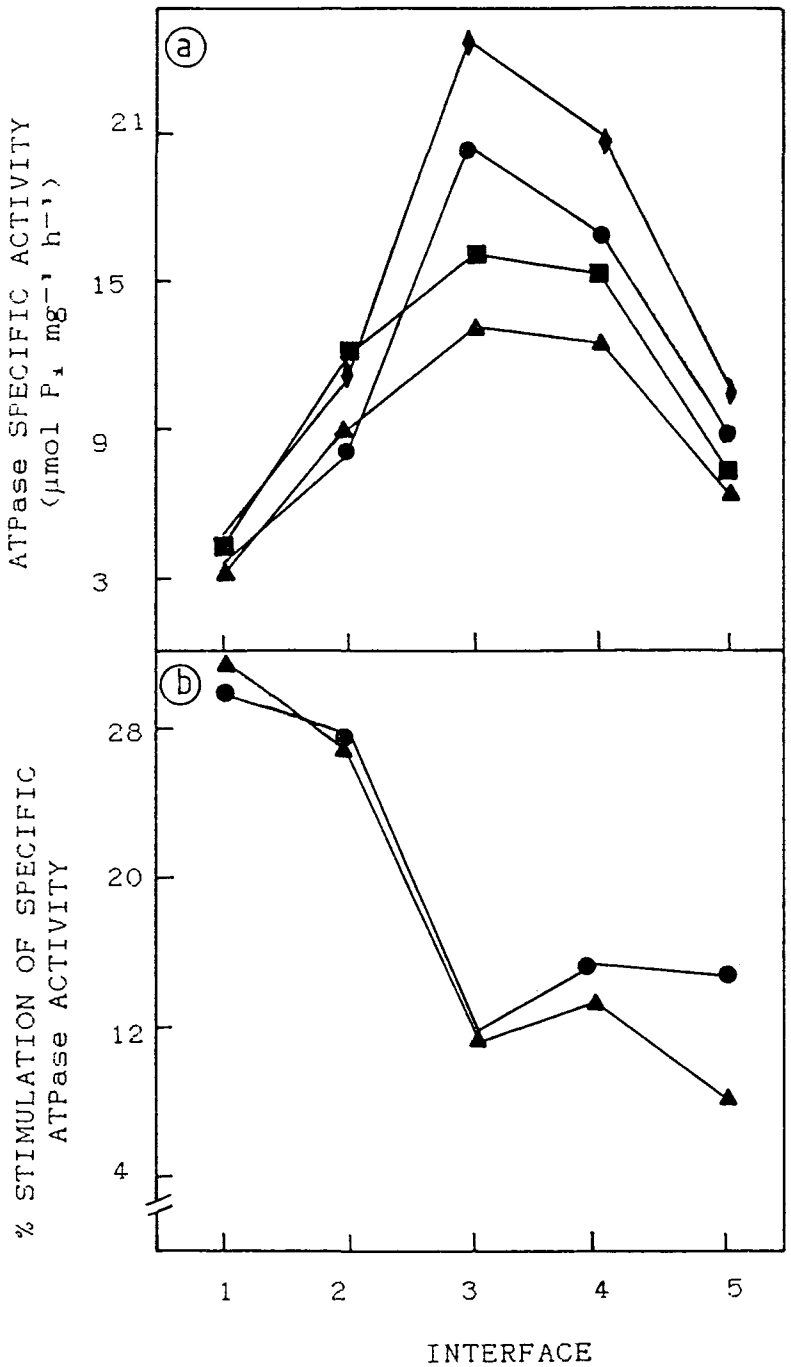

a
b
ATPase SPECIFIC ACTIVITY
(μmol P_i mg^{-1} h^{-1})
% STIMULATION OF SPECIFIC
ATPase ACTIVITY
21
15
9
3
28
20
12
4
1
2
3
4
5
INTERFACE

Figure 4.15a. Effect of incubation temperature on ATPase activity prepared from tissue that had been incubated for 2½ h in IAA. Interfaces 1 - 5 were examined. Tissue was incubated *in vivo* in distilled water (▲■), or 10^{-1}mol m^{-3} IAA (●◆). ATPase assay was carried out with the standard assay at 37°C in the absence of KCl (▲●) and with 50mol m^{-3} KCl (■◆). ATPase assay was also carried out at 25°C with tissue that had been incubated in distilled water (△□), or 10^{-1}mol m^{-3} IAA (○◊). The assay was carried out without KCl (△○) and with 50 mol m^{-3} KCl (□◊). Result represents the mean of two experiments.

Figure 4.15b. Percentage stimulation of ATPase activity following an *in vivo* incubation in distilled water (◆■), or 10^{-1}mol m^{-3} IAA (●▲). ATPase specific activity was measured using the standard assay mixture at 37°C (◆●) and 25°C (▲■).

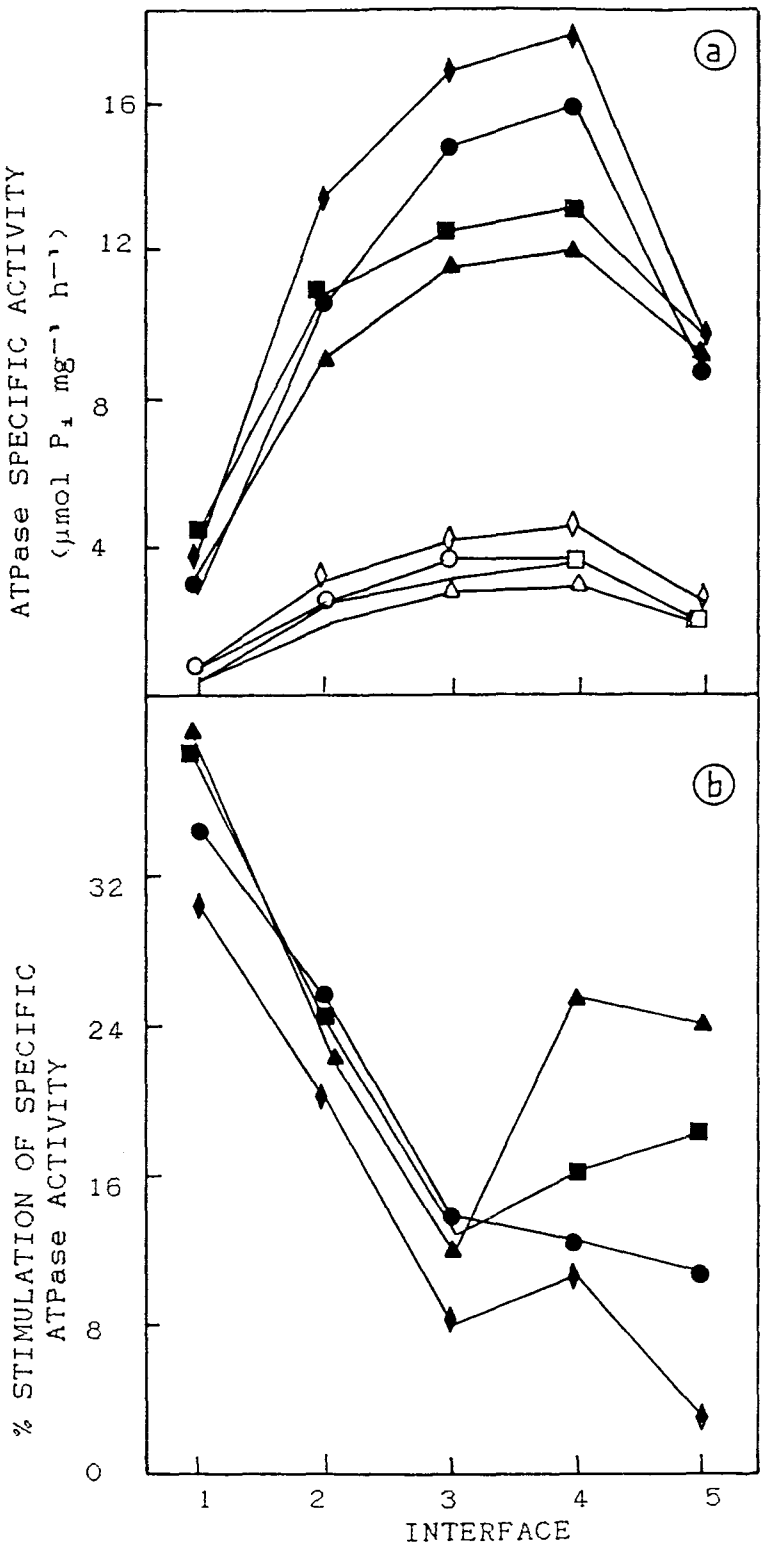
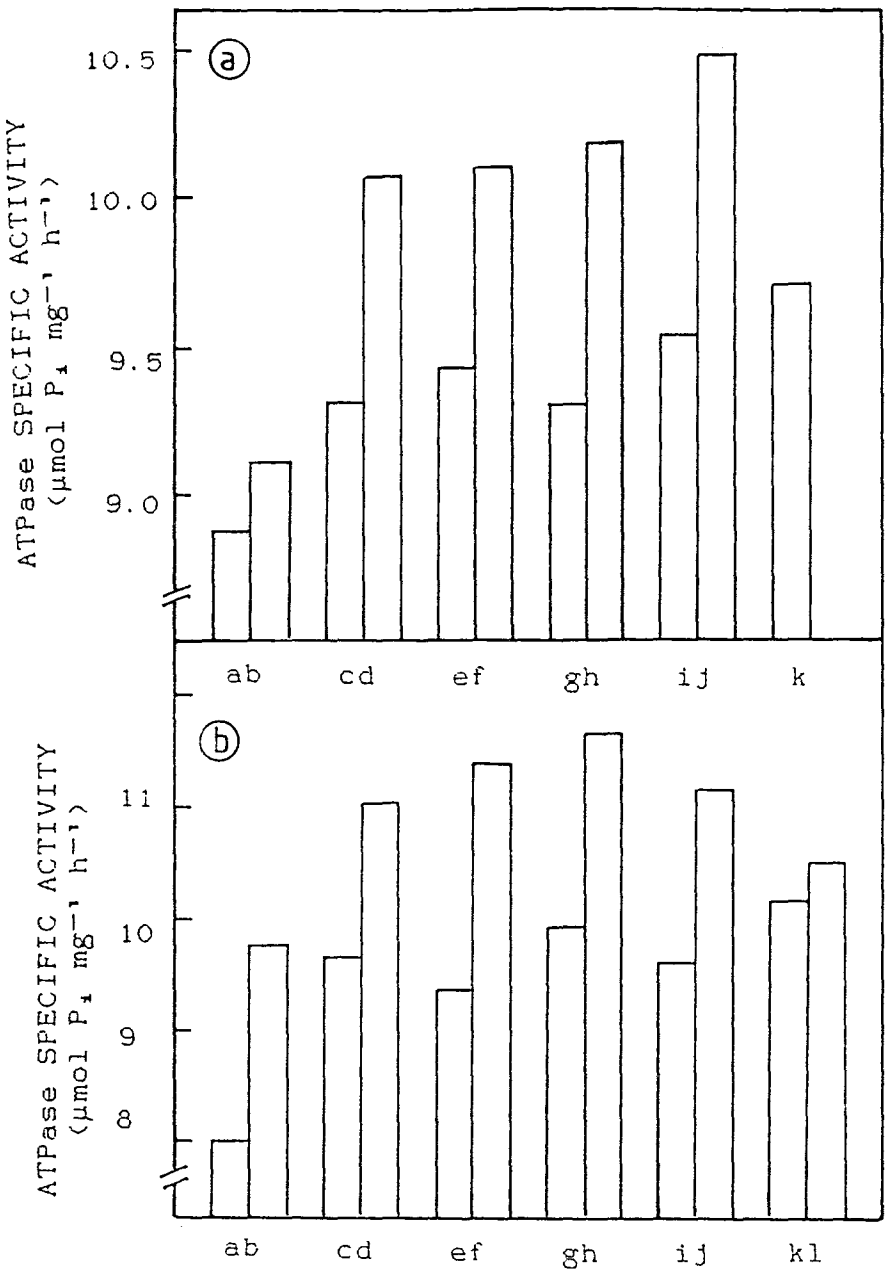

a
b
ATPase SPECIFIC ACTIVITY
(μmol P_i mg^{-1} h^{-1})
16
12
8
4
% STIMULATION OF SPECIFIC
ATPase ACTIVITY
32
24
16
8
0
1
2
3
4
5
INTERFACE

Figure 4.16a. Effect of IAA *in vitro* on ATPase specific activity from tissue that had been IAA pretreated. Tissue was incubated for 2½ h in (a,c,e,g,i,k) distilled water or (b,d,f,h,j) 10^{-3}mol m^{-3} IAA and interface 4 examined. The standard ATPase assay was used (a,b) in the absence of KCl, (c,d) with 50mol m^{-3} KCl, (e,f) 10^{-1}mol m^{-1} IAA, (g,h) 10^{-3} mol m^{-3} IAA, (i,j) 10^{-5}mol m^{-3} IAA and (k)10^{-7}mol m^{-3} IAA.

Figure 4.16b. ATPase specific activity of interface 4 prepared from tissue that had been incubated for 2½ h in (a,c,e,g,i,k) distilled water or (b,d,f,h,j,l) 10^{-1}mol m^{-3} PAA. The standard ATPase assay was used (a,b) in the absence of KCl, (c,d) in the presence of 50mol m^{-3} KCl, and (e,f) with the addition of 10^{-1}mol m^{-1} PAA, (g,h) 10^{-3}mol m^{-3} PAA, (i,j) 10^{-5}mol m^{-3} PAA and (k,l) 10^{-7}mol m^{-3} PAA.


ATPase SPECIFIC ACTIVITY
(μmol P_i mg^{-1} h^{-1})
10.5
10.0
9.5
9.0
a
ab
cd
ef
gh
ij
k
ATPase SPECIFIC ACTIVITY
(μmol P_i mg^{-1} h^{-1})
11
10
9
8
b
ab
cd
ef
gh
ij
kl

to the incubation medium *in vitro* caused a small increase in ATPase activity at some concentrations (Fig.4.16b). Tissue that had been incubated in PAA *in vitro*, PAA *in vivo* was stimulatory over the concentration range of 10^{-1} to 10^{-3}mol m^{-3}, higher concentrations of 10^{-5} to 10^{-7}mol m^{-3} inhibited activity.

The effect of IAA and PAA *in vitro* on ATPase activity following an *in vivo* incubation in IAA and PAA were examined in enriched membrane fractions from the apical hook region, 1A, in five experiments. In four cases, stimulation of activity was observed with the results of one experiment shown in Fig.4.17a; in the other no stimulation occurred. Stimulation of ATPase activity associated with interface 4 by 10^{-3}mol m^{-3} IAA *in vivo* was increased by 15% over that of the control. In the presence of K^+, incubation with PAA at 10^{-1}mol m^{-3} *in vivo* for 2½ h caused a 22% stimulation of ATPase activity associated with interface 4 over that of the control. Examination of the Mg^{2+}-, and K^+-stimulated activity of the ATPase from interface 4 indicated that IAA and PAA had similar effects on the stimulation of basal Mg-ATPase activity, but in the presence of K^+, the stimulation caused by PAA exceeded that by IAA (Fig.4.17b).

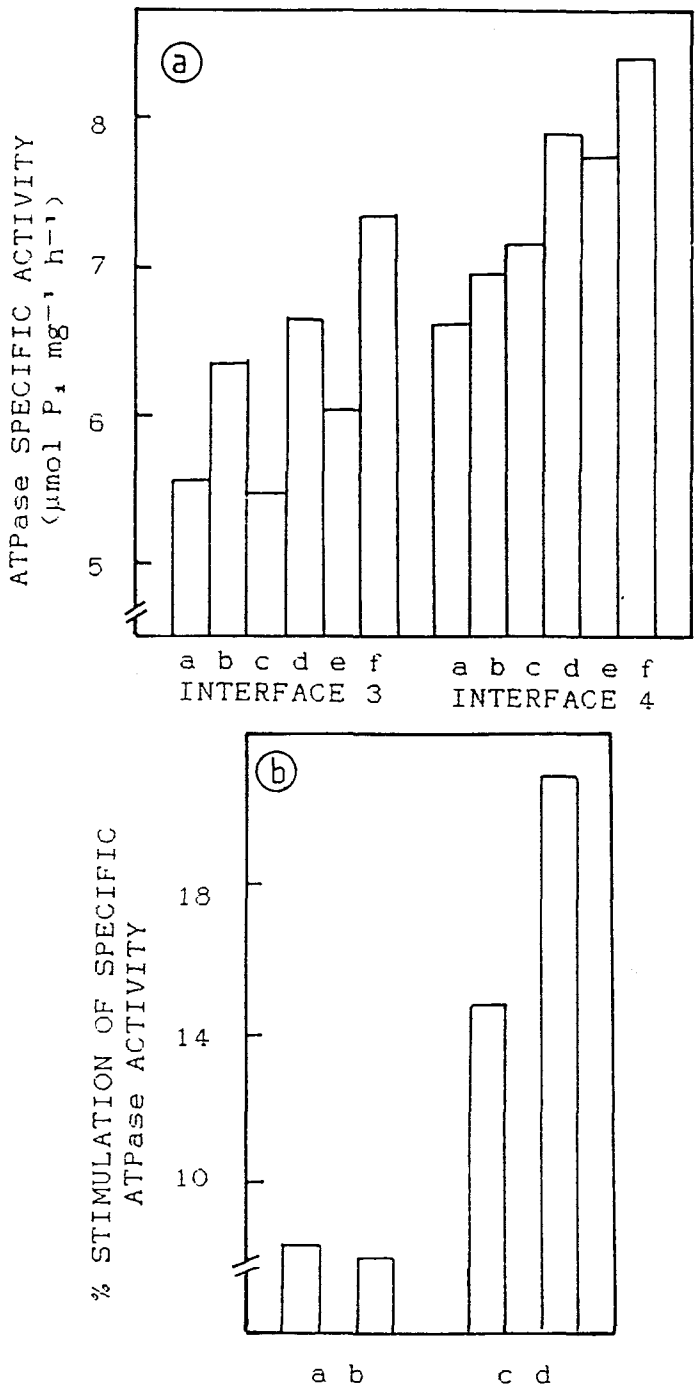
4.2.8 Effect of plant hormones on ATPase activity in purified membrane fractions from different regions of the hypocotyl.

The effect of IAA (10^{-3}molm^{-3}) applied *in vivo* on the specific ATPase activity associated with interface 4 from regions 1 and 111 of the hypocotyl was examined in five experiments. The ATPase from region 1A of the hypocotyl was stimulated in the range of 5 - 35% after incubation in IAA *in vivo*. IAA stimulated ATPase activity from region 1A by 18%, and inhibited it in region 111 by 28% (Fig.4.18a).

ATPase activity associated with interface 1V prepared from complete hypocotyls was measured in one experiment following incubation of the hypocotyls in 10^{-1}mol m^{-3} 2,4-D and 10^{-3}mol m^{-3} IAA for 2½ h (Fig.4.18b). 2,4-D stimulated the ATPase to a lesser extent.

Figure 4.17a. Effect of IAA and PAA on ATPase specific activity *in vitro* associated with interfaces 3 and 4 prepared from region 1A of the hypocotyl following an *in vivo* incubation in 10^{-1}mol m^{-3} IAA for 2½ h. The standard ATPase assay mixture was used (a,c,e) without KCl, (b,d,f) with 50mol m^{-3} KCl, (c,d) 10^{-3}molm^{-3} IAA and (e,f) 10^{-1}mol m^{-3} PAA.

Figure 4.17b. Percentage stimulation of ATPase activity by IAA and PAA *in vitro*. Tissue (region 1A) was incubated in 10^{-1}mol m^{-3} IAA for 2½ h. The standard ATPase assay mixture was used (a,b) without the incorporation of KCl and (c,d) with the inclusion of 50mol m^{-3} KCl, (a,c) 10^{-3}mol m^{-3} IAA and (b,d) 10^{-1}mol m^{-3} PAA.


a
ATPase SPECIFIC ACTIVITY
(μmol P_i mg^{-1} h^{-1})
8
7
6
5
a b c d e f
INTERFACE 3
a b c d e f
INTERFACE 4
b
% STIMULATION OF SPECIFIC
ATPase ACTIVITY
18
14
10
a b
c d

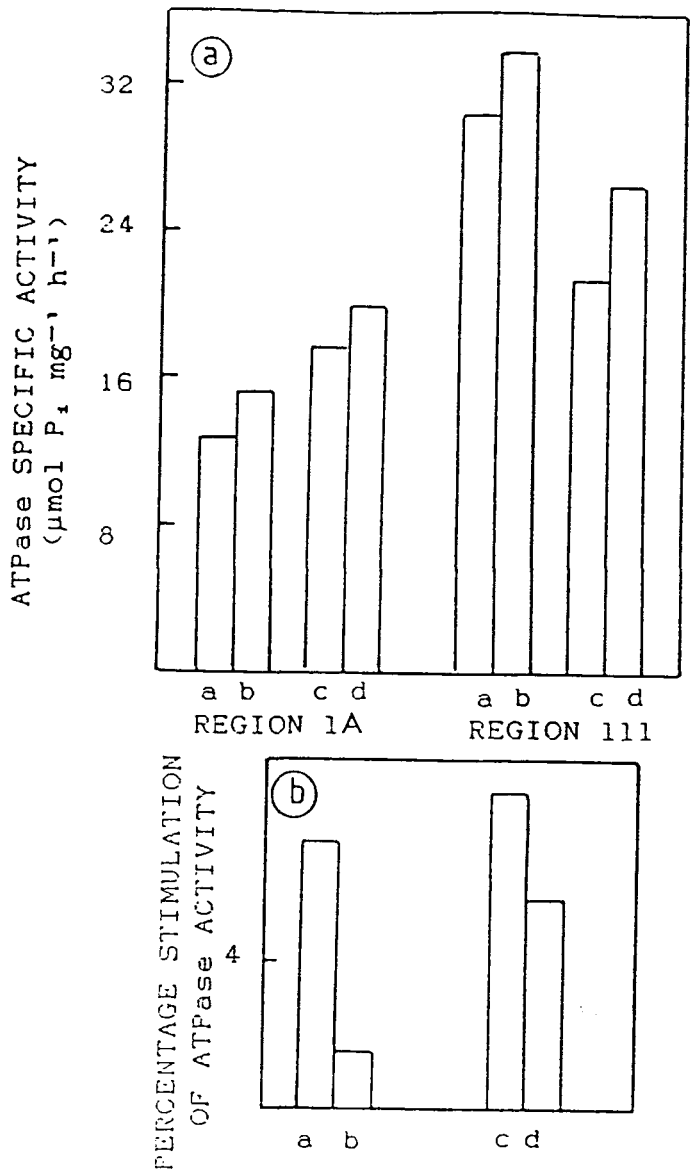


Figure 4.18a. Effect of IAA *in vitro* on ATPase specific activity in interface 4 prepared from regions 1A and 111 of the hypocotyl following an *in vivo* incubation in 10^{-1}mol m^{-3} IAA for 2½ h. The standard ATPase assay mixture was used (a,c) without the incorporation of KCl, (b,d) with 50mol m^{-3} KCl and (c,d) with 10^{-3}molm^{-3} IAA.

Figure 4.18b. Effect of IAA and 2,4-D *in vitro* on percentage stimulation of ATPase specific activity in interface 4 prepared from region 1 of the hypocotyl following an *in vivo* incubation in 10^{-1}mol m^{-3} IAA for 2½ h. The standard ATPase assay was used (a,b) without the incorporation of KCl, and (c,d) with 50mol m^{-3} KCl, (a,c) with 10^{-3}molm^{-3} IAA and (b,d) 10^{-1}mol m^{-3} 2,4-D.

4.2.9 Effect of growth regulators on hypocotyl elongation.

The effect of buffer strength and type was examined on cell elongation of hypocotyl segments. The inclusion of 40mol m^{-3} Tris-Mes buffer (pH 6.5) in the incubation medium totally inhibited auxin-stimulated elongation. When the concentration was lowered to 1 or 5mol m^{-3}, significant auxin-stimulated elongation was observed. The inclusion of 1mol m^{-3} Tris-Mes buffer made no difference to hypocotyl elongation, but with 1mol m^{-3} sodium phosphate buffer, IAA stimulated elongation was reduced by 22% (Fig.4.19). In all subsequent experiments, 5mol m^{-3} Tris-Mes buffer (pH 6.5) was used.

The effect of growth regulators on hypocotyl elongation in the 5 regions of the hypocotyl was examined using auxin-depleted tissue. Region 1 was found to have the highest potential for elongation in the presence and absence of all of the growth regulators tested (Fig.4.20a). Substantial growth was induced by IAA at concentrations of 10^{-3}mol m^{-3} and at a concentration of 10^{-1}molm^{-3} elongation was further induced (Fig.4.21a). PAA (10^{-1}mol m^{-3}) significantly induced hypocotyl elongation compared with the water control (Fig.4.20b), though to a lesser extent than that caused by IAA. 2,4-D markedly stimulated growth in the upper regions of the hypocotyl at a concentration of 10^{-1}mol m^{-3} (Fig.4.21b). A lower concentration was found to be less effective on tissue elongation in all regions.

4.2.10 Effect of auxin stimulated hypocotyl growth by SW26 and Erythrosin B.

The effects of SW26 and erythrosin B on hypocotyl elongation were examined in the presence and absence of IAA (10^{-3}mol m^{-3}) and expressed as a percentage change in hypocotyl length (Fig.4.22a, 4.22b). The data is also expressed as the percentage inhibition of elongation caused by the inhibitors over that of the auxin induced growth (Fig.4.23). Erythrosin B (50mol m^{-3}) markedly reduced non-auxin-stimulated growth. SW26 at the same concentration reduced growth in the presence and absence of IAA, but to a lesser extent than erythrosin B. Inhibition of

Figure 4.19. Effect of buffer composition on hypocotyl extension. Length was measured after 4 h incubation in (a,b,c,d) 1mol m^{-3} Tris-Mes buffer (pH 6.5) containing (a) distilled water (b) 10^{-3}mol m^{-3} IAA, (c) 10^{-3}mol m^{-3} IAA, $50x10^{-3}$mol m^{-3} SW26 (d) 10^{-3}mol m^{-3} IAA, $50x10^{-3}$mol m^{-3} erythrosin B. Percentage increase in hypocotyl length after 4 h incubation in (e.f.g.h) distilled water containing (f) 10^{-3}mol m^{-3} IAA, (g) 10^{-3}mol m^{-3} IAA, $50x10^{-3}$mol m^{-3} SW26 (h) 10^{-3}mol m^{-3} IAA, $50x10^{-3}$mol m^{-3} erythrosin B and in an incubation medium (i,j) containing 1mol m^{-3} sodium phosphate buffer (pH 6.5) containing (i) H_2O j) 10^{-3}mol m^{-3} IAA. The one way ANOVA gave an F value at time 0 h of 4.13 for groups a,b,c,d. At time 4 h, an F value of 67.87 was calculated indicating significant ($p<0.05$) differences between these groups. Duncan analysis was used to indicate significant differences between the groups and are illustrated. Groups e.f.g.h.i.j at time 0 h, F was equal to 1.336 indicating no significant difference between the groups. At time 4 h, the F value was equal to 43.5 indicating significant differences existed between the groups ($p<0.05$) and Duncan analysis was used to indicate significant differences between the groups which are illustrated,

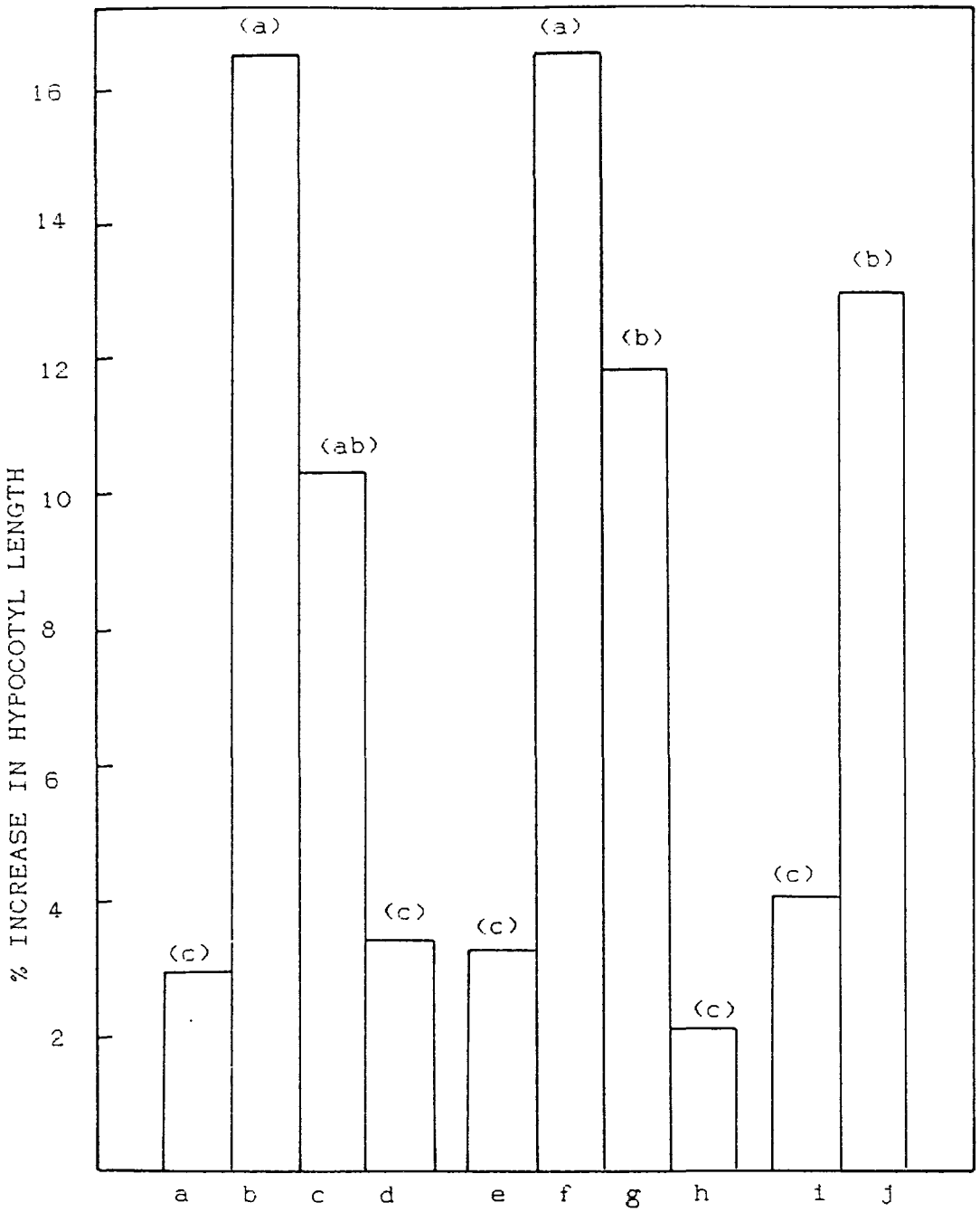

% INCREASE IN HYPOCOTYL LENGTH
16
14
12
10
8
6
4
2
(a)
(a)
(b)
(b)
(ab)
(c)
(c)
(c)
(c)
(c)
a
b
c
d
e
f
g
h
i
j

Figure 4.20a. Percentage increase in length of each region of the hypocotyl after 2½ h incubation in the standard incubation medium containing (a,c,e,g,i,k) distilled water or (b,d,f,h,j,l) 10^{-3}mol m^{-3} IAA. In this experiment 60 mm hypocotyl segments were used and were cut equally into 10 mm segments to give regions 1 (a,b), 11 (c,d), 111 (e,f), 1V (g,h), V (i,j), V1 (k,l). The one way ANOVA gave an F value of 1.93 indicating no significant ($p<0.05$) differences existed between groups (a - l) of hypocotyl segments at time 0 h. At 2½ h the calculated F ratio was equal to 24.73 ($p<0.001$) indicating that significant differences existed between groups a - l. Duncan analysis was used to indicate significant differences between the groups and are illustrated.

Figure 4.20b. Percentage increase in length of each region of the hypocotyl after 2½ h incubation in the standard incubation medium without IAA (a,c,e) and with 10^{-3}mol m^{-3} IAA (b,d,g). In this experiment 60 mm hypocotyls were used and were divided equally into 10 mm segments to give regions 1 - V1. At time 0 h, an F value of 4.63 which indicated that there was a significant difference ($p<0.05$) between the initial group lengths. Analysis was run using each region. Region 1 at time 0 h, an F value of 0.074 was calculated, indicating that no significant difference ($p<0.05$) existed between groups a & b. At time 4 h, an F value of 15.71 was calculated, which indicated that significant differences existed between the groups. Duncan analysis was used to indicated significant differences between the groups, which are illustrated. The same procedure was carried out with region 11 (groups c & d) and 111 (groups e & f). No significant differences were found at time 4 h.

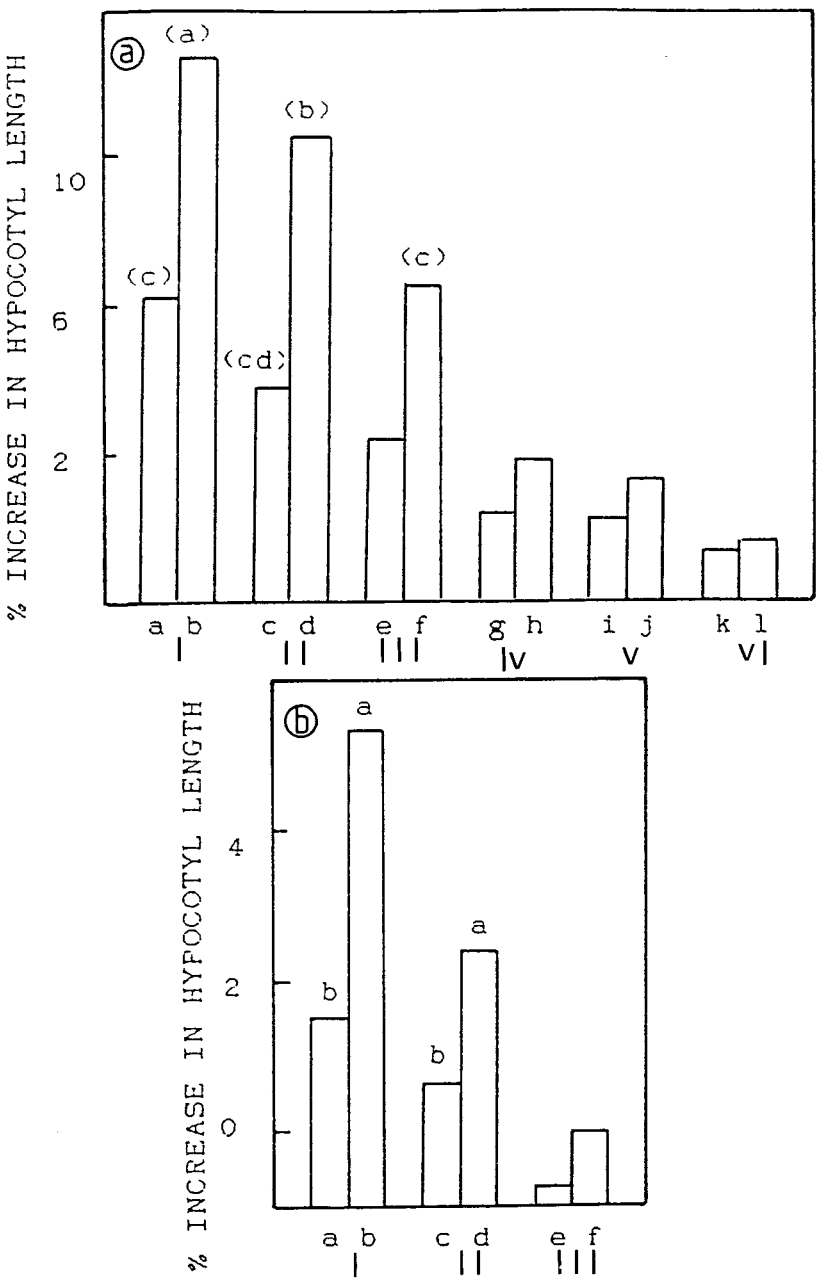

ⓐ
% INCREASE IN HYPOCOTYL LENGTH
10
6
2
(a)
(b)
(c)
(c)
(cd)
a b c d e f g h i j k l
I II III IV V VI
ⓑ
% INCREASE IN HYPOCOTYL LENGTH
4
2
0
a
b
a
b
a b c d e f
I II III

Figure 4.21a. Effect of IAA concentration on hypocotyl extension in regions 1 - V. Tissue was incubated in the standard incubation medium containing (a) no IAA, and with IAA at concentrations of (b) 10^{-1}mol m^{-3} and (c) 10^{-3}mol m^{-3}. An F ratio of 5.88 was calculated at time 0 h which indicated that large differences existed between groups at this time. F values were calculated at time 4 h and Duncan analysis was used to indicate significance differences between groups. Groups in common at time 0 and 4 h were ignored. Significant differences are illustrated.
Figure 4.21b. Percentage increase in length in regions 1 - V of the hypocotyl in a range of 2,4-D concentrations. Tissue was incubated in the standard incubation medium containing (a) no IAA, and with 2,4-D at concentrations of (b) 10^{-1}mol m^{-3} and (c) 10^{-3}mol m^{-3}. The one way ANOVA gave an F value at time 0 h of 1.611 which indicated that there was no significant differences ($p<0.05$) between the groups at this time. At time 4 h a calculated F value of 48.39 was obtained and Duncan analysis was used to indicate significant differences between the groups which are illustrated.

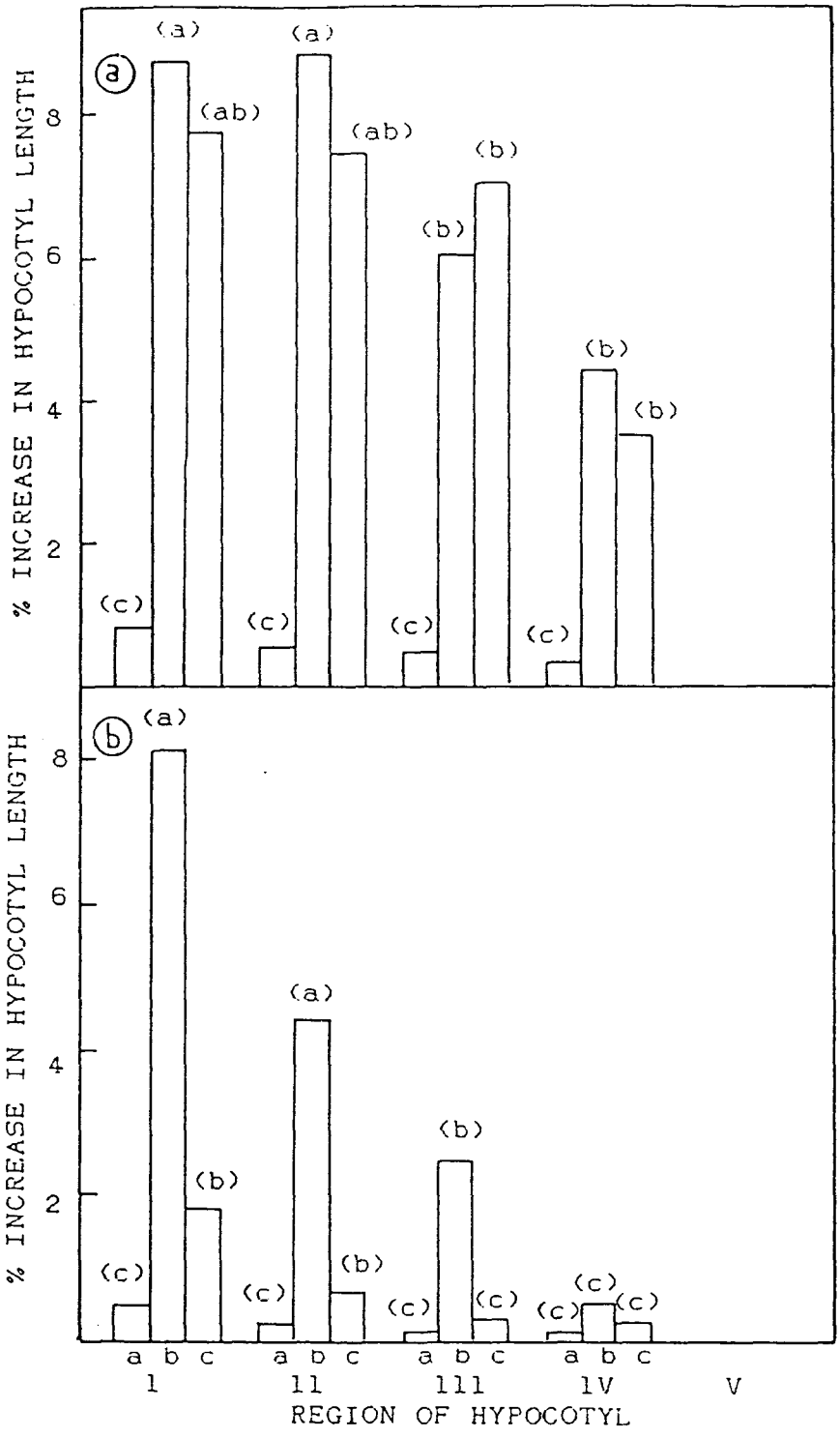
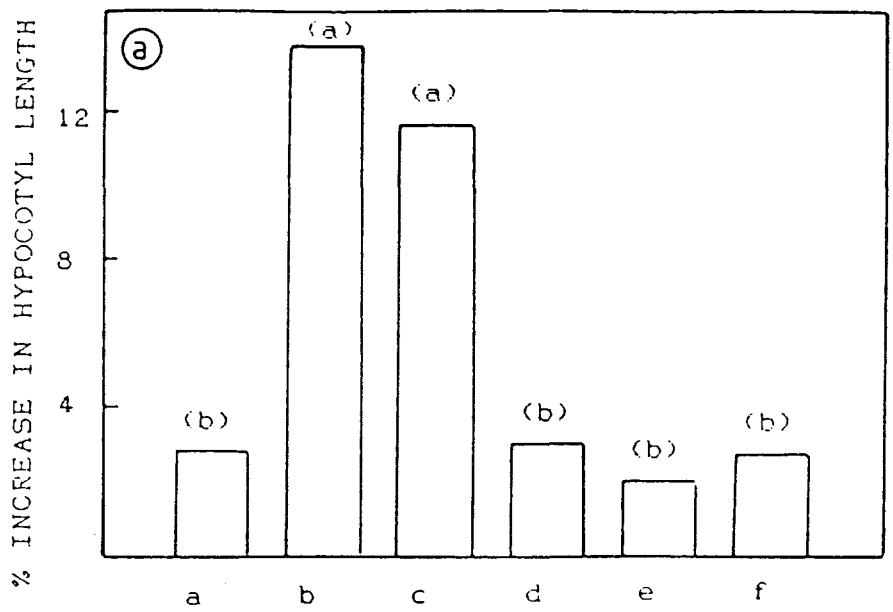
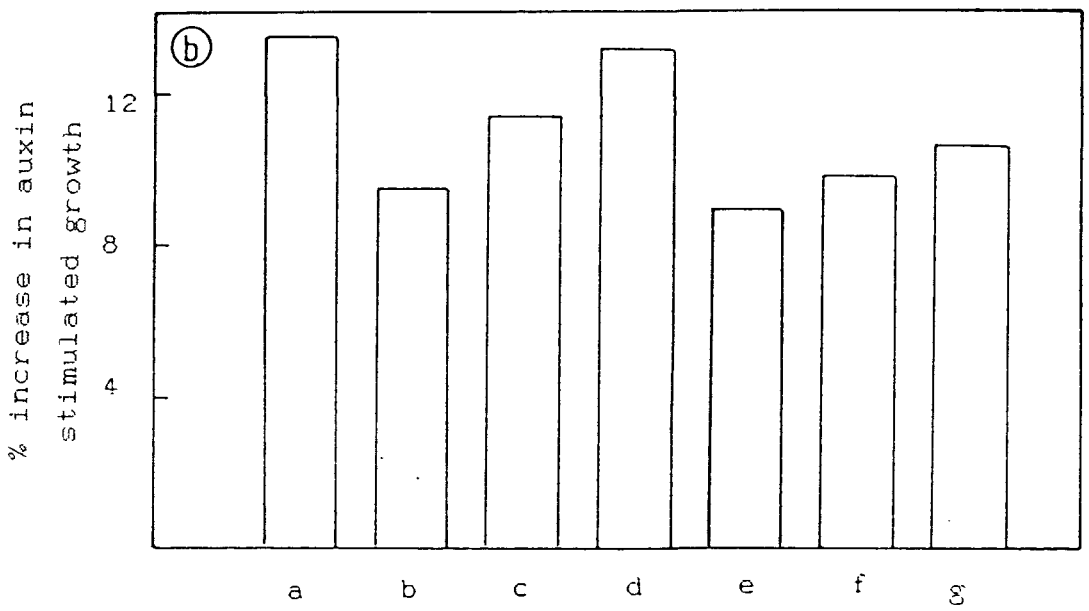

a
b
% INCREASE IN HYPOCOTYL LENGTH
8
6
4
2
(a)
(ab)
(b)
(c)
a b c
I
II
III
IV
V
REGION OF HYPOCOTYL

Figure 4.22a. Effect of SW26 and erythrosin B on hypocotyl extension in the absence of IAA. Growth was measured in regions 1 and expressed as a percentage. The standard incubation medium was used (a) without IAA, (b) in the presence of 10^{-3}mol m^{-3} IAA, (c) 10^{-1}mol m^{-3} PAA, (d) 1% (v/v) DMSO, (e) $50x10^{-3}$mol m^{-3} erythrosin B, (f) $50x10^{-3}$mol m^{-3} SW26. The one way ANOVA gave an F value at time 0 h of 0.81 which indicated that there was no significant difference ($p<0.05$) between groups a - f. At time 4 h, the F value was equal to 14.98 indicating significant differences existed ($p<0.05$) and Duncan analysis was used to indicate significant differences between the groups which are illustrated.

Figure 4.22b. Effect of SW26 and erythrosin B on auxin induced hypocotyl extension of region 1 during 4 h. The standard incubation medium (a) was used, (b) with $50x10^{-3}$mol m^{-3} SW26, (c) $25x10^{-3}$mol m^{-3} SW26, (d) $12.5x10^{-3}$mol m^{-3} SW26 (e) $50x10^{-3}$mol m^{-3} erythrosin B, (f) $25x10^{-3}$mol m^{-3} SW26 (g) $12.5x10^{-3}$mol m^{-3} SW26. The one way ANOVA gave an F value at time 0 h of 1.83 which indicated that there was no significant difference ($p<0.05$) between the initial lengths in each treatment. After 4 h an F value of 1.96 was calculated, indicating that no significant difference existed between any of the groups.


a
% INCREASE IN HYPOCOTYL LENGTH
12
8
4
(a)
(a)
(b)
(b)
(b)
(b)
a
b
c
d
e
f


b
% increase in auxin
stimulated growth
12
8
4
a
b
c
d
e
f
g

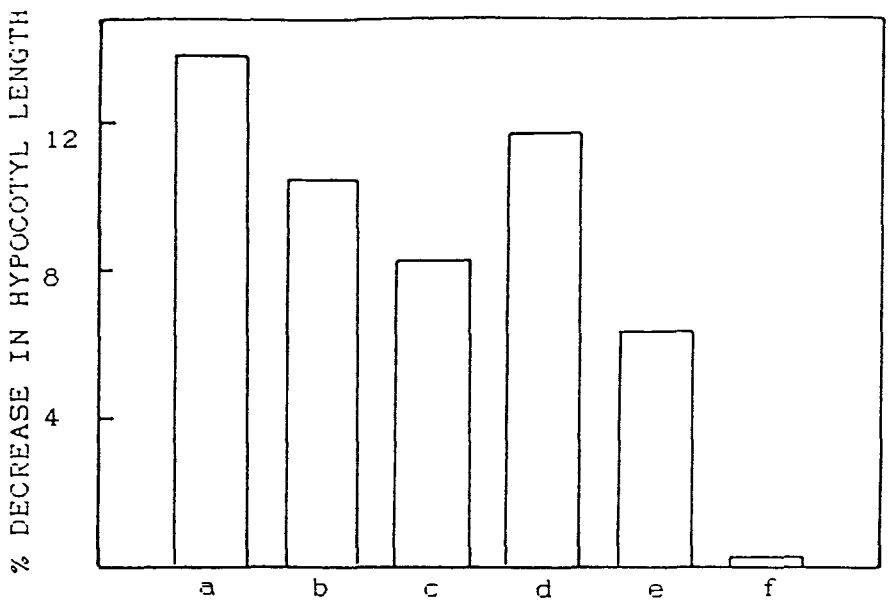


Figure 4.23b. Percentage decrease by inhibitors of auxin stimulated growth. Data is calculated from results presented in Figure 4.22b. Percentage decrease in hypocotyl length by (a) $50x10^{-3}$mol m^{-3} erythrosin B, (b) with $25x10^{-3}$mol m^{-3} erythrosin B, (c) $12.5x10^{-3}$mol m^{-3} erythrosin B, (d) $50x10^{-3}$mol m^{-3} SW26 (e) $25x10^{-3}$mol m^{-3} erythrosin B, (f) $12.5x10^{-3}$mol m^{-3} SW26.

auxin-induced growth by SW26 was much greater than the inhibition of non-auxin stimulated growth. To test that both inhibitors were actually penetrating the tissue, a light abrasion was given to the hypocotyls by four strokes with a very dilute carborundum solution. The effect of SW26 and erythrosin B on this tissue was examined (data not shown). Auxin-induced growth was greatly reduced in the abraded tissue (5% of the control rate was observed after 4 h). Erythrosin B (50mol m^{-3}) caused a much greater inhibition of growth in this tissue than in non-abraded tissue. Inhibition by SW26 was not significantly increased in abraded tissue at $50x10^{-3}$mol m^{-3}, but at $25x10^{-3}$mol m^{-3} greater inhibition was observed.

4.2.11 Effect of growth regulators and inhibitors on hypocotyl growth rate.

Initial calibration of the linear displacement transducer showed that maximum displacement of the transducer (1.68V) is equal to 1000μm (Fig.4.24). This was then used to monitor the effect of IAA and PAA on hypocotyl growth and growth rate (Fig.4.25a,b & 4.26a,b). The characteristic biphasic growth rate produced by exogenous IAA was observed. In the presence of PAA, the initial phase was present, but the second phase was absent (Fig.4.26).

The effect of erythrosin B ($5x10^{-2}$mol m^{-3} and $2.5x10^{-2}$mol m^{-3}) on auxin-stimulated elongation (Fig.4.27a & 4.28a) and growth rate were examined (Fig.4.27b & 4.28b). At the higher concentration erythrosin caused a marked inhibition of both the initial and second phases of growth. At $2.5x10^{-3}$mol m^{-3} erythrosin B, both the first and second phases of growth were detectable. The effect of SW26 on hypocotyl elongation (Fig.4.29a) and growth rate (Fig.4.29b) was also investigated. SW26 caused a very marked inhibition of elongation at a concentration of $5x10^{-2}$mol m^{-3} and both phases of growth were inhibited.

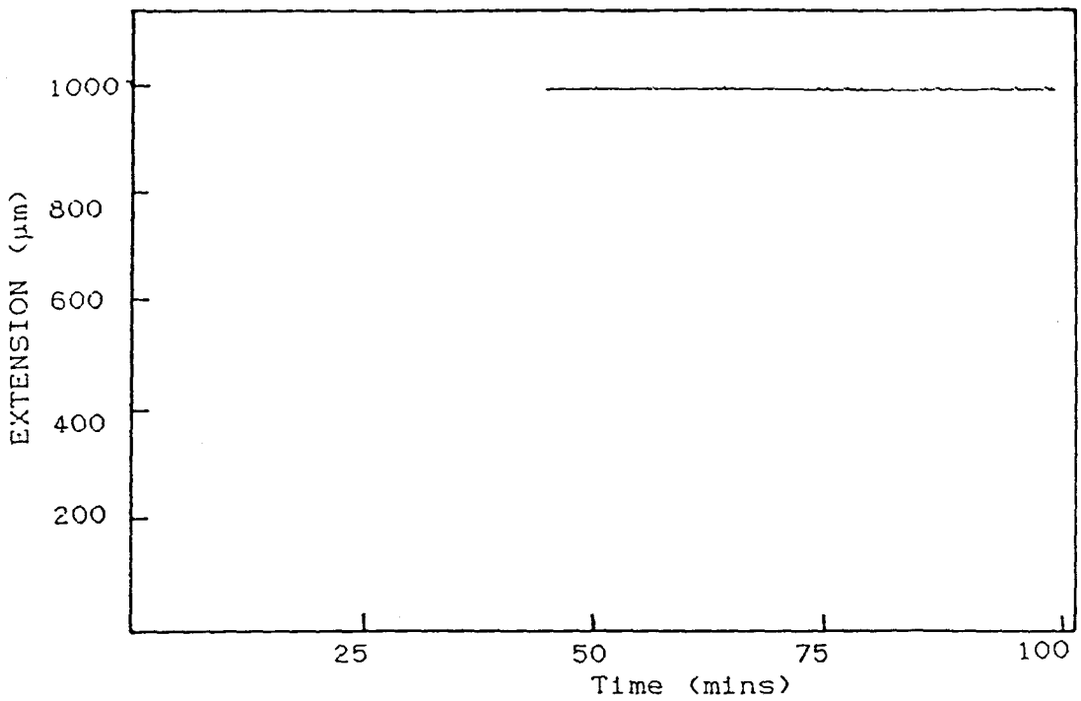


Figure 4.24 The transducer was maximally displaced (1000µm) and the voltage obtained (1.68) was used to convert the output voltage into µm.

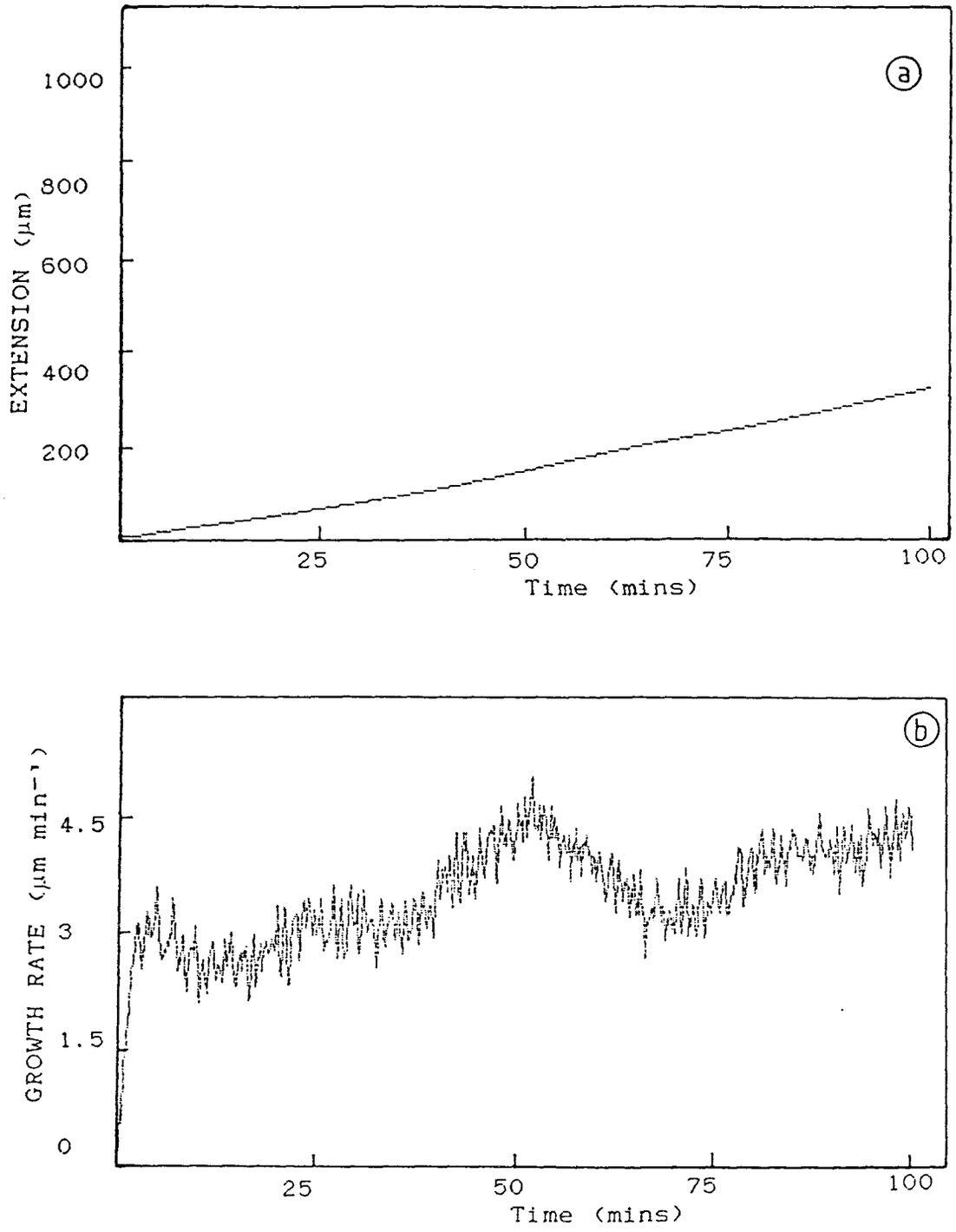


Figure 4.25a. Effect of 10^{-1}mol m^{-3} IAA on hypocotyl extension. Hypocotyl segments were incubated in distilled water for 2½ h and the extension measured every 10 sec for 100 min. All runs were repeated at least six times and a representative trace is shown.

Figure 4.25b. Growth rate calculated from the data of Figure 4.25a over the 100 min period.

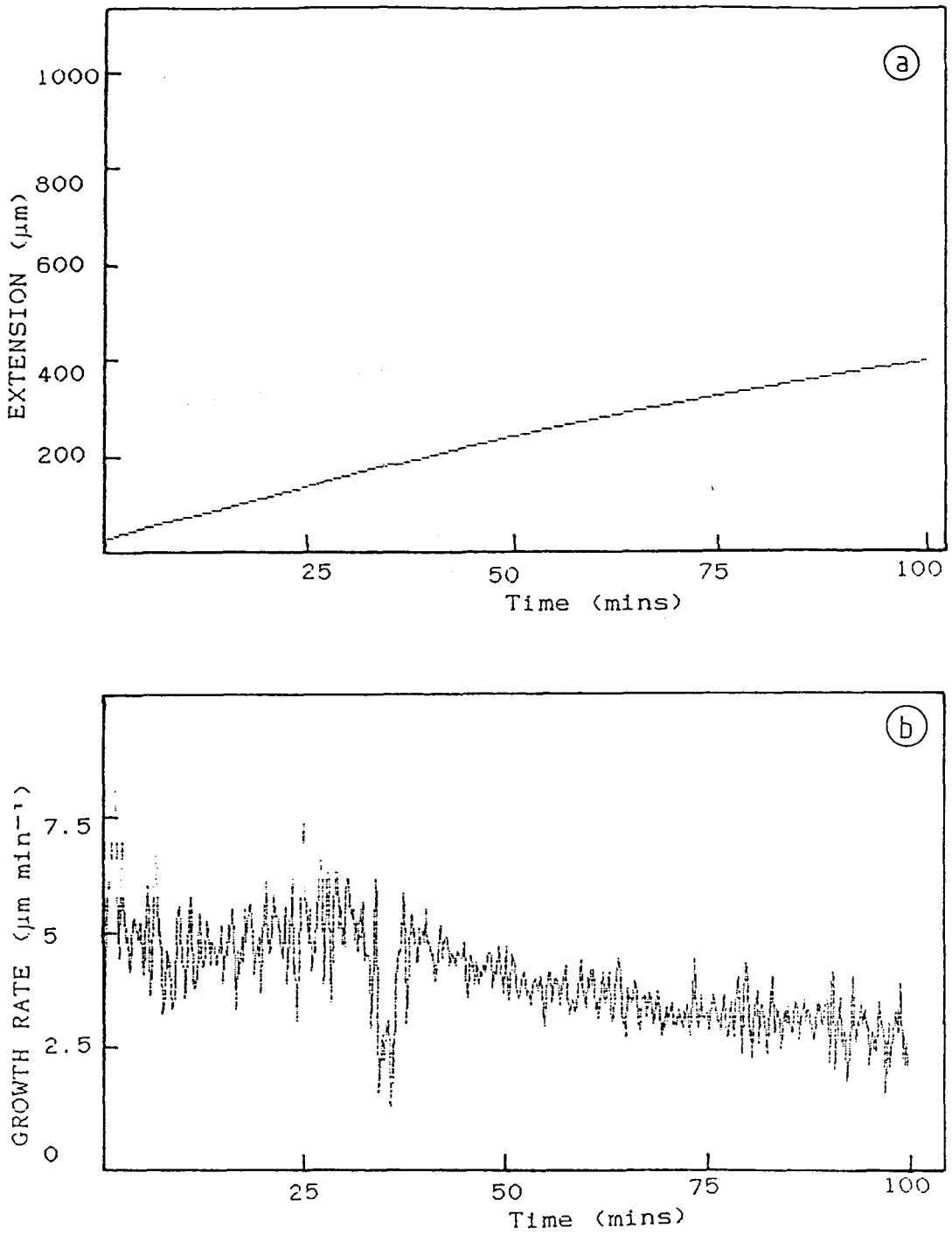


Figure 4.26a. Effect of 10^{-1}mol m^{-3} PAA on hypocotyl extension. Segments were incubated in distilled water for 2½ h and extension measured every 10 sec for 100 min using a linear displacement transducer.

Figure 4.26b. Growth rate calculated from the data of Figure 4.26a over the 100 min period.

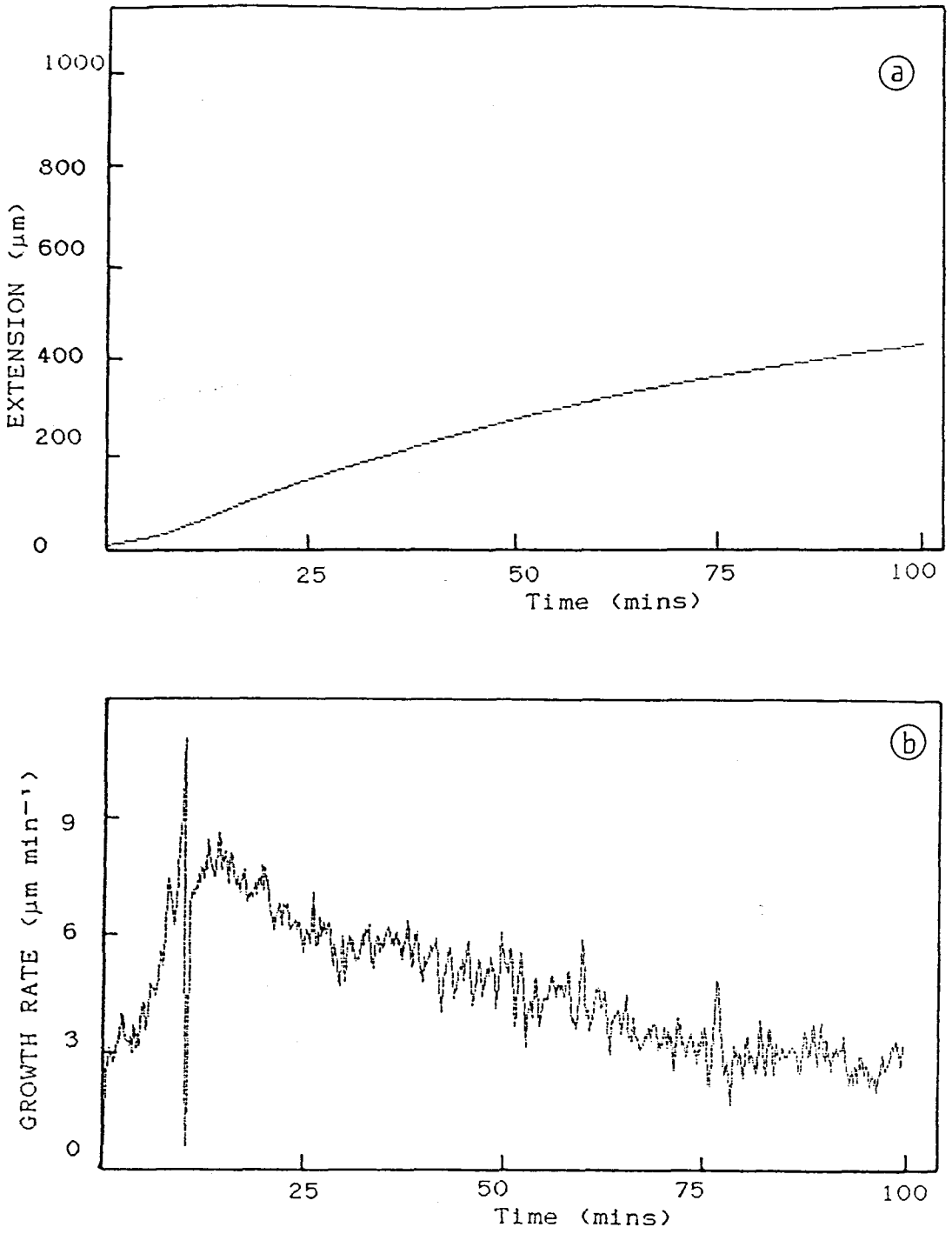
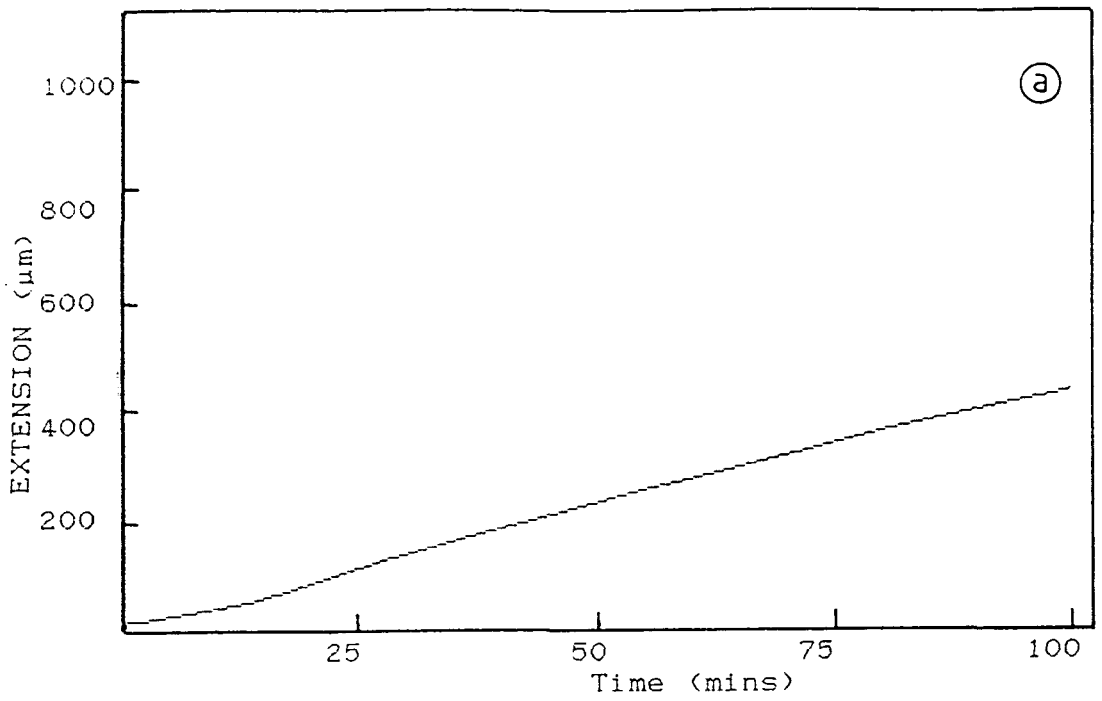


Figure 4.27a. Effect of 10^{-1}mol m^{-3} IAA and $5x10^{-2}$mol m^{-3} erythrosin B on hypocotyl extension. Segments were incubated in distilled water for 2½ h and extension measured every 10 sec for 100 min with a linear displacement transducer.

Figure 4.27b. Growth rate calculated from the data of Figure 4.27a over the 100 min period.



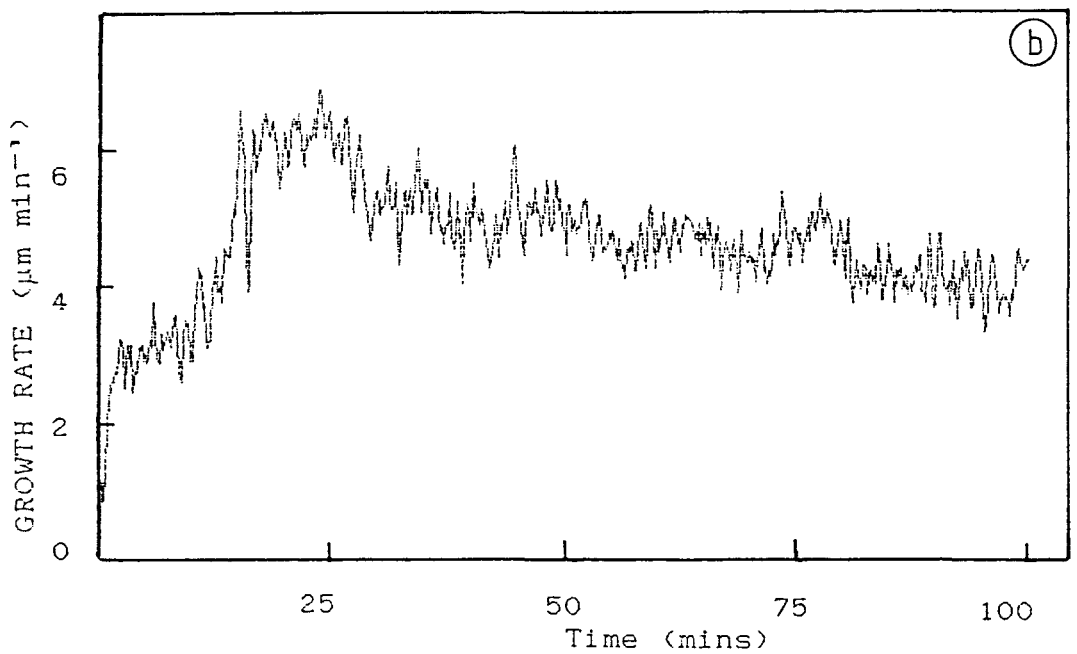
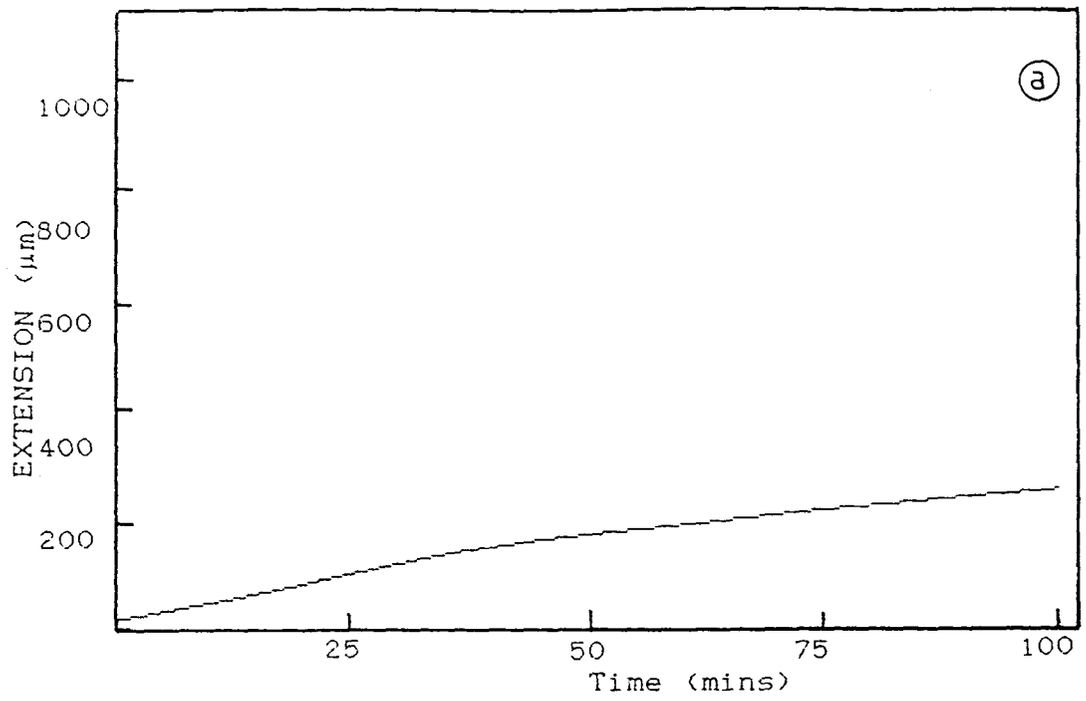


Figure 4.28a. Effect of 10^{-1}mol m^{-3} IAA and $2.5x10^{-2}$mol m^{-3} erythrosin B on hypocotyl extension. Segments were incubated in distilled water for 2½ h and extension subsequently measured every 10 sec for 100 min with a linear displacement transducer.

Figure 4.28b. Growth rate calculated from the data of Figure 4.28a over the 100 min period.



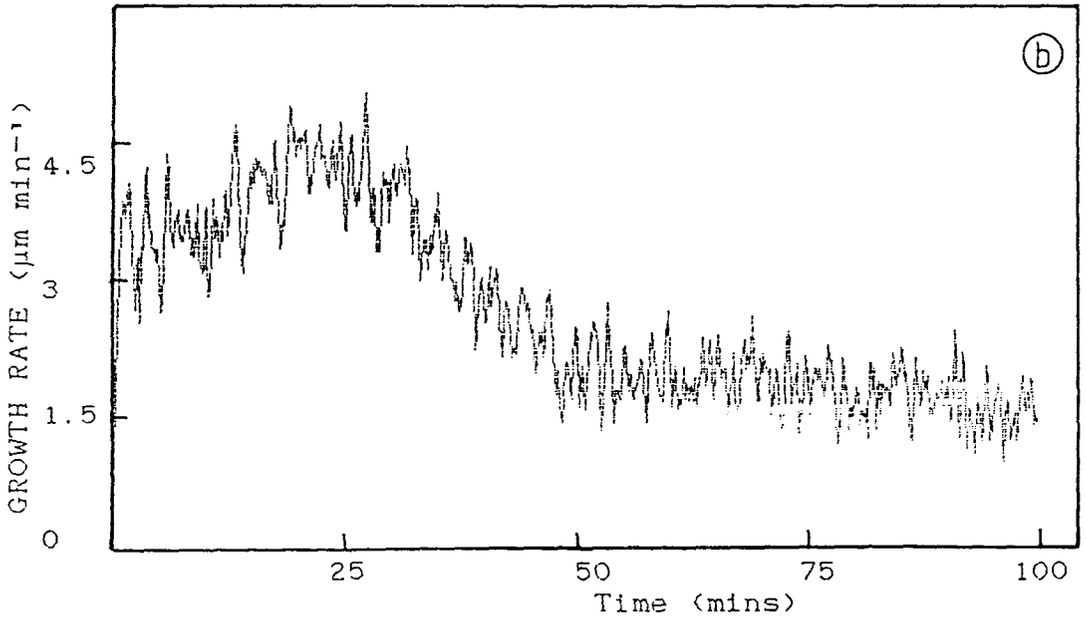


Figure 4.29a. Effect of 10^{-1}mol m^{-3} IAA and $5x10^{-2}$mol m^{-3} SW26 on hypocotyl extension. Segments were incubated in distilled water for 2½ h and extension measured every 10 sec for 100 min with a linear displacement transducer.
Figure 4.29b. Growth rate calculated from the data of Figure 4.29a over the 100 min period.

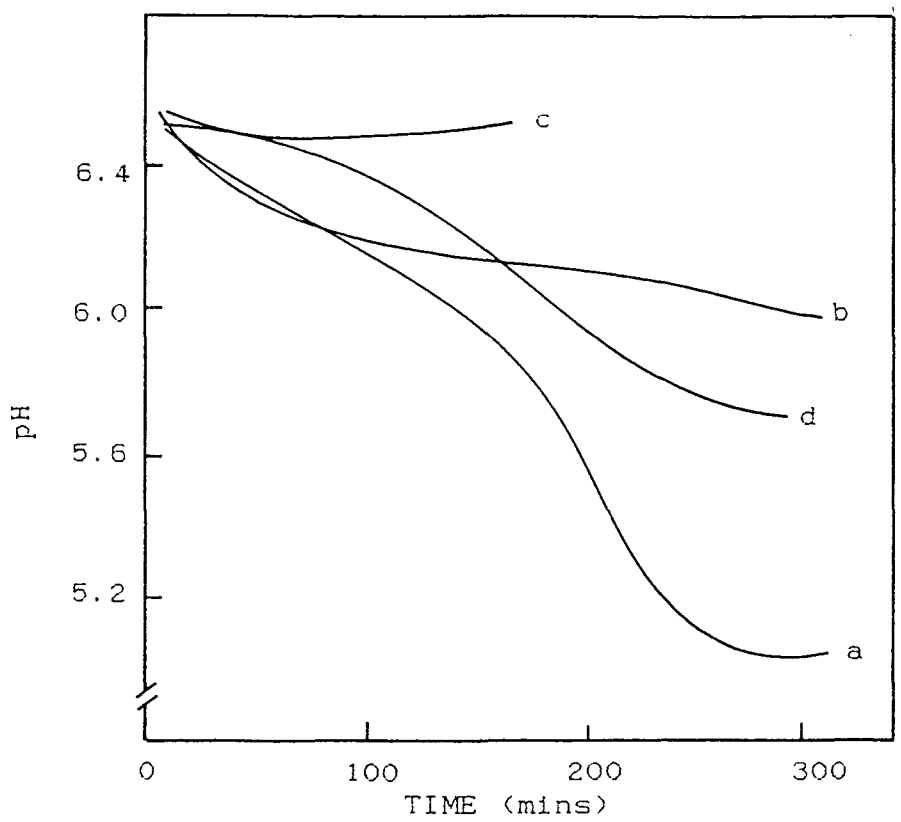


Figure 4.30. Inhibition of medium acidification by vanadate, erythrosin B and SW26. The assay was performed as described in "Material and Methods" with (a) the standard incubation mixture, (b) with the inclusion of $50x10^{-3}$mol m^{-3} vanadate, (c) $50x10^{-3}$mol m^{-3} erythrosin B, (d) $50x10^{-3}$mol m^{-3} SW26.

4.2.12 Effect of ATPase inhibitors on medium acidification by hypocotyls.

The effect of the inhibitors vanadate, SW26 and erythrosin B ($50x10^{-3}$mol m^{-3}) on acidification of the external bathing medium by abraded hypocotyl segments in the presence of IAA was examined. Each inhibitor was tested at least four times and the acidification obtained is illustrated in Figure 4.30. Of the three inhibitors, erythrosin was found to be the most potent, totally preventing acidification of the external bathing medium (Fig.4.3). Both vanadate and Sw26 significantly reduced acidification of the medium, compared to the control.

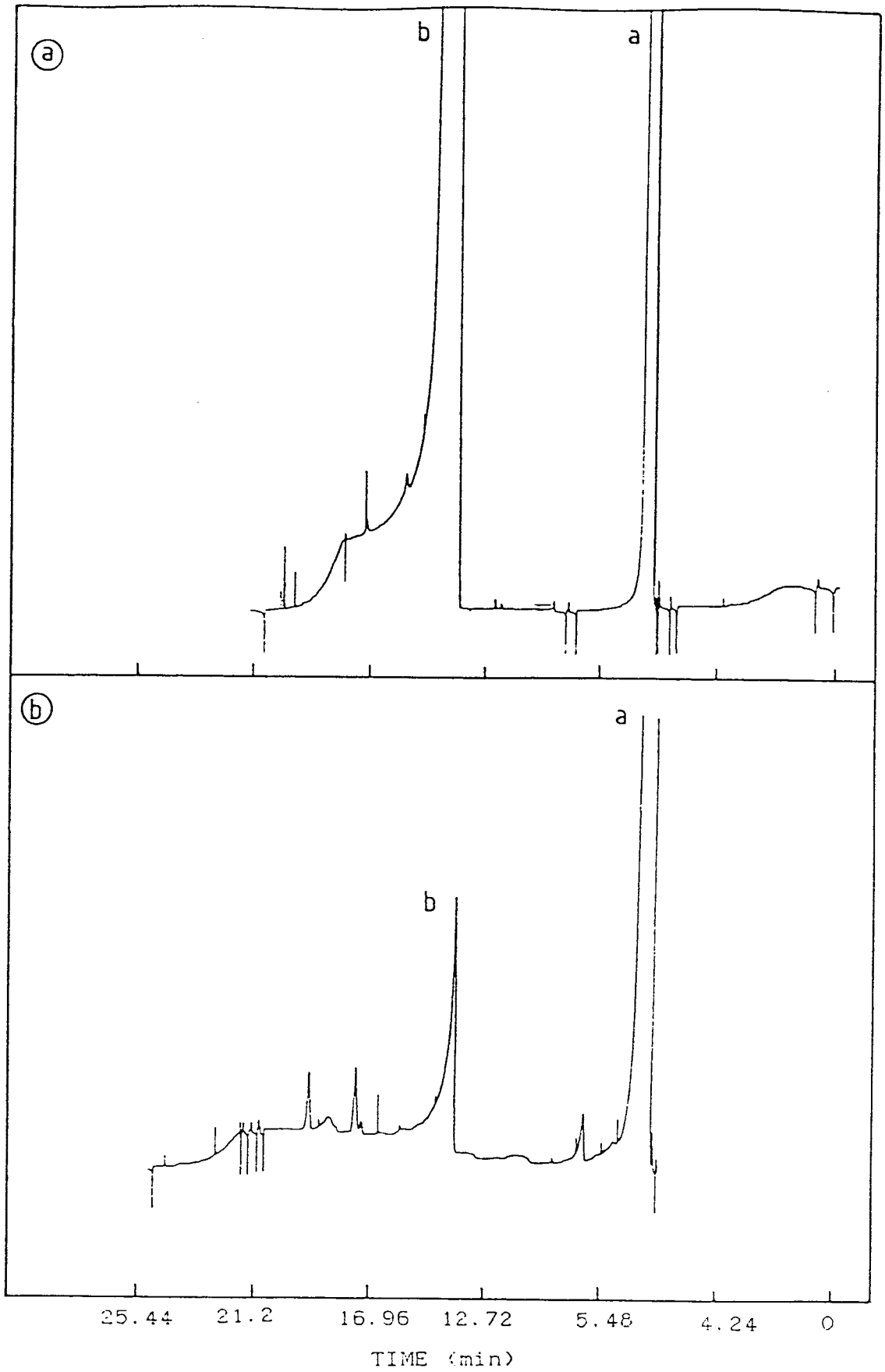
4.2.13 Detection of phenyl acetic acid in hypocotyls of *Cucumis.*

An acidic ether extract was prepared from hypocotyls as outlined in "Materials and Methods". The standard run of PAA (Fig.4.31a) gave two peaks, one being the solvent front (a) and the other PAA (b). Two peaks were obtained for the extract (31b), (a) being the solvent front and (b) corresponded exactly to that obtained for the PAA standard.

4.3 Discussion.

ATPase levels in regions of the hypocotyl were determined to examine the possibility that the growth potential of a cell may be related to its membrane ATPase activity. ATPase specific activities associated with the tonoplast- and plasma membrane-enriched fractions were found to increase basipetally. Similarly, nitrate- and vanadate-sensitive ATPase associated with the two membrane types and potassium-stimulated ATPase activity associated with the tonoplast, increased basipetally. The vanadate-resistant ATPase activity showed a decrease in the region of the hypocotyl that had a corresponding large increase in vanadate-sensitive activity. A possible explanation is that a conversion occurs down the hypocotyl from a resistant form into a vanadate-sensitive species or, alternatively, it represents a different proportion of

Figure 4.31. Gas liquid chromatogram of (a) PAA control and (b) an acid ether extract from 100 g fresh weight of *Cucumis* hypocotyls. An ignition temperature of 100°C was run for two min and ramped at 16°C/min up to 250°C. An injection temperature of 200°C and detonation temperature of 260°C was used. A flow rate of 25cm^3/min was used and the column was an OV 101.


a
b
a
b
a
b
25.44
21.2
16.96
12.72
5.48
4.24
0
TIME (min)

membrane types being purified from the regions of the hypocotyl.

The growth experiments indicated that the apical region of the hypocotyl underwent the maximum auxin stimulated growth, which declined basipetally. Thus no apparent link may be made between levels of specific ATPase activity in the hypocotyl and potential for growth. At least three possible explanations may be put forward to explain the basipetal increase in vanadate-sensitive ATPase. Either enzymes within the hypocotyl capable of degrading ATPase decline basipetally or the total membrane associated protein decreases. Alternatively, and a likely possibility is that the ATPase component may be increasing for transport purposes rather than for elongation growth (e.g.Williams & Hall 1987). ATPase specific activity is a measure of the protein/ATPase ratio, and protein changes within the hypocotyl were reported and therefore influence levels of calculated ATPase specific activity. Total ATPase activity in regions of the hypocotyl indicated that activity did not follow the same trend as the specific activity and peaked in the upper regions of the hypocotyl. Changes in ATPase activity may therefore be associated with the developmental stages of the hypocotyl, but interpretation depends on the method used to express activity.

Other workers have reported changes in levels of specific ATPase activity associated with developing cells. For example, in maize roots (Dupont *et al.* 1982b). ATPase activity was found to be similar in membrane fractions prepared from the developing root, except for the apical 5 mm which had a four-fold higher specific ATPase activity. ATP-dependent H^+ transport was also examined by these workers in microsomal vesicles prepared from different regions. Activity was higher in the apical 5 mm than in regions further from the tip. The vesicles though were likely to be mainly of tonoplast origin. The meristematic region in the root extends back 1 - 2 mm from the tip (Lyndon 1976), and therefore it may well be that those cells undergoing rapid division have a higher ATPase specific activity. In contrast to the root, the meristem of the shoot consists of tens of millions of cells and

extends back 10 - 20 mm from the apex. Similarly these meristematic cells may have contributed to the elevated ATPase activity detected in the more basipetal area of the hypocotyl meristem.

There have been other reports of levels of ATPase activity being linked to the developmental status of organs. For example, ATPase activity associated with the tonoplast and plasma membrane have been reported to vary during maturation of kiwi fruit (Chedhomme & Rona 1986). Activity was low at the onset of ripening and increased during the ensuing month of maturation and then was lost in the mature fruits. Similarly, in artichoke tubers, plasma membrane associated ATPase was highest during the months September to December (Ishikawa & Yoshida 1985). It is possible that physiological changes may be related to these changes in levels of ATPase activity reported. Williams and Hall (1987) reported that sucrose uptake varied with the developmental stage of *Ricinus* cotyledons. Uptake capacity was found to be highest in the third size class and the cotyledons at this stage were also found to have the highest microsomal ATPase specific activity.

The percentage stimulation of the tonoplast-associated ATPase by KCl varied markedly in this tissue and may be related to a transport function. This result may indicate that a change of the ATP catalytic activity occurs within the hypocotyl. Other workers have reported that a specific interaction exists between K^+ and a specific phosphoenzymic form of the ATPase (Briskin & Poole 198[illegible]) and this may develop basipetally in the hypocotyl. The plasma membrane ATPase isolated from several plant species is stimulated by K^+ (e.g. Leonard & Hotchkiss 19[illegible]). Similarly the animal cell Na,K-ATPase (Cantley *et al.* 19[illegible]), and gastric mucosal H,K-ATPase (Faller *et al.* 1982) both show stimulation of activity by K^+ and this reflects a direct transport of this cation. Plant ATPases may have a similar role (Hodges 1973) which is supported by the reported K^+ stimulation and K^+ uptake kinetics (Leonard 1984). In plants K^+ transport is likely to occur *via* an indirect mechanism driven by the electrical component of the membrane potential. Several mechanisms support this proposal for K^+ uptake at the plasma

membrane. For example, the movement of protons out of the cell is enhanced in the presence of K^+ an effect which can be interpreted in terms of K^+ reducing the membrane potential through its entrance into the cell (Marré & Ballarin-Denti 1985).

In this tissue, auxin and PAA were reported to have no stimulatory effect on membrane bound ATPase *in vitro* over a wide concentration range, if the tissue received no pre-treatment. ATP-dependent H^+ transport was found to be enhanced in some experiments by auxin. Only at low ATP concentrations was this effect observed and therefore it is unlikely to be of physiological significance. There have been similar reports with pea vesicles and again IAA was found to increase ATP-dependent H^+ transport only at low ATP concentrations. Vanadate was found to inhibit this effect (Gabathuler & Cleland 1985). Two possible explanations may be presented for this observed activation by IAA of H^+ transport alone. Either IAA is directly affecting the pump, or it is entering the vesicle *via* the anion efflux carrier, reducing the H^+ gradient and stimulating further H^+ influx. The lack of stimulation of ATPase *in vitro* provides no evidence for the first proposal. The finding that IAA did not collapse the H^+ gradient established within a vesicle would suggest that auxin is not entering *via* the anion efflux carrier either. However, this data does give a little support to the acid growth theory (Rayle & Cleland 1970) which postulates that acidification of the cell wall is provided for by ATP-dependent H^+ transport.

In some tissues and preparations IAA has been reported to affect ATPase activity, while in others no effect has been found. For example, IAA *in vitro* was found to have no effect on ATPase activity (Gabathuler & Cleland 1985) while Scherer (1981, 1984a) found IAA *in vitro* to be stimulatory in a plasma membrane enriched fraction. This lack of affect has been tentatively resolved by the suggestion that auxin only affects ATPase activity when it is associated with tightly sealed inside-out membrane vesicles. The proportion of such vesicles in a microsomal fraction containing total membrane-bearing ATPase is likely to be small, so the lack of inducible

ATPase activity associated with non-vesicular membranes would mask that of the vesicular form. The lack of stimulation of ATPase *in vitro* by IAA might be due to IAA being at supraoptimal levels, by remaining attached to the membranes during isolation.

An *in vivo* incubation in IAA was found to be necessary to obtain stimulation of ATPase specific activity in this work. Addition of IAA *in vitro* following this pretreatment was found to further stimulate activity. Addition of IAA *in vitro* to the water control caused a much lower change in ATPase activity, therefore the result is not a consequence of auxin depletion in the system. Similar reports have been made in which an *in vivo* incubation in IAA followed by *in vitro* incubation of a microsomal fraction in IAA from *Phaseolus mungo* hypocotyls produced enhanced ATPase activity (Kasamo & Yamaki 1974). This increase may suggest that auxin is mediating an effect *via* two different mechanisms. The first may be due to a gene activation which is supported by the finding that activity was found to increase with incubation time *in vivo* and the second, a direct stimulation of the H^+ pump. Examination of the growth rate in the presence of either the first or second phase inhibitors would of been of interest. The two inhibitors examined in this chapter, SW26 and erythrosin B caused no differential affect of these two phases.

Gene activation by auxin is proposed to occur by IAA binding and inhibition of a short-lived repressor of mRNA synthesis, which results in the transcription of certain genes (Theologis 1986). This induction of mRNA synthesis is believed to be the primary auxin event with all other cellular changes and growth resulting from this. Auxin-stimulated H^+ extrusion is suggested not to be involved directly in growth regulation by acidification of the cell wall, but to be associated with the secretory vesicles and to provide part of the energy requirement for the transport and incorporation process.

In this work, PAA was found to stimulate growth to a lesser extent than IAA, but gave the initial fast acid inducible phase of growth. In this respect, the action of PAA is similar to that of an acid. PAA is a naturally

occurring plant regulator and its growth promotive action might suggest that certain hormones in plants may be used to induce growth by a direct mechanism which is only transient, while other hormones have a role in sustained growth.

4.4 Conclusions.

Based on the membrane protein estimations made by coomassie binding, levels of ATPase within the hypocotyl were found to increase basipetally, both in tonoplast- and plasma membrane-enriched fractions. These levels did not correspond to the meristematic regions, but may represent an increase for transport purposes. However, ATPase activity based on a per fraction of the hypocotyl, was found to peak in the meristematic region.

Auxin was found to have no affect on plasma membrane associated ATPase activity when applied *in vitro*. When tissue was given either a water or auxin pretreatment *in vivo*, auxin *in vitro* caused a stimulation of the plasma membrane associated ATPase in particular. This finding indicated that auxin does play a role in the second phase of growth.

The initial fast phase of growth which is acid and auxin inducible and believed to occur *via* auxin stimulated H^+ excretion *via* a plasma membrane located ATPase was not readily demonstrated in vesicles that were actively transporting H^+, monitored by the fluorescence quenching of quinacrine. Stimulation was only observed at low non-physiological ATP concentrations.

CHAPTER FIVE.

Effect of R and FR treatments *in vivo* and *in vitro* on membrane associated ATPase.

5.1 Introduction.

Seasonal changes in the environment are accompanied by alterations in light composition and duration. Many plants respond to climatic changes and use these light alterations as signalling devices. Major adaptations in plant growth and development are brought about in the process of photomorphogenesis whereby light is absorbed by a chromophore and subsequently amplified. Changes brought about *via* phytochrome include the promotion of seed germination, epicotyl hook opening in etiolated dicotyledonous seedlings, anthocyanin synthesis, initiation of flowering by light break in long day plants and inhibition of stem elongation (e.g. Smith 1975).

Photomorphogenesis begins with the absorption of light by specialized plant pigments often by the universally occurring pigment, phytochrome. Many photomorphogenetic responses are saturated at very low irradiances, making the system very sensitive to the environment. Our understanding of the role that phytochrome plays in the initial and subsequent stages is still very limited.

It is thought that one of the early events under control by phytochrome involves ion transport across membranes *via* either a passive alteration of membrane properties or an active mechanism (Hendricks & Borthwick 1967) and there is considerable evidence to support the latter (e.g. Marmé 1977). More recently Roux (1984) has proposed that calcium controls many, if not all of the phytochrome responses, *via* the following transduction sequence:

1. Photoactivation of phytochrome;
2. The conversion of phytochrome into an ion carrier, so that as a result of the prevailing negative membrane PD and high external Ca^{2+} concentration, cytosolic Ca^{2+} levels rise;

3. Activation of calmodulin and/or other calcium-binding modulator proteins (CMPs);
4. Activation of CMP-dependent enzymes.
Two plant proteins have been found to be regulated by phytochrome *via* calmodulin, namely, NAD kinase and Ca-ATPase.

This work is related to earlier reports of phytochrome regulation of ion transport in plants.

5.1.1 Phytochrome regulation of ion fluxes at the multicellular level.

This aspect has been approached using a variety of *in vivo* studies and the first reports of the involvement of phytochrome in ion transport came from the study of nyctinastic movements of *Mimosa* leaflets. These were recognized to be controlled *via* ion fluxes which are influenced by R and FR (Fondeville *et al.* 1966). More specifically, phytochrome modulation of K^+ fluxes have been examined by means of radiotracer techniques using $^{86}Rb^+$ in mung bean (Brownlee & Kendrick 1979a, b) pea (Köhler *et al.* 1968), etiolated oat shoots (Pike & Richardson 1977) and mungbean (Tezuka & Yamamoto 1975). P_{fr} has been found to enhance K^+ ($^{86}Rb^+$) uptake by apical oat coleoptile segments (Pike & Richardson 1977). In sub-hypocotyl hook segments from mung bean, K^+ uptake was inhibited by P_{fr} (Tezuka & Yamamoto 1975) while in apical segments, P_{fr} enhanced uptake (Brownlee & Kendrick 1977). These reports provide evidence for the involvement of P_{fr} in a direct K^+ exchange or indirectly through an increase in the passive permeability of the plasma membrane (Brownlee & Kendrick 1979a).

Phytochrome has been reported to regulate wheat leaf protoplast swelling (Blakeley *et al.* 1983), treatment with R yielding protoplasts which were larger than those treated with FR. This was thought to be brought about by an increase in solute concentration. This increase may be the result of hydrolysis of storage compounds or of solute release from internal compartments. However, K^+ was found to be always required in the external medium for a strong response, suggesting that uptake from the bathing medium occurs. However, $^{86}Rb^+$ uptake in isolated wheat leaf

protoplasts was not altered by light treatments, arguing against this view (Blakeley *et al.* 1983).

5.1.2 Mechanism of phytochrome-induced protoplast swelling and medium acidification.

The plasma membrane is typically reported to be involved in eliciting many of the phytochrome responses. Thus phytochrome control of protoplast swelling in oat (Kim *et al.* 1986) and etiolated wheat leaves (Blakeley *et al.* 1983) is likely to be elicited at the plasma membrane.

Another approach used to monitor and determine the location of the phytochrome response has been to examine active H^+ extrusion across the plasma membrane from isolated hypocotyl segments. H^+ extrusion was found to be under phytochrome control in *Phaseolus aureus*, indicating the intimate involvement of the plasma membrane. Proton extrusion was increased by P_{fr} in the apical hypocotyl region of *Phaseolus aureus* and decreased in the subapical region (Brownlee & Kendrick 1979b). Work with cucumber hypocotyls has shown that phytochrome elicits a response *via* the plasma membrane. In the presence of Mg^{2+} and K^+ with phytochrome in the P_{fr} form, the pH of the bathing medium fell by 2.89 units $g^{-1}FW^{-1}$, whereas with P_r, the pH fell only by 1.2 units $g^{-1}FW^{-1}$ (Roth-Bejerano & Hall 1986b). The regulation of protoplast swelling and medium acidification by phytochrome both provide evidence for the involvement of phytochrome mediated responses occurring *via* the plasma membrane of epidermal leaf cells.

Indirect methods have been employed to determine if phytochrome directly modulates a plasma membrane integral enzyme. Sodium orthovanadate, an inhibitor of plasma membrane ATPase, was found to inhibit wheat leaf protoplast swelling in response to R in the absence of K^+ and this was completely prevented in the presence of K^+ (Blakeley *et al.* 1987). Additionally, the reduction in protoplast size following transfer from $0.5x10^{-3}$mol m^{-3} to $0.6x10^{-3}$mol m^{-3} sorbitol was also prevented by vanadate. Acidification of the external bathing medium by *Cucumis* hypocotyl segments was increased in the presence of Mg^{2+} and K^+ (Roth-Bejerano & Hall 1986b) and inhibited by vanadate (Brummell *et al.* 1986). These reports indicate

firstly that protoplast swelling occurs *via* an active process and not a passive osmotic effect and secondly, that phytochrome is eliciting an effect *via* a membrane bound enzyme that is the Mg^{2+} dependent K^{+} stimulated ATPase associated with the plasma membrane (Sze 1984). The observation that GA3 may be substituted for R during induction of protoplast swelling in the presence of KCl has prompted the hypothesis that GA's may act as intermediates in phytochrome-regulated responses (Lockhart 1956, Loveys & Wareing 1971).

Phytochrome occurs predominantly as a soluble cytosolic protein in dark-grown plant cells, but upon light activation associates with subcellular structures (Mackenzie *et al.* 1975) and acts as a regulatory protein at the organelle level. P_{fr} has been suggested to associate with more than one type of subcellular structure (Coleman & Pratt 1974). Using gold coupled second antibodies it has been demonstrated that following R irradiation, phytochrome is sequestered into special electron-dense areas within the cytoplasm. These areas are not found in dark grown tissues and are not surrounded by a membrane (Speth *et al.* 1987).

5.1.3 Phytochrome modulation of enzyme activity.

Direct approaches have been made to determine if phytochrome modulates certain enzymic activities associated with the plasma membrane *in vitro*. An ATPase in an unidentified membrane fraction was first reported to be modulated by R and FR from hypocotyl hook tissue of *Phaseolus aureus* (Jose 1977). Activity was influenced by the photostationary state of phytochrome established *in vivo* or *in vitro* provided the tissue was subjected to R prior to membrane isolation. Subsequently, adenylate kinase activity was similarly found to be regulated by R and FR irradiation *in vitro* in *Phaseolus aureus* hypocotyls (Jose & Schäfer 1979). Both enzymes were regulated by the form of phytochrome established *in vitro*. R lowered enzyme activity compared with FR, and the system was photoreversible for at least six successive irradiations. Regulation by R and FR was also observed during the linear phase of enzyme reactions with hexokinase, adenylate

kinase and ATPase. No enzyme regulation was found in extracts from completely etiolated material (Jose & Schäfer 1979).

R and FR irradiations were found to have an effect on ATPase activity in plant membranes *in vitro* (Thomas & Tull 1981). Membranes isolated from *Cucumis sativus*, when exposed to R *in vivo* for 2 min showed an increase in the k_m for ATP by FR *in vitro*. This effect was partially reversed by R. Changes in V_{max} were very small following treatments with FR or R *in vitro*, suggesting a competitive inhibition of the ATPase by FR. Similarly, *in vivo* irradiation with R was found to be necessary for any light effect to be observed *in vitro* (Roth-Bejerano & Hall 1986a). In this work, R *in vitro* was found to stimulate ATPase activity by 37% over a FR treatment *in vitro*. V_{max} and K_m were both altered, suggesting a non-competitive inhibition of the ATPase by R. The difference in the effects of R and FR on ATPase activity was significant in the presence of K^+, but little difference was observed in its absence which suggests that it is the K^+ stimulated component of the ATPase that is being altered.

There is strong evidence for stomatal movement being phytochrome modulated *via* K^+ fluxes into and out of the guard cells, *via* a plasma membrane associated ATPase (Roth-Bejerano *et al.* 1983). Stomatal opening is driven by K^+ accumulation in the guard cells, (Penny & Bowling 1974). R triggers the opening of isolated guard cells, which can be reversed by a subsequent short irradiation with FR in *Commelina communis, Pisum sativum, Vicia faba* and *Pelargonium* sp. B also induces stomatal opening which is FR reversible, indicating that B may act through phytochrome (Roth-Bejerano & Itai 1987). Exhaustive attempts have been made to photo-induce a phase shift in the rhythm of stomatal opening or closure by phytochrome in *Phaseolus vulgaris* (Holmes & Klein 1986). Although unsuccessful, phytochrome was found to reduce the amplitude of opening in darkness if given FR at the end of the day. This provides evidence for the involvement of phytochrome in membrane transport, but not in the circadian clock.

5.1.4 Phytochrome regulation of NAD kinase.

There have been reports that illumination of seedlings causes an increase in NADP/NAD ratio (Muto *et al.* (1981). NAD kinase is the only enzyme known to catalyze the phosphorylation of NAD to NADP. Similar reports that NAD kinase activity in cell-free extracts is photoregulated *via* the action of phytochrome have been made (Tezuka & Yamamoto 1975), but these results could not be verified by other workers (Hopkins & Briggs 1973, Dieter & Marmé 1986). Ca^{2+} was found to be a requisite for NAD kinase activity which was further stimulated by the protein activator calmodulin (Anderson & Cormier 1978). Subsequently, calmodulin-dependent NAD kinases have been identified in several plants such as corn and spinach (Dieter 1984) and have all been found to be either cytosolic (Simon *et al.* 1982, Dieter & Marme 1984), or associated with an organelle membrane with the regulatory site located on the cytoplasmic face and so able to detect changes in cytoplasmic Ca^{2+} levels (Simon *et al.* 1984). On the basis of this data and the assumption that cellular Ca^{2+} concentration can be changed by irradiation with FR (Marmé & Dieter 1983), it has been suggested that the effect of light on NAD kinase is mediated *via* Ca^{2+} and calmodulin (Dieter 1984).

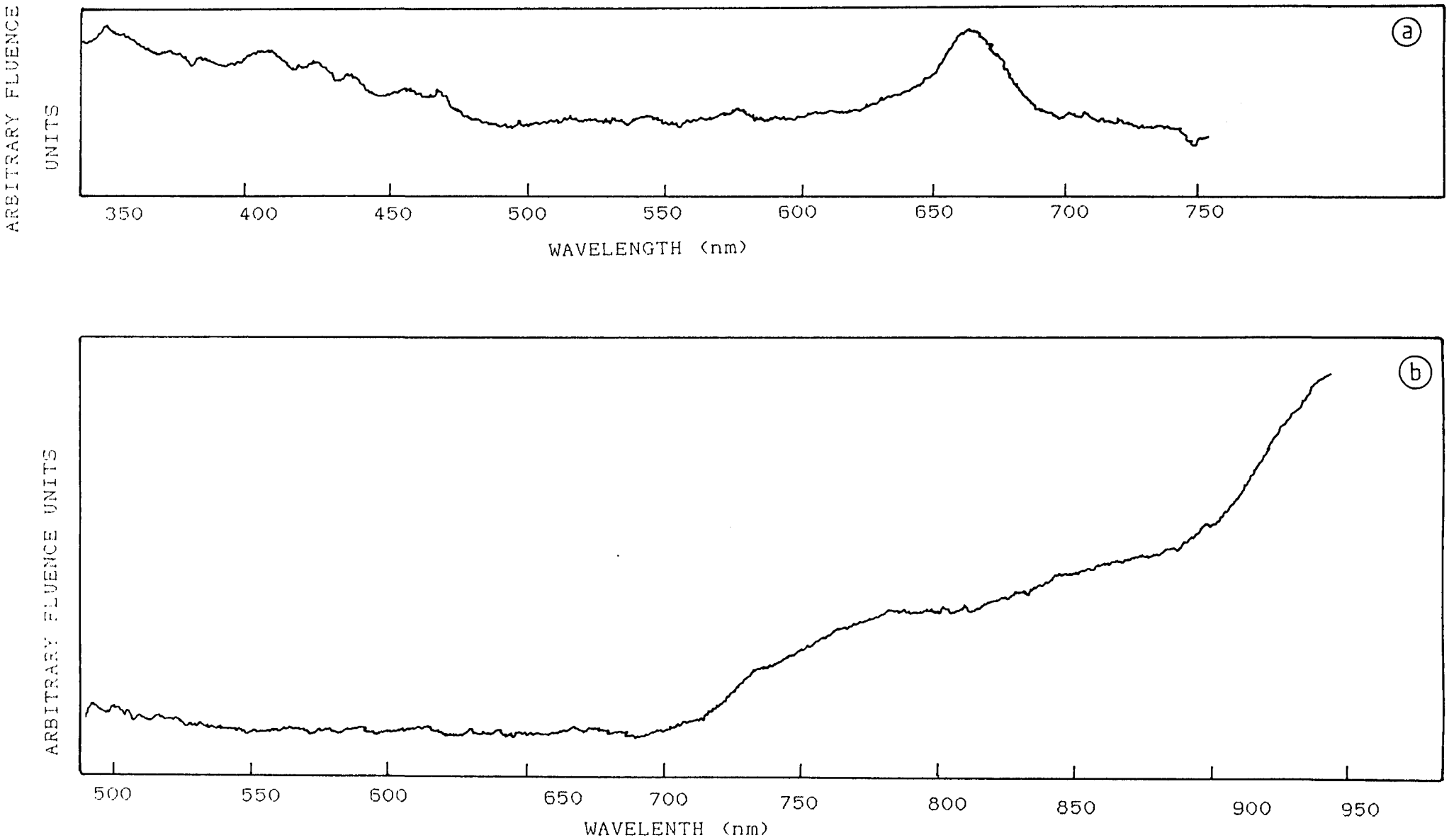
5.2 Results.

5.2.1 Light source characterization.

The spectral composition of the R and FR sources obtained using the light box was examined (Fig.5.1a,b). The R source gave a peak at 666 nm, with a cut-off at 690 nm (Fig.5.1a). The FR source showed relatively low emission spectra below 700 nm, but above this wavelength the emission was biphasic with the initial plateau at 750 nm and the second at 950 nm (Fig.5.1b).

Figure 5.1. Wavelength emission scan of the (a) red light source and (b) far red light source obtained from the light box with the filter combinations as described in "Materials and Methods".

Measurements were made using a Hilger Watts 0.5m grating monochrometer with a silicon photodiode. The fluence units obtained were linear.


ARBITRARY FLUENCE UNITS
a
350
400
450
500
550
600
650
700
750
WAVELENGTH (nm)
ARBITRARY FLUENCE UNITS
b
500
550
600
650
700
750
800
850
900
950
WAVELENTH (nm)

5.2.2 Whole hypocotyl experiments.

Initial experiments involved an *in vivo* illumination of intact plants and extraction of a microsomal fraction from the complete hypocotyl. The change in ATPase activity in isolated membrane fractions resulting from R and FR treatments was measured with H_3BO_3 (5mol m^{-3}) in the homogenization medium. Incorporation of boric acid enhanced the overall ATPase specific activity (Fig.5.2). K^+ stimulation of ATPase activity was increased by R treatments whether or not boric acid was present in the homogenization medium. When these experiments were repeated in the absence of boric acid, an increase of specific activity by R was usually observed, but the extent of this increase varied. Levels of specific ATPase activity in these experiments were not significantly affected by either treatment, or else were slightly enhanced by FR treatment.

The effect of *in vitro* R and FR on ATPase activity was examined at pH 6.0, 6.5 and 6.75 and at ATP concentrations of 2.0, 1.0 and 0.5mol m^{-3}. Seven consecutive experiments showed there to be no consistent affect of R or FR on ATPase activity with both inhibition and stimulation of activity by R being demonstrated.

5.2.3 Apical hook experiments.

5.2.3.1 Effect of ATP concentration, temperature and pH on R/FR modulation of ATPase activity.

Tissue from the apical hook region of the hypocotyl (Region 1A) was excised under the green safe-light, placed in homogenization buffer on ice and given 30 min exposure to R. With the inclusion of H_3BO_3 (5mol m^{-3}) in the homogenization medium, the effect of R or FR treatments on ATPase activities was examined at varying ATP concentrations. The Mg^{2+} concentration was kept equal to that of ATP.

The effect of varying ATP concentrations was examined in five separate experiments. Figures 5.3a, 5.3b, 5.4a and 5.5a show typical results from two experiments when carried out in an ATPase incubation medium at pH 6.5 and 25°C. Out of five experiments, stimulation of ATPase

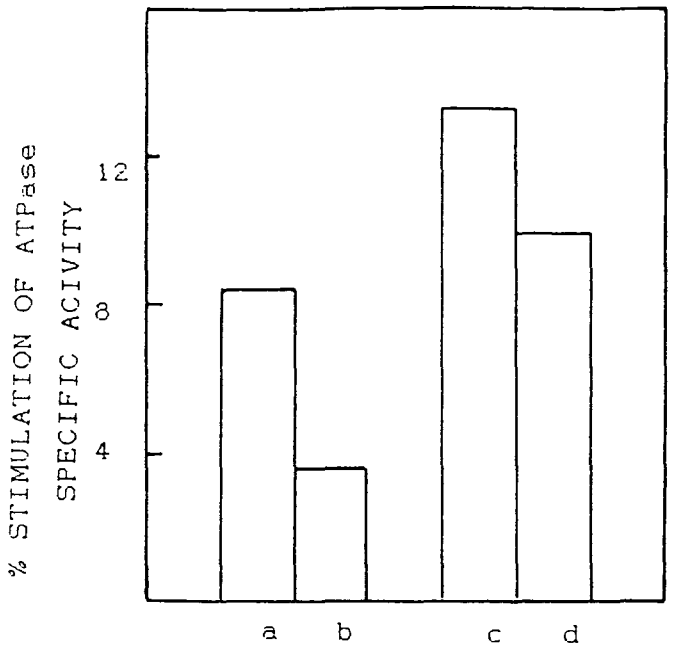


Figure 5.2. Effect of R and FR treatment on the percentage stimulation of ATPase activity in a microsomal fraction by 50mol m^{-3} KCl prepared from regions I - IV of the hypocotyl. Tissue was ground with (a,b) the standard homogenization medium and (c,d) with the incorporation of 5mol m^{-3} boric acid. An *in vivo* R treatment was given for 30 min and the microsomal fractions were given either (a,c) R for 10 min or (b,d) FR for 5 min. The standard ATPase assay was used. Each result is from one experiment and the mean of three replicates.

Figure 5.3a. Effect of ATP assay concentration on percentage stimulation of ATPase activity in a microsomal fraction at pH 6.5 from region 1A of the hypocotyl. The tissue was illuminated with R for 30 min and ground in the standard homogenization medium with the incorporation of boric acid as described in "Materials and Methods". Microsomal fractions were illuminated with R for 10 min or FR for 5 min. The standard ATPase assay was used with the following modifications. An assay temperature of 25°C and pH of 6.5 were used. The assay was carried out at ATP concentrations of (a,b) 2mol m^{-3}, (c,d) 1mol m^{-3} and, (e,f) 0.5mol m^{-3}. Percentage change of activity by R was calculated (a,c,e) in the absence of potassium and (b,d,f) in the presence of 50mol m^{-3} KCl.

Figure 5.3b. Effect of ATP concentration on percentage stimulation of ATPase activity in a microsomal fraction prepared from region 1A of the hypocotyl. Tissue was illuminated with R for 30 min and ground in the standard homogenization medium as described in "Materials and Methods". Microsomal fractions were either illuminated with R for 10 min or FR for 5 min. The standard ATPase assay was used with the following modifications. An incubation temperature of 25°C and assay pH of 6.5 were used. The assay was carried out at ATP concentrations of (a,b) 2mol m^{-3}, (c,d) 1mol m^{-3}. Percentage change of activity by R was calculated (a,c) in the absence of KCl and (b,d) in the presence of 50mol m^{-3} KCl.

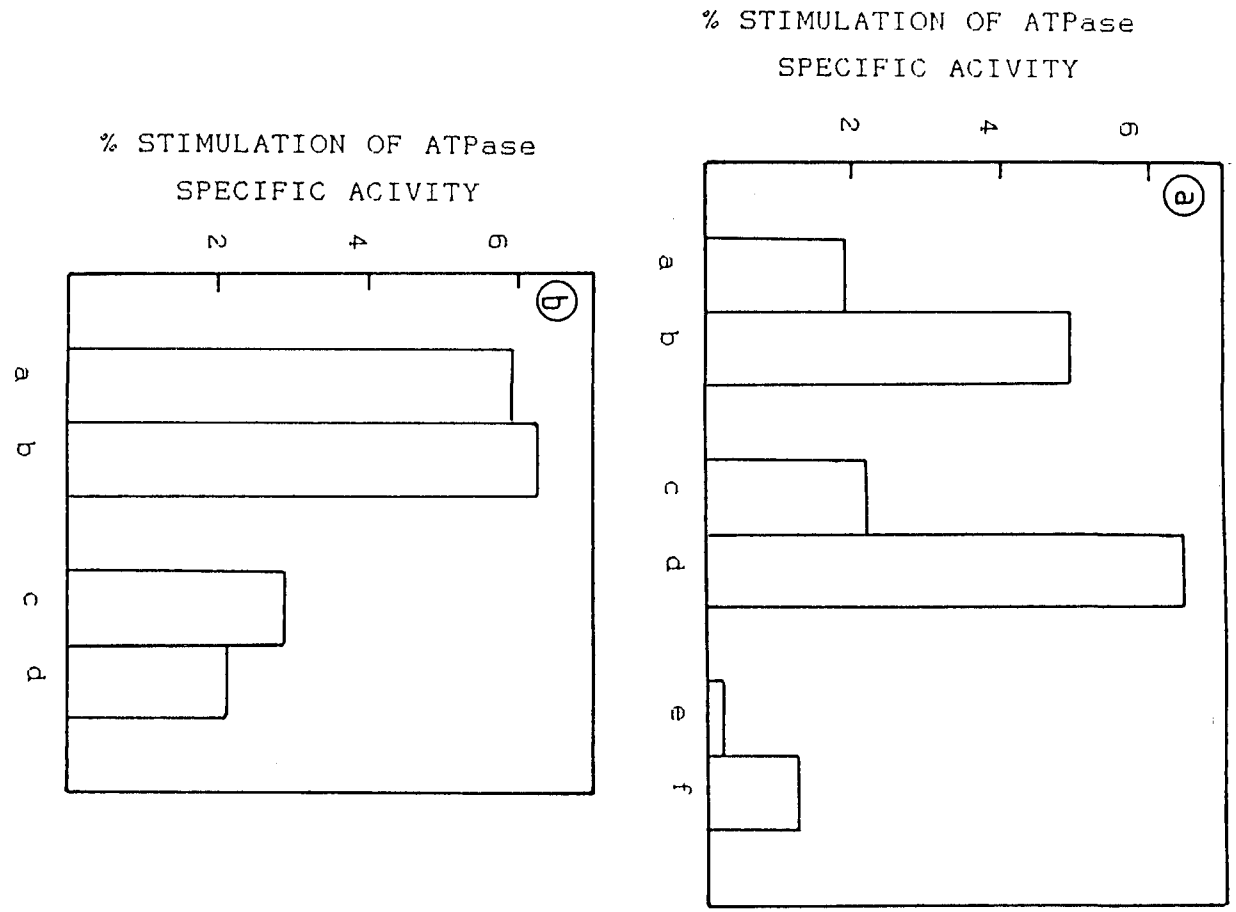
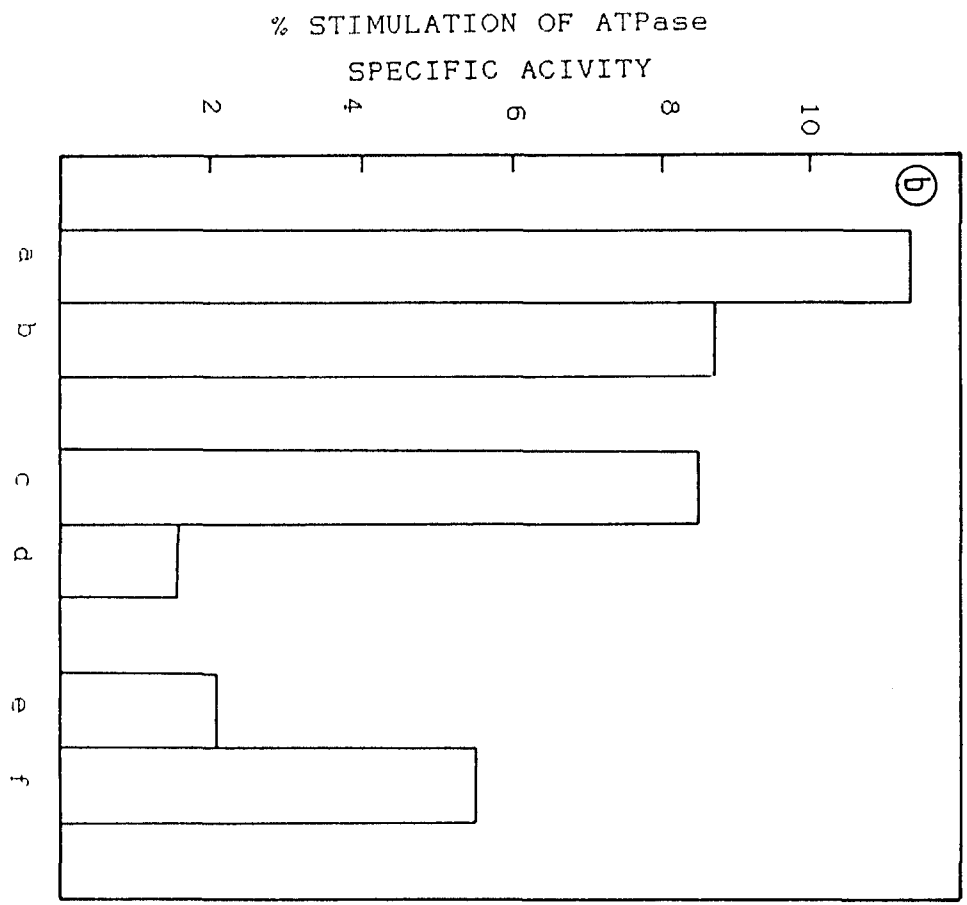

% STIMULATION OF ATPase
SPECIFIC ACIVITY
2
4
6
(b)
a
b
c
d
% STIMULATION OF ATPase
SPECIFIC ACIVITY
2
4
6
(a)
a
b
c
d
e
f

Figure 5.4a. Effect of ATP concentration in the incubation medium on the percentage stimulation of ATPase activity at pH 6.5 in a microsomal fraction prepared from region 1A of the hypocotyl. Tissue was illuminated with R for 30 min, ground in the standard homogenization medium as described in "Materials and Methods". Microsomal fractions were either illuminated with R for 10 min or FR for 5 min. The standard ATPase assay was used with the following modifications. An incubation temperature of 25°C and assay pH of 6.5 were used. The assay was carried out at ATP concentrations of (a,b) 3mol m^{-3}, (c,d) 2mol m^{-3}, (e,) 1mol m^{-3}. Percentage change of ATPase activity caused by R over FR was calculated (a,c,e) in the absence of KCl and (b,d) in the presence of 50mol m^{-3} KCl.

Figure 5.4b. Effect of ATP concentration on percentage stimulation of ATPase activity at pH 6.0 in a microsomal fraction prepared from region 1A of the hypocotyl. Tissue was illuminated for 30 min R and ground in the standard homogenization medium as described in "Materials and Methods". Microsomal fractions were either illuminated with R for 10 min or FR for 5 min. The standard ATPase assay was used with the following modifications. An incubation temperature of 25°C was used and the assay pH used was 6.0. The assay was carried out at ATP concentrations of (a,b) 3.6mol m^{-3}, (c,d) 2.7mol m^{-3}, (e,f) 1.8mol m^{-3}. Percentage change of ATPase activity was calculated (a,c,e) in the absence of KCl and (b,d,f) in the presence of 50mol m^{-3}.


% STIMULATION OF ATPase
SPECIFIC ACIVITY
2
4
6
8
10
a
b
c
d
e
f
(b)

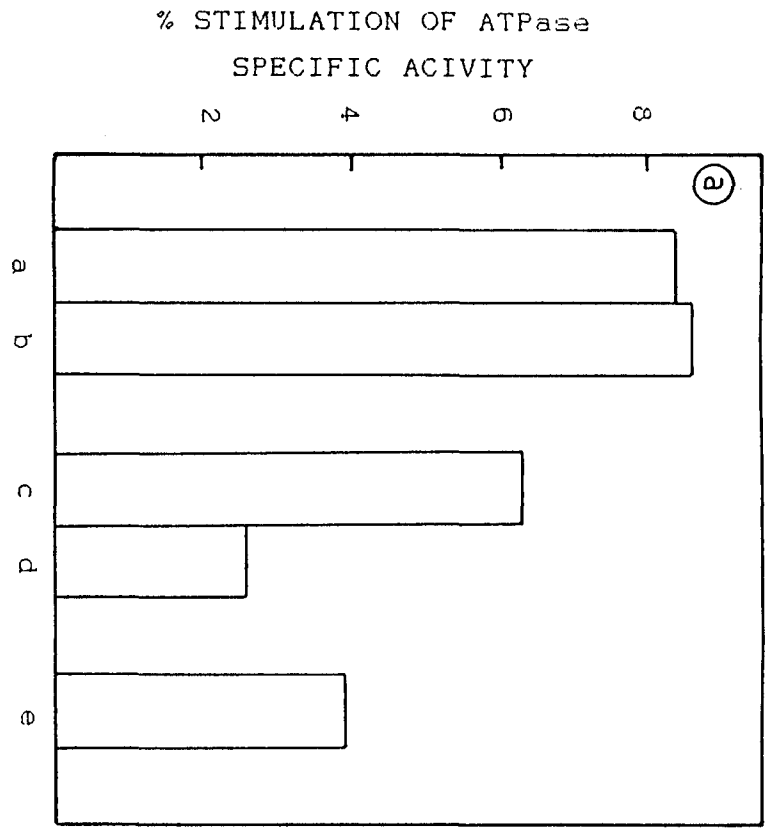
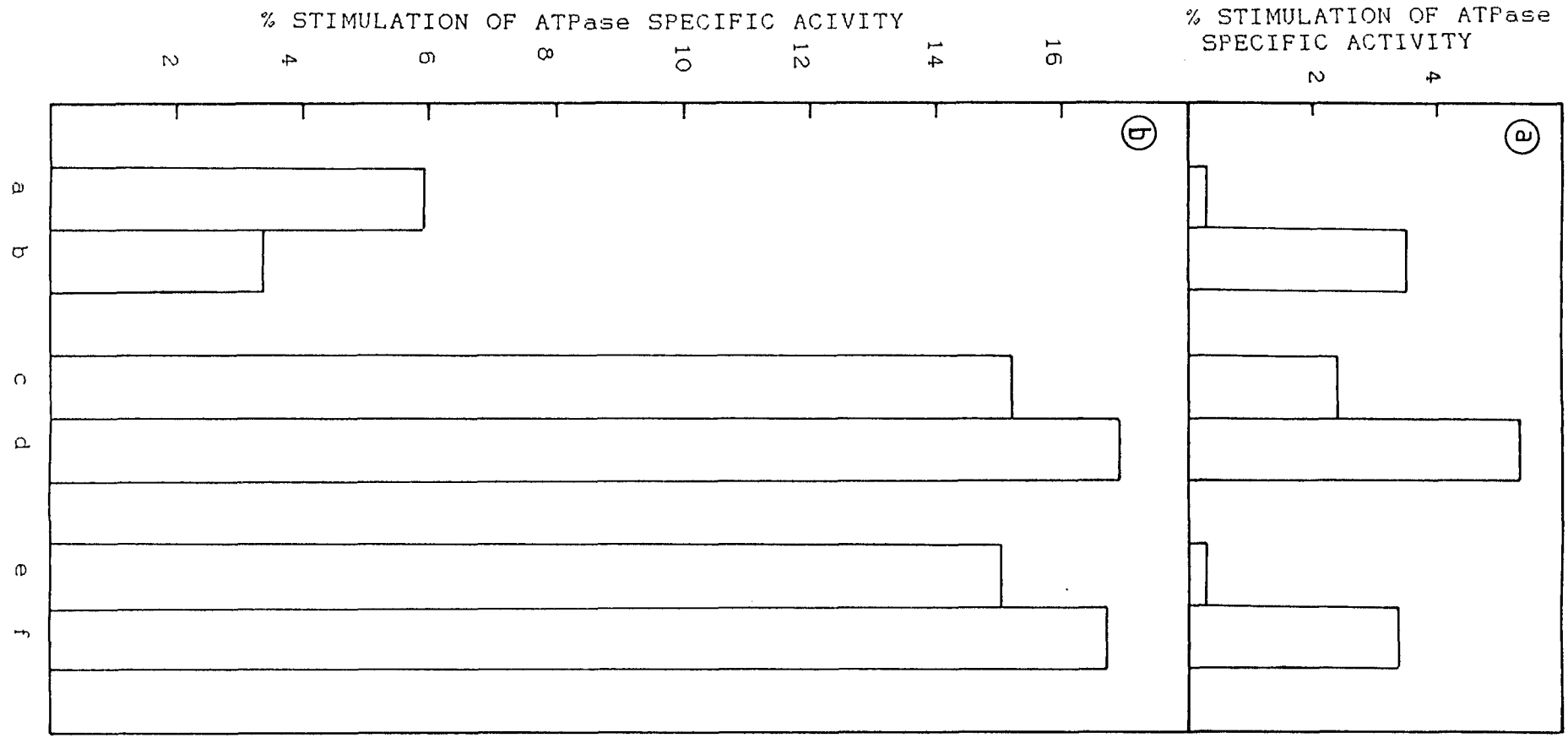

% STIMULATION OF ATPase
SPECIFIC ACIVITY
2
4
6
8
a
b
c
d
e
(a)

Figure 5.5a,b. Effect of ATP concentration on percentage stimulation of ATPase activity (a) at pH 6.5 and (b) 6.0 in a microsomal fraction prepared from region 1A of the hypocotyl that had been illuminated for 30 min with R. Following illumination the tissue was ground in the standard homogenization medium as described in "Materials and Methods". Microsomal fractions were either illuminated with R for 10 min or FR for 5 min. The standard ATPase assay was used with the following modifications. An assay temperature of 25°C was used. ATP concentrations of (a,b) 4mol m^{-3}, (c,d) 3mol m^{-3} (e,f) 2mol m^{-3} were used. Percentage change of activity was calculated (a,c,e) in the absence of KCl and (b,d,f) in the presence of 50mol m^{-3} KCl.


% STIMULATION OF ATPase SPECIFIC ACIVITY
2
4
6
8
10
12
14
16
b
a
b
c
d
e
f
% STIMULATION OF ATPase SPECIFIC ACTIVITY
2
4
a

activity by R was observed on four occasions, but no consistent differences were seen between any of the ATP concentrations examined. On the other occasion no effect was observed by R or FR. K^+ stimulation of the ATPase at the ATP concentrations examined, showed there to be no differential effect by R or FR treatment.

Four identical experiments were carried out, but at pH 6.0, and results from two such experiments are illustrated (Figs.5.4b, 5.5b). K^+ stimulated ATPase activity was increased at this pH, but the light treatments caused no change of specific activity. No consistent difference was seen of the light treatments on ATPase activity at the different ATP concentrations at this pH.

When the experiment was repeated with 2.5mol m^{-3} H_3BO_3 in the homogenization medium, no effect of light on ATPase activity was found.

5.2.3.2 Effect of reducing the length of *in vitro* light treatments.

In vitro irradiation of the microsomal fraction was reduced to 5 min exposure to R and 10 min FR. The standard ATPase assay was altered to include a range of Mg^{2+} and K^+ concentrations. When 2mol m^{-3} Mg^{2+} and 50 and 25mol m^{-3} KCl were included in the assay, R had no effect on ATPase activity. At 3mol m^{-3} Mg^{2+} and 50mol m^{-3} KCl, R was found to be stimulatory to a small degree, but when KCl was reduced to 25mol m^{-3} this effect was lost. Identical experiments were performed, using apical hooks which had been grown at 25°C. R or FR treatments were found to be without effect on the ATPase activity.

5.2.3.3 Effect of using a different filter combination.

ATPase activity was measured in tissue that had been illuminated with R *in vivo* and either R or FR *in vitro* using light sources and filter combination as described by Roth-Bejerano & Hall (1986). R was found to have no stimulatory effect on ATPase activity at 2,3 or 4mol m^{-3} ATP.

5.2.3.4 Modulation of ATPase from enriched membrane fractions.

The effect of R illumination *in vivo* and R or FR *in vitro* on ATPase activity was examined in the five enriched membrane fractions from the six step sucrose gradient prepared as described in "Materials and Methods". No effect of R was found at ATP concentrations of 4 or 3mol m^{-3} and 50 and 25mol m^{-3} KCl.

5.2.3.5 Modulation of active H^+ transport by R or FR.

Following R treatments *in vivo*, R and FR treatments were given *in vitro* and their effect on H^+-pumping ATPase in a microsomal fraction from apical hooks was examined. The fluorescence mixture was prepared and vesicles added, equilibrated and illuminated for 5 min with R or FR from the fluorimeter at 660 or 720nm or with FR using a Balzer filter. The standard assay was used with either 1 or 0.2mol m^{-3} concentrations of ATP-BTP. No consistent differences were observed in the initial slopes or the extent of proton pumping following either a R or FR treatment.

5.2.5 Effect of R and FR on membrane-associated ATPase activity prepared from cotyledon tissue.

Microsomal membrane fractions were prepared from from *Cucumis* cotyledons that had been given a 5 min R treatment *in vivo*. ATPase activity was assayed at pH 6.0 and at ATP concentrations of 4.0, 3.0 and 2.0molm^{-3}. R had a small stimulatory effect on ATPase activity in one experiment at 2mol m^{-3} only, but in the following two experiments ATPase activity was unaffected by the light treatments.

5.3 Discussion.

It was anticipated that a varying effect of ATPase from different regions of the hypocotyl might be obtained by R and FR treatments (Jose 1977); membrane-associated ATPase activity prepared from regions 1 - V of the *Cucumis* hypocotyl showed no stimulation in response to R or FR, while the apical hook was found to be R stimulated. Roth-Bejerano & Hall (1986a) obtained similar results with

apical hooks from *Cucumis*. The hypocotyl may contain distinct populations of ATPase some of which are regulated by light. The apical hook region may contain an enriched population of ATPase, that is R activated, while the lower region may contain two populations, one that is R inhibited and a larger population that is unaffected by light. This population would mask any affect that light may have on the ATPase associated with the smaller population.

The incorporation of boric acid into the homogenization medium appeared to enhance the stimulatory effect of an *in vivo* FR and R treatment on ATPase activity. Boron may interact directly with the membrane *via* glycoprotein and glycolipid components to maintain an active conformation (Parr & Loughman 1983).

It is assumed that P_{fr} remains reversible by FR after isolation of membrane fractions. Blakeley *et al.* (1983) reported that protoplast swelling in wheat is photoreversible if the period between R and FR treatments is less than 5 min. However, the escape from FR reversibility was relatively rapid, being lost after approximately 1 min at 4°C in experiments with maize. Jose (1977) carried out irradiation treatments at room temperature, as did Roth-Bejerano & Hall (1986b) which were long after the initial R treatment and obtained FR reversals. Similar FR reversions were carried out 2 h after homogenization with *Cucumis* in this work at room temperature and on ice and neither enhanced the light affect on ATPase activity.

Examination of the effect of light treatments on ATPase activity at different ATP concentrations gave no consistent results. In this area there is conflict with previous reports using the same *Cucumis* tissue. Roth Bejerano & Hall (1986a), reported that the V_{max} is altered by light treatments while Thomas & Tull (1981) reported that the K_m for ATP is altered rather than V_{max}.

Varying reports of the effect of R treatments while on ATPase activity have been made. Blakeley *et al.* (1987) found no evidence for any R or FR effect on ATPase activity *in vitro* in wheat leaf protoplasts. They suggested that this lack of effect might be due to loss or

proteolytic degradation of phytochrome during preparation of the microsomal fraction. Alternatively, the plasma membrane could be altered during protoplast isolation. On the other hand Blakeley *et al.* (1987) reported that changes in the solute potential within protoplasts were modulated by light and inhibited by vanadate. This suggests that uptake was occurring *via* a plasma membrane located ATPase, but could provide no strong evidence of $^{86}Rb^{+}$ uptake being enhanced by R.

The absolute amount of phytochrome in embryonic axes of cucumber seeds (Harrison 1986) was found to be low compared with concentrations in etiolated seedlings of the same species (Angelakais & Frankland 1986). Three to 5 h after imbibition a rise in the phytochrome concentration occurs which is due to rehydration of pre-existing phytochrome and this has been found to be unaffected by light (Hilton & Thomas 1985). Another large increase follows after 5 h and represents *de novo* synthesis of P_r and may be prevented by irradiation with R or B in *Amaranthus* (Kendrick *et al.* 1969). Thus at the time of harvesting *Cucumis*, large pools of P_r would be present. Levels of phytochrome within an etiolated hypocotyl would be expected to be higher in the apical hook region. A monoclonal antiphytochrome antibody has been used to detect bound phytochrome and its distribution in dark grown maize seedling has been assessed (Schwarz & Schneider 1987). Regions of high phytochrome accumulation were found in the coleoptile tip, root cap and shoot apex: mesocotyl and leaves contained relatively low amounts of phytochrome, which was almost uniformly distributed throughout the organs. Distribution of phytochrome was found not to alter during the development of the seedlings. The results obtained in this work support this finding in that only the ATPase associated with the apical tip was found to be responsive to phytochrome *via* R and FR.

The finding that FR treatment *in vitro* had no effect on ATPase activity whereas R enhanced activity, is in agreement with the data obtained with *Cucumis* (Roth Bejerano & Hall 1986b) and provides support for the control of ATPase *via* phytochrome.

Strong evidence is provided that phytochrome modulation is *via* a plasma membrane located ATPase. Its activity, as indicated by medium acidification, was found to increase with R treatment (Roth-Bejerano & Hall 1986a), suggesting that hypocotyl elongation may be stimulated by R.

FR is also inhibitory and R to a lesser extent to growth of etiolated five-day-old seedlings (Black & Shuttleworth 1974) R and FR were equally inhibitory to growth of four-day-old seedlings. This data for de-etiolated tissue suggests that P_{fr} is the form that is inhibitory to growth and it would be expected that ATPase would be stimulated by FR.

The effect of cotyledons on the growth of de-etiolated *Cucumis sativus* hypocotyls has been examined (Gaba & Black 1985). With the cotyledon attached, recovery from light inhibition started at 8.5 h, whereas when the cotyledon is removed, recovery of the hypocotyl from inhibition begins after 4 h. With etiolated tissue , covering of the cotyledons reduced the inhibition of hypocotyl growth by continual R (Black & Shuttleworth 1974). Whether or not the cotyledons are covered, FR is inhibitory. The behaviour of de-etiolated tissue in response to R and FR is usually an inhibition response, with FR becoming less inhibitory as the plant ages. It is therefore surprising that any stimulation of ATPase by R is observed.

5.4 Conclusions.

R was found to stimulate membrane bound ATPase activity in a microsomal fraction derived from the apical hook region of *Cucumis* hypocotyls (Region 1A). No R or FR effect was found in a microsomal fraction when the complete hypocotyl was used and no effect of R or FR on ATPase activity was found in any membrane enriched fraction, possibly because any differential activity was lost during the centrifugation period. R and FR treatments were found to have no effect on H^+-pumping ATPase activity.

CHAPTER SIX.

Evidence for an active Ca^{2+} transport mechanism operating in membrane vesicles from *Cucumis* hypocotyls.

6.1 Introduction.

6.1.1 Chlorotetracycline as a Ca^{2+} probe.

Many of the experimental techniques used initially for monitoring the movement of Ca^{2+} into cells gave only sequential measurements. More recently fluorescence methods have been used to study the time course of Ca^{2+} movement, and offer the dual advantage of a rapid and continuous measurement of Ca^{2+}-associated fluctuations. The fluorescent Ca^{2+} probe, chlorotetracycline (CTC) has seen widespread use in a large number of systems (e.g. Caswell 1972) and has been shown to be an accurate and quantitative measure of the internal calcium concentration (Dixon *et al.* 1984). Thus Caswell & Hutchison (1971b) showed that CTC fluorescence increased as a result of active Ca^{2+} accumulation into vesicles of sarcoplasmic reticulum and mitochondria origin.

The fluorescence response of CTC to Ca^{2+} has been monitored by pre-equilibrating vesicles with solutions of known Ca^{2+} concentration followed by dilution in a Ca^{2+} free medium containing CTC (Dixon *et al.* 1984). The mechanism was shown to be based on passive accumulation of CTC as a Ca^{2+}-CTC complex and its association with the organelle. The technique has seen widespread application in studies of the sarcoplasmic reticulum (Caswell 1972, Caravalho & Caravalho 1977). Caswell (1979) has also discussed the use of CTC as a Ca^{2+} probe for use in the study of islets of Langerhan cells, excocrine acinar cells, platelets, human sperm cells and insect salivary glands.

Dixon *et al.* (1984) observed that CTC does not measure membrane-bound Ca^{2+} as such. For a Ca^{2+} enhanced signal to be monitored the Ca^{2+}-CTC complex must bind to the membrane, but the binding is more appropriately considered as an intermediate step in the indicator

reaction. It is not necessary to have a Ca^{2+} binding site on the membrane for an enhancement of CTC fluorescence in response to elevations in cytoplasmic Ca^{2+} concentrations. Effects of Ca^{2+} on CTC fluorescence can be observed on lecithin membranes which are electrically neutral and have virtually no ability to bind Ca^{2+}. In this case, the reaction clearly involves binding of Ca^{2+}-CTC to a neutral membrane at non-specific binding sites.

Tetracycline antibiotics show a propensity for chelation with divalent cations. The selectivity of chelation with a range of cations in aqueous solutions has been determined, and it is considered that this chelation plays a role in its reported bacteriostatic properties, (Doluisio & Martin 1963, Cammarate & Yau 1970) and the deleterious action on bone and tooth growth (Saxen 1965).

In an aqueous solution, the emission spectrum for Ca^{2+}-CTC or Mg^{2+} chelate is similar, but in 90% methanol a difference is observed (Caswell & Hutchison 1971a). The emission spectrum of the Mg2+-chelate has a peak at 520nm while that of the Ca^{2+}-chelate peaks at 530nm. This phase change might be used to determine the concentration of the Ca^{2+}-chelate in methanol. CTC not only binds to Ca^{2+}, but binds the following cations with the order of affinity in the aqueous phase of $Zn^{2+} > Sr^{2+} > Mg^{2+} > Ca^{2+}$ and thus fluorescence changes must be taken into account if concentrations of these ions are altered.

Chlorotetracycline has been used as a probe for Ca^{2+} uptake into microsomal vesicles from zucchini (Lew *et al.* 1986). Uptake was found to be dependent on ATP and release of Ca^{2+} from the vesicles was possible with the Ca^{2+} ionophore A23187. Characterization of a Ca^{2+} transport system in endoplasmic reticulum vesicles from garden cress was also carried out using $^{45}CaCl_2$ (Buckhout 1984). The characteristics described by these two groups agreed with regard to K_m values for ATP and Ca^{2+} and for pH dependence. These reports suggest that chlorotetracycline may be used to monitor real-time changes in Ca^{2+} levels.

6.1.2 Use of inhibitors to determine the mechanism of Ca^{2+} transport and modes of regulation.

The characteristics of plasma membrane/endoplasmic reticulum and tonoplast associated Ca^{2+} transport has been reviewed in the general introduction. Membrane inhibitors and protonophores have been employed to determine if Ca^{2+} transport is *via* a direct coupling to ATP hydrolysis or by a H^+ antiport/symport.

Calmodulin has been implicated in the regulation of Ca^{2+} transport. Calmodulin, an intracellular Ca^{2+} receptor, is widely distributed in many plant cell types (Cheung 1980). The involvement of calmodulin in Ca^{2+} transport has been investigated primarily in intact red blood cell membranes and in purified, reconstituted vesicles (Niggli *et al.* 1981). In the presence of micromolar concentrations of Ca^{2+}, calmodulin has been found to bind directly to the transport protein of red cell membranes and to regulate activity by increasing the transport rate and the affinity of the enzyme for Ca^{2+}. In animal systems, several varying reports exist concerning the responses of Ca^{2+} transport to calmodulin. Stimulation has been reported in gastric smooth muscle, plasma membrane vesicles and pancreatic islet cell transport, but not in the endoplasmic reticulum from gastric smooth muscle or pancreatic islet cells (Raeymaekers *et al.* 1983, Colca *et al.* 1983). Stimulation of Ca^{2+} uptake by native calmodulin has been reported in zucchini hypocotyl hooks (Dieter & Marmé 1980,1981) but no effect was reported with endoplasmic reticulum vesicles from Lepidium sativum (Buckhout 1984).

Investigations into the action of calmodulin have been helped by the use of the antipsychotic agents such as trifluoperazine, benperidol and pimozide which are all potent competitive calmodulin antagonists. For example, TFP is a competitive antagonist of cyclic AMP phosphodiesterase of brain (Levin & Weiss 1976). Phenothiazine neuroleptics have been used to examine if calmodulin regulates Ca^{2+} uptake into vesicles prepared from *Lepidium sativum* (Buckhout 1984).

Although phenothiazines inhibit calmodulin binding,

these local anaesthetics bind by a hydrophobic interaction. Because of this hydrophobicity, they may affect processes other than those mediated by calmodulin (Roufogalis 1981, Schatzman *et al.* 1981). Examination has been made of such nonspecific hydrophobic interactions on active Ca^{2+} uptake by the use of other local anaesthetics for example dibucaine and tetracaine which interact hydrophobically with membranes (Schatzman *et al.* 1981). Ca^{2+} uptake into vesicles was inhibited by both these anaesthetics (Buckhout 1984).

Cytoplasmic Ca^{2+} concentrations have been manipulated using inhibitors which prevent Ca^{2+} sequestration into membranes and vesicles. For example, ruthenium red has been reported to inhibit Ca^{2+} uptake into mitochondria (Vasington *et al.* 1972, Ash & Bygrave 1977), while fluorescein isothiocyanate is thought to inhibit calcium uptake into isolated sarcoplasmic reticulum vesicles (Pick & Kalish 1980, Mitchinson *et al.* 1982). Lanthanum acts as a competitive inhibitor in the presence of Ca^{2+}, due to its similar ionic radius and high charge density (Martin & Richardson 1979). In plants, these reagents have been used to study the role of calcium in various physiological processes e.g. pollen tube growth (Picton & Steer 1985) and auxin-induced growth (Cunninghame & Hall 1986).

In this thesis the fluorescent probe chlorotetracycline has been used to monitor temporal changes in Ca^{2+} uptake and binding using vesicle preparations from *Cucumis* hypocotyls. The characteristics of active Ca^{2+} transport systems associated with microsomal vesicles and purified membrane fractions have been examined to ascribe a cellular location to the membranes containing an active Ca^{2+} transport mechanism. Following the work of Buckhout *et al.* (1980) of an auxin stimulated Ca^{2+} release from plant membranes, this has been assessed further in our system, together with the specificity of ruthenium red and fluorescein isothiocyanate and the effect of lanthanum on active Ca^{2+} transport. Investigations were also made into the possible occurrence of a Ca^{2+} stimulated ATPase.

6.2 Results.

6.2.1 Hypocotyl Ca^{2+} status and homogenization media required to obtain vesicles which actively transport Ca^{2+}.

Various homogenization media were used to obtain stable vesicles which transported Ca^{2+} in the presence of ATP. The factors affecting transport appeared to be the Ca^{2+} concentration within the tissue and the concentration that remained associated with the vesicles following isolation. Various concentrations of EGTA in the homogenization medium were used to remove Ca^{2+} and it was found that a concentration of 13mol m^{-3} allowed the production of vesicles which were capable of active Ca^{2+} transport. The initial calcium status of the hypocotyl tissue appeared to be variable but this homogenization medium produced vesicles which actively transported Ca^{2+}, if the Ca^{2+} concentration of the hypocotyl was about 5mol m^{-3} (Fig.6.1). This tissue level was obtained if the plants were watered with distilled water (0.1mol m^{-3} Ca^{2+}). When watered with tap water (2mol m^{-3}), plants had a calcium status of over 10mol m^{-3} in the tissue and ATP-dependent transport was not detectable.

The calcium concentration in each of the five regions of the hypocotyl was determined and was found to increase basipetally in plants that had been watered with tap water (Fig.6.2). The calcium status of the cotyledons (Fig.6.1) was found to be lower than that of the hypocotyl (<1mol m^{-3}). ATP-dependent Ca^{2+} transport occurred to a small extent in vesicles prepared from the cotyledons, which was reversible by the ionophore A23187.

Vesicles prepared from the hypocotyl were found to be very labile, with considerable loss of Ca^{2+} transport capacity occurring after 30 min of reannealing (Fig.6.3). Incorporation of 0.5% - 1% (w/v) BSA into the homogenization medium (Lew *et al.* 1986) had little effect on the retention of activity. Lability was overcome by incorporating 10% (w/v) glycerol into the resuspension medium and immediately freezing aliquots in liquid nitrogen. These were then used in sequence to maintain activity. The incorporation of glycerol into the assay mixture was found to be without effect on Ca^{2+} transport.

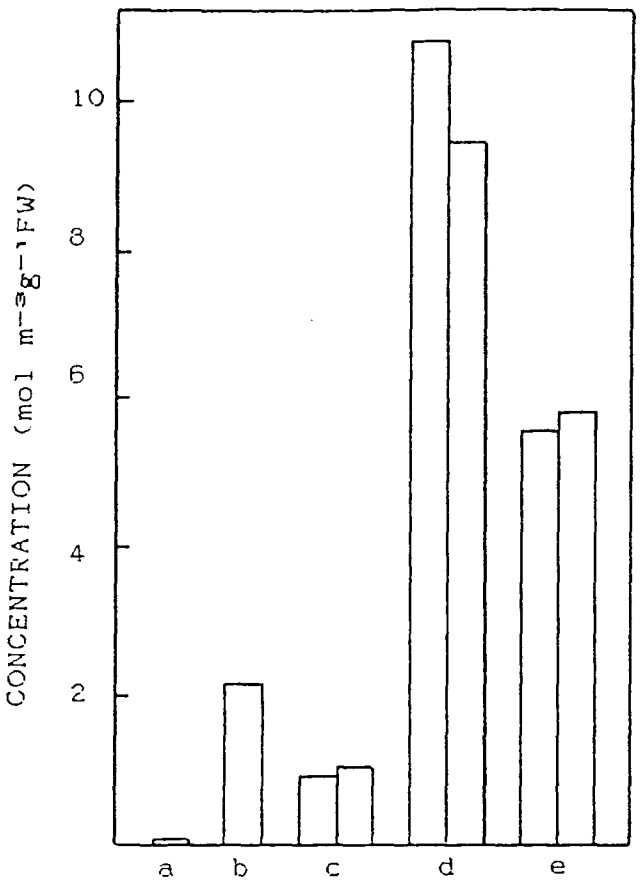


Figure 6.1. Calcium concentration of (a) distilled water and (b) laboratory tapwater. Determinations were made as outlined in "Materials and Methods". Five readings were taken per sample and an average calculated. Calcium concentration (mol m^{-3} g^{-1}FW) (c) of a 10,000*g* supernatant (Materials and Methods) (c) from cotyledons that had been watered with tap water, and (d) from hypocotyls that were watered with either tapwater, or (e) distilled water during a seven day growth period. Results illustrated are taken from two different experiments and each bar represents the mean of five readings.

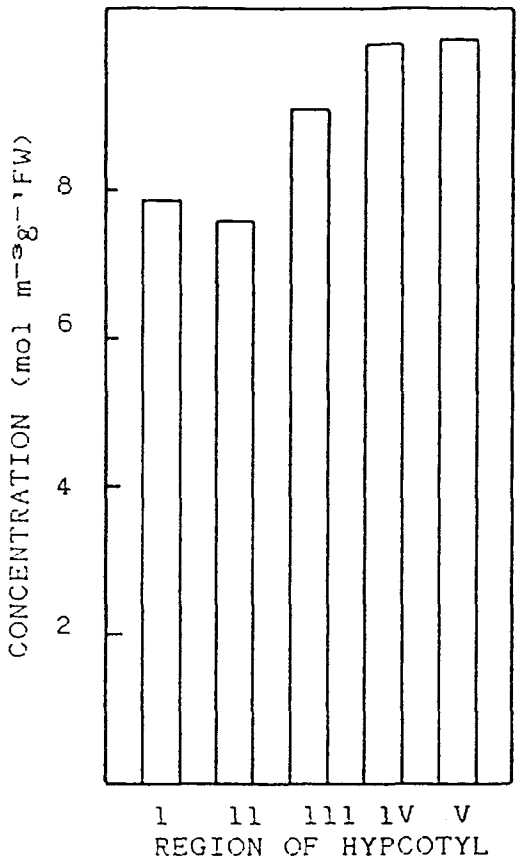


Figure 6.2. Calcium concentration of the 10,000*g* supernatant (Materials and Methods) prepared from the five hypocotyl regions (I - V) that had been watered with tap water for seven days during growth. Each result is the mean of five readings from one experiment.

6.2.2 Characterization of Ca^{2+} transport into membrane vesicles from the hypocotyl.

An example of non-ATP and ATP-dependent increase in chlorotetracycline fluorescence in a microsomal fraction is shown in Fig.6.3. Ca^{2+} transport did not occur when the calcium ionophore, A23187 was present. When A23187 was added during the steady state phase, Ca^{2+} re-equilibrated and the fluorescence level returned to near the initial value. Following the steady phase of Ca^{2+} transport, Ca^{2+} was found to gradually equilibrate with the external solution (Fig.6.17a). The concentration of protein per assay was found to be very critical, with optimal levels being in the order order of 0 $\mu g/0.5cm^3$ (Fig.6.4a). Higher levels were inhibitory to Ca^{2+} transport.

When chlorotetracycline concentrations within the assay mix were varied (Fig.6.4b), $25x10^{-3}$ mol m^{-3} was found to be optimal. Concentrations above and below this level were inhibitory to transport.

Pyrophosphate was tested for its ability to drive the movement of Ca^{2+} into the vesicles at pH values of 7.0, 7.5 and 8.0, but only minor Ca^{2+} transport was observed with this substrate (Fig.6.4b).

The optimum pH for Ca^{2+} transport in membrane vesicles was found to be pH 7.5 with ATP as substrate (Fig.6.5a). Sodium azide (1mol m^{-3}) caused no significant reduction of Ca^{2+} transport at pH 7.5 (Fig.6.5a). Oxalate stimulated calcium uptake into the vesicles with an optimum level of 10mol m^{-3} (Fig. 6.5b).

6.2.3 Effect of inhibitors on Ca^{2+} transport.

Vanadate was found to greatly reduce calcium uptake into the vesicles at concentrations of 1 - $20x10^{-3}$mol m^{-3}, while $250x10^{-6}$mol m^{-3} vanadate stimulated this movement (Fig.6.6a). Nitrate similarly reduced Ca^{2+} transport (Fig.6.6b).

Calcium uptake was also studied in tonoplast and plasma membrane-enriched fractions from a sucrose gradient. ATP-dependent Ca^{2+} transport was inhibited by nitrate and vanadate to the same extent in both fractions (Fig.6.7a,b). Vesicles which were prepared in the

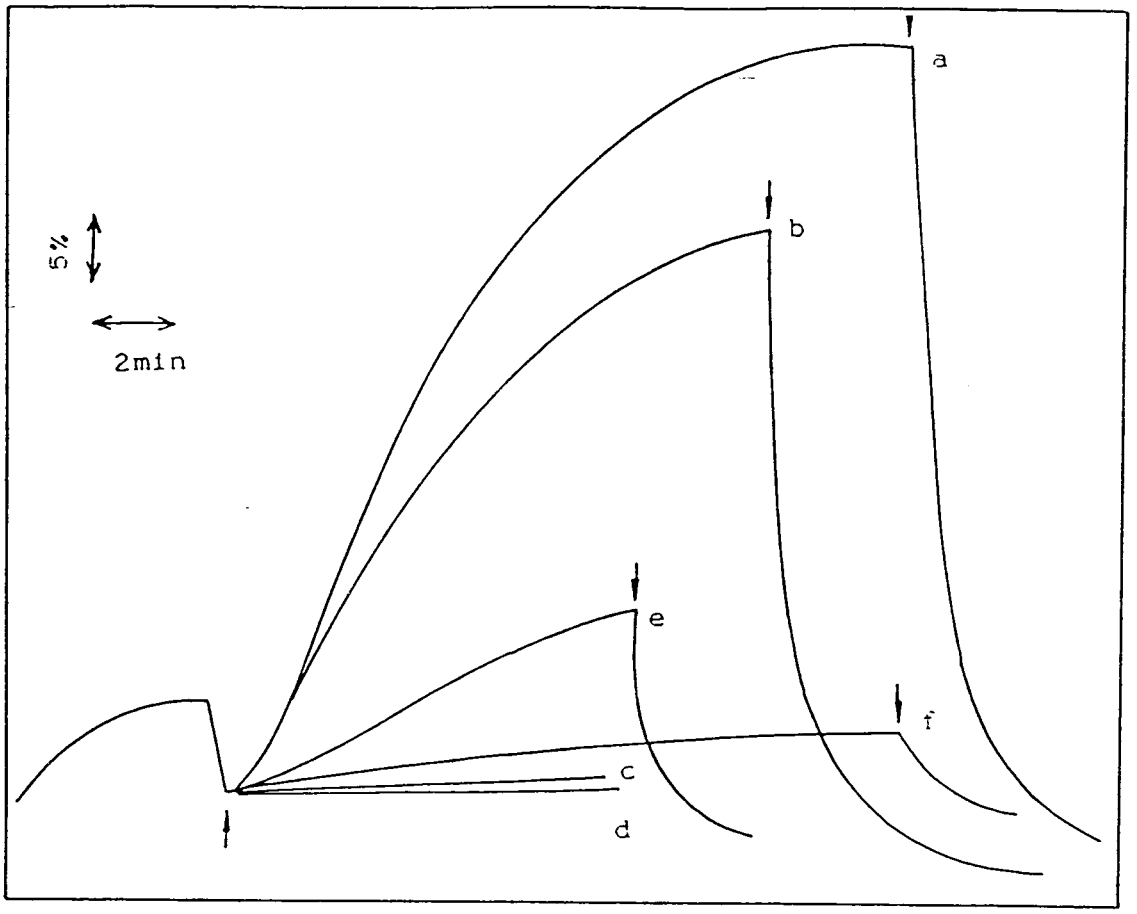
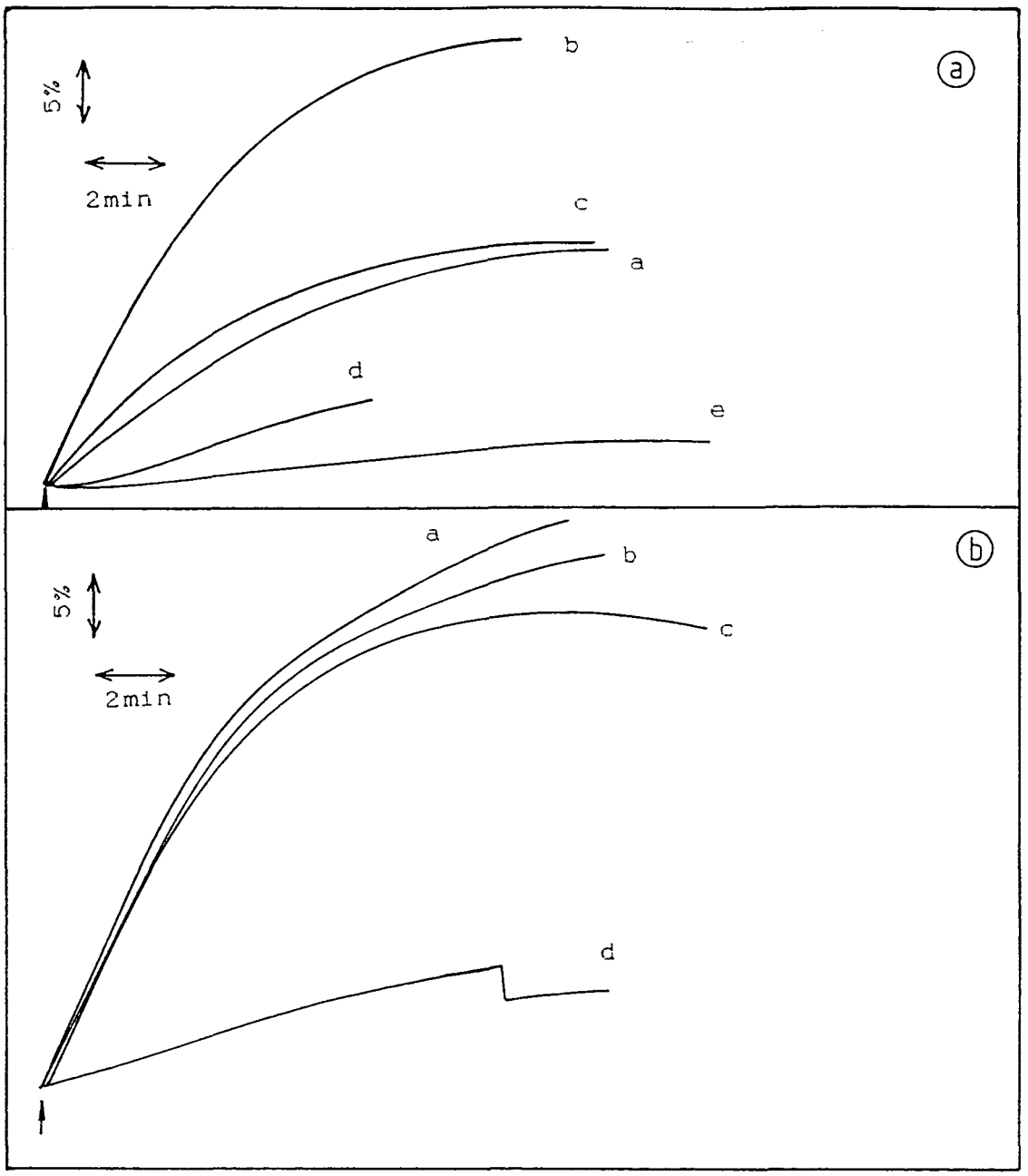


Figure 6.3. ATP-dependent increase in chlorotetracycline fluorescence in a microsomal fraction from *Cucumis* hypocotyls at various times after annealing. Vesicles were incubated in the standard assay medium as described in "Materials and Methods". Following equilibration the reaction was initiated by the addition of ATP (upward arrow) and Ca^{2+} equilibration was achieved by the addition of the calcium ionophore A23187 (5mm^3) at a final concentration of 7 µgcm^3 (downward arrows). The relative change in fluorescence is shown. In all subsequent experiments, each run was repeated at least twice and the whole experiment two times and a typical trace is illustrated. The assay was carried out (a) immediately after annealing and at subsequent time intervals of (b) 30 min, (c) 60 min without ATP, (d) 75 min in the presence of ATP and the ionophore A23187 (7µgcm^3) and, (e) 95 min in the presence of ATP. ATP-dependent increase in chlorotetracycline fluorescence in a microsomal fraction from *Cucumis* cotyledons (f).

Figure 6.4a. The effect of protein concentration on ATP dependent Ca^{2+} transport into microsomal vesicles. The reaction was initiated by the addition of ATP (upward arrow). The final protein concentrations used were (a) 15.9µg/0.5cm^3, (b) 31.7µg/0.5cm^3, (c) 63.5µg/0.5cm^3, (d) 126.9µg/0.5cm^3, and a control (e) with 31.7µg/0.5cm^3 and in the absence of ATP.

Figure 6.4b. The effect of chlorotetracycline concentrations on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard assay mixture was used with chlorotetracycline at concentrations of (a) 25x10^{-3}mol m^{-3}, (b) 35x10^{-3}mol m^{-3} and (c) 55x10^{-3}mol m^{-3}. PPi-dependent Ca^{2+} transport (d) was assayed using the standard assay but at pH 7.5 and with pyrophosphate (1mol m^{-3}) replacing ATP. The reaction was initiated by the addition of ATP or PPi (upward arrow).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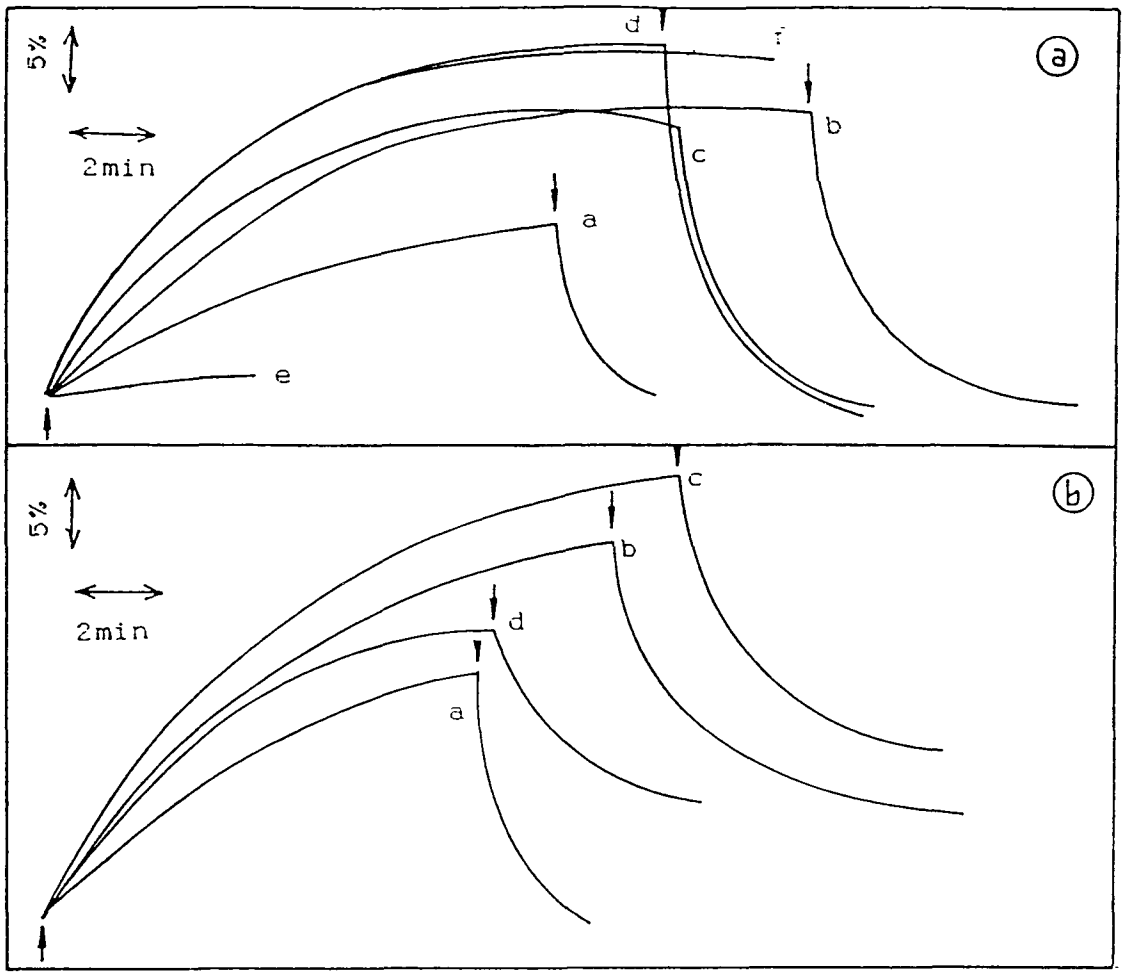
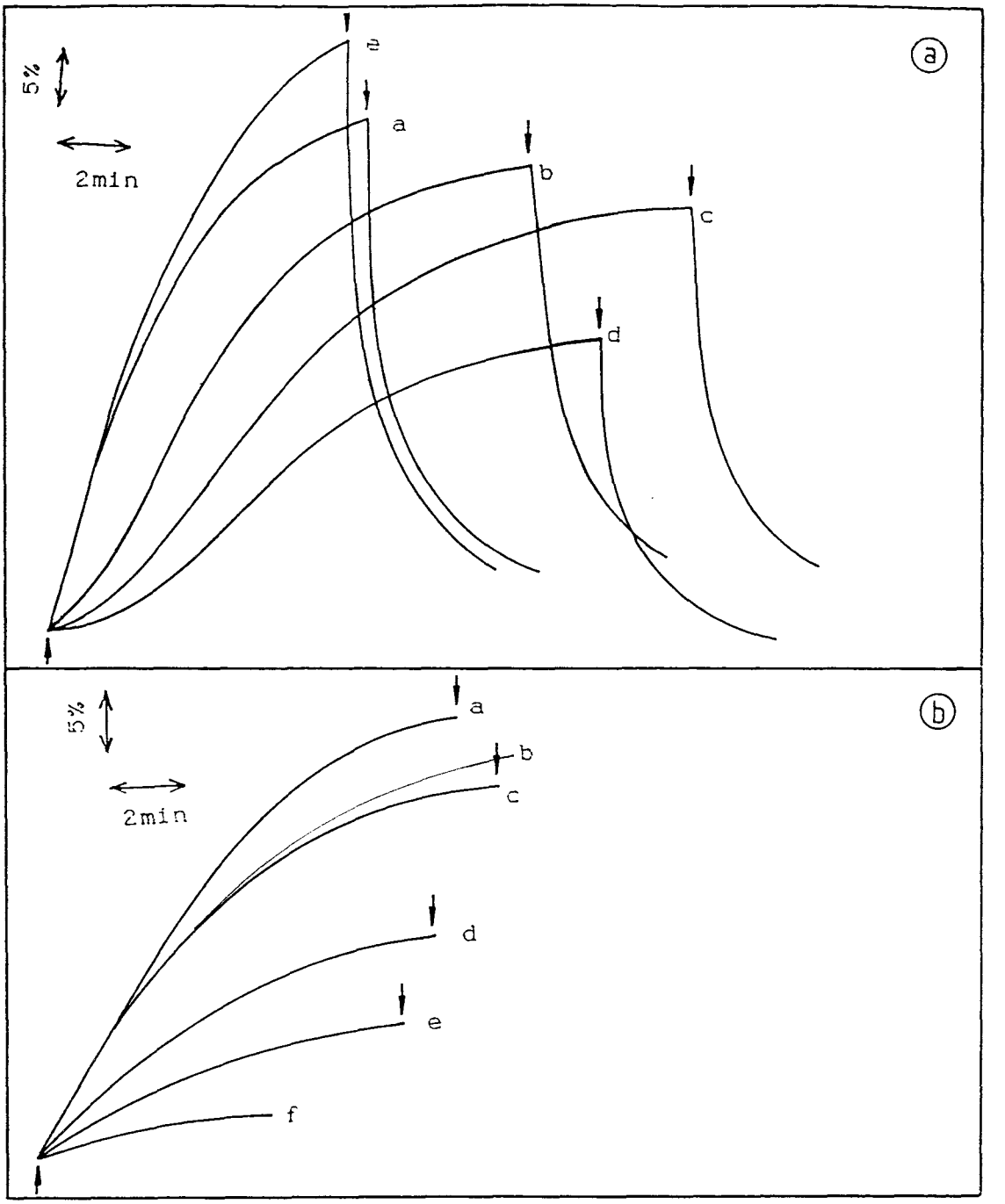


Figure 6.5a. The effect of pH on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used with the reaction being initiated by the addition of ATP (upward arrow) and reversed by the addition of A23187 (downward arrow). Transport was carried out at pH values of (a) 6.5, (b) 7.0, (c) 7.5, (d) 8.0 and (e) 8.5. Ca^{2+} transport was examined using the standard assay mixture with (f) the addition of sodium azide at a final concentration of 1mol m^{-3}.

Figure 6.5b. The effect of oxalate on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a) with the addition of oxalate at final concentrations of (b) 5mol m^{-3}, (c) 10mol m^{-3} and (d) 20mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow) and reversed by the addition of A23187 (downward arrows).

Figure 6.6a. The effect of vanadate on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a), with the addition of vanadate at final concentrations of (b) 10^{-3}mol m^{-3}, (c) $5x10^{-3}$mol m^{-3}, (d) $20x10^{-3}$mol m^{-3} and (e) $250x10^{-6}$mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow) and equilibrium restored of transported Ca^{2+} was made by the addition of A23187 (downward arrows).

Figure 6.6b. The effect of nitrate on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay mixture was used (a), with the addition of nitrate at final concentrations of (b) 25mol m^{-3}, (c) 50mol m^{-3}, (d) 100mol m^{-3}, (e) 150mol m^{-3} and (f) 200mol m^{-3} (f). The reaction was initiated by the addition of ATP (upward arrow).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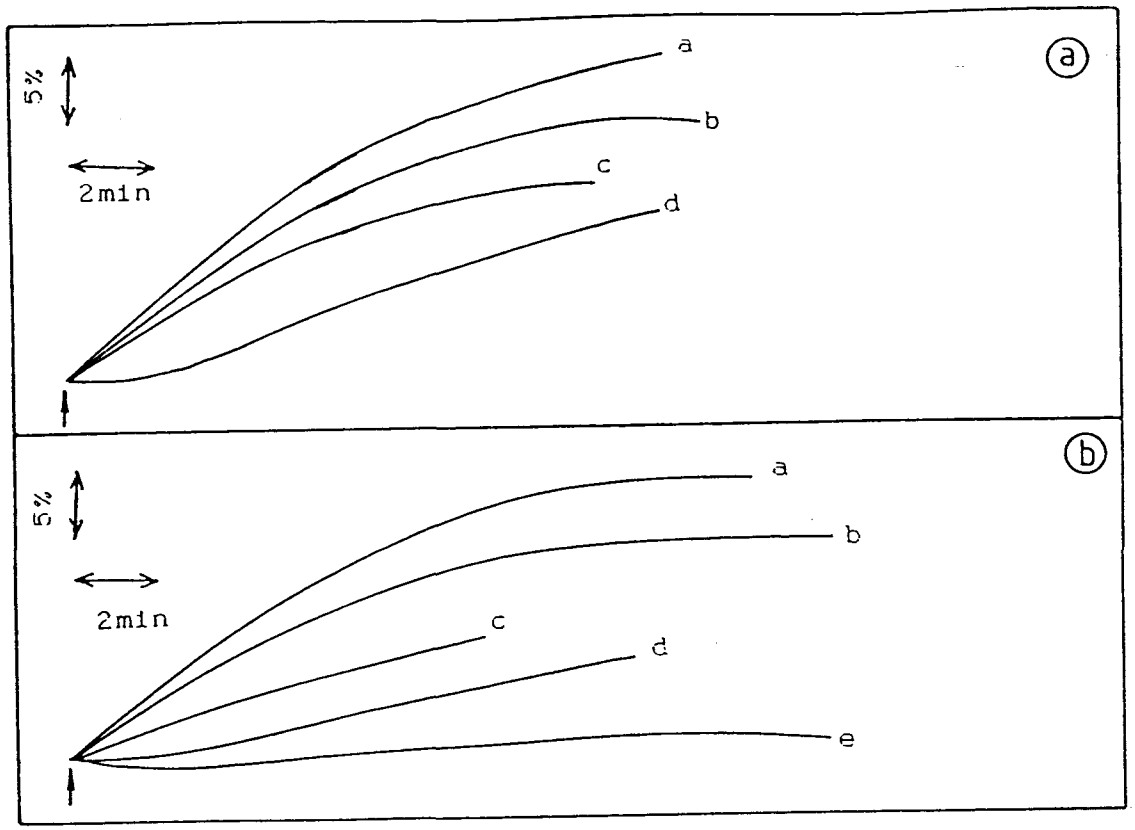


Figure 6.7a. The effect of nitrate and vanadate on ATP-dependent Ca^{2+} transport into vesicle fractions taken from interface 1 of the sucrose gradient. The standard chlorotetracycline assay was used (a) with the addition at final concentrations of (b) 25mol m^{-3} nitrate, (c) 50mol m^{-3} nitrate and (d) $5x10^{-3}$mol m^{-3} vanadate. The reaction was initiated by the addition of ATP (upward arrow).

Figure 6.7b. The effect of nitrate and vanadate on ATP-dependent Ca^{2+} transport into membrane vesicles derived from interface 4 of the sucrose gradient. The standard chlorotetracycline assay was used, (a) with the addition at final concentrations of (b) 25mol m^{-3} nitrate, (c) 50mol m^{-3} nitrate, (d) $5x10^{-3}$mol m^{-3} vanadate and (e) $20x10^{-3}$mol m^{-3} vanadate.

presence of Mg^{2+} and purified on a gradient with Mg^{2+} and Ca^{2+}, the cations being finally chelated with EGTA (Materials and Methods). These vesicles failed to transport Ca^{2+} in the presence of ATP.

Ca^{2+} uptake into microsomal vesicles was sensitive to erythrosin B at low concentrations. A concentration of $5x10^{-3}$mol m^{-3} gave approximately 50% inhibition of transport (Fig.6.8a) and complete inhibition at 10^{-2}mol m^{-3}. SW26 was also inhibitory to active Ca^{2+} transport into membrane vesicles (Fig.6.8b).

As noted earlier, azide was found to have no effect on calcium uptake at 1mol m^{-3} (Fig.6.5a). Ruthenium red, an inhibitor of calcium uptake into mitochondria, was found to inhibit ATP-dependent Ca^{2+} transport into microsomal vesicles (Fig.6.9). The Ca^{2+} uptake inhibitor fluorescein isothiocyanate was found to have an emission wavelength very close to that of chlorotetracycline; the output signal was enhanced many thousand-fold and under these conditions. Fluorescein isothiocyanate at a concentration of 10^{-2}mol m^{-3} completely inhibited active Ca^{2+} uptake. Lanthanum chloride markedly increased the initial binding of Ca^{2+} to the vesicles in the absence of ATP (Fig.6.10a). However, on the addition of ATP, lanthanum inhibited Ca^{2+} transport into the vesicles, with $50x10^{-3}$mol m^{-3} being totally inhibitory (Fig.6.10b).

6.2.4 Effect of ionophores on Ca^{2+} transport.

The H^+ gradient ionophore CCCP reduced active calcium uptake into microsomal vesicles, with complete inhibition occurring at a concentration of $50x10^{-3}$mol m^{-3} (Fig.6.11a). Similarly, transport was sensitive to the H^+/K^+ ionophore nigericin, with near full inhibition occurring at a concentration of 10^{-2}mol m^{-3} (Fig.6.11b).

DCCD increased ATP-dependent Ca^{2+} transport into microsomal vesicles in the concentration range 0.1 - 0.4 mol m^{-3} (Fig.6.12a) but became inhibitory at 0.6mol m^{-3}. Verapamil, an inhibitor of ion movement through voltage-gated channels when added to the preparation following equilibration in the presence of ATP, Ca^{2+} efflux was reduced (Fig.6.12b).

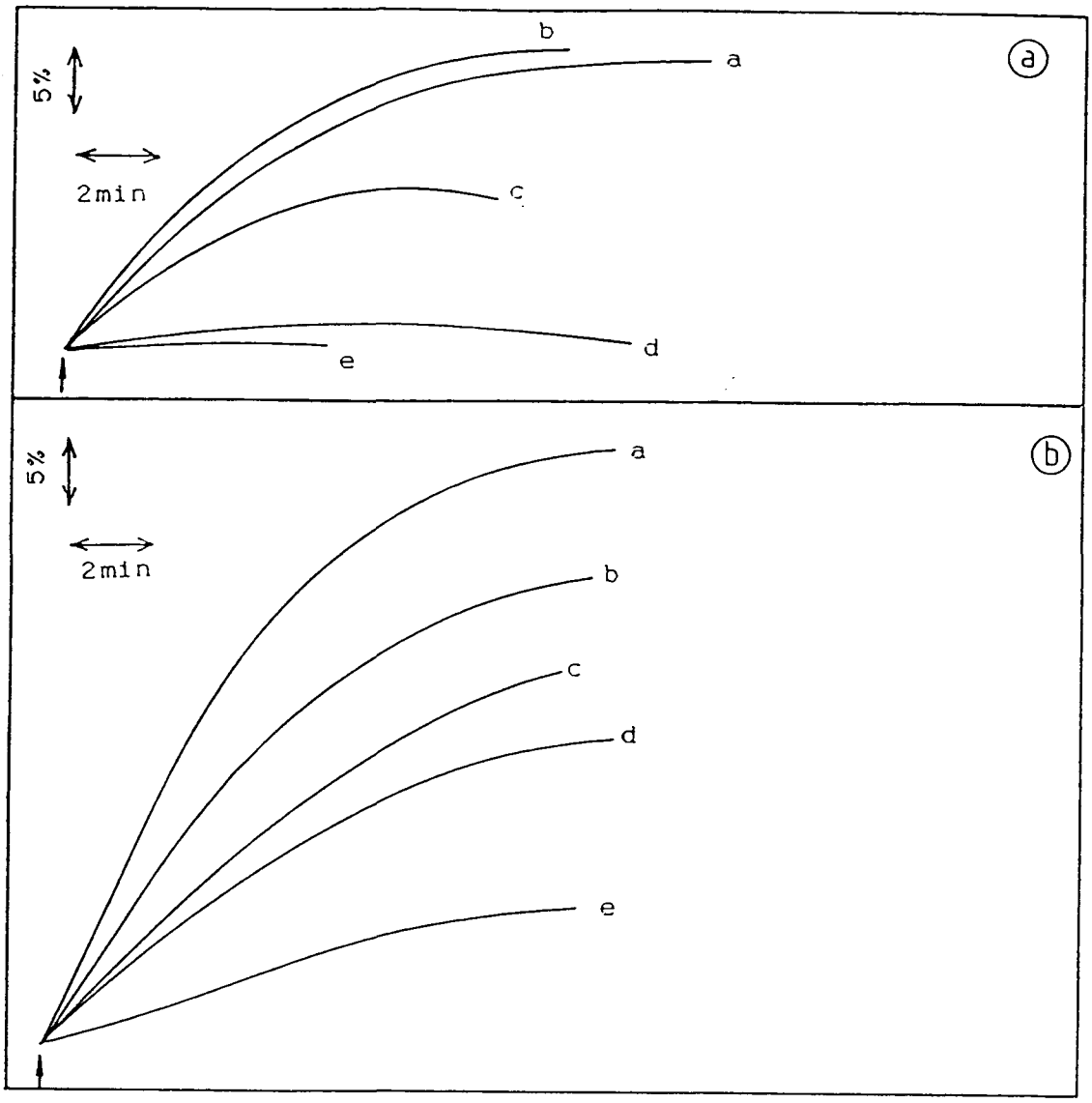


Figure 6.8a. The effect of the inhibitor erythrosin B on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a), with erythrosin B at final concentrations of (b) 2.5×10^{-3}mol m^{-3}, (c) 5×10^{-3}mol m^{-3}, (d) 7.5×10^{-3}mol m^{-3} and (e) 10^{-2}mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow).

Figure 6.8b. The effect of SW26 dissolved in DMSO on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a) with a final concentration of 1% (v/v) DMSO, and 1% DMSO in subsequent assays with the addition of SW26 at final concentrations of (b) 6.25×10^{-3}mol m^{-3}, (c) 12.5×10^{-3}mol m^{-3}, (d) 25×10^{-3}mol m^{-3} and (e) 50×10^{-3}mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow).

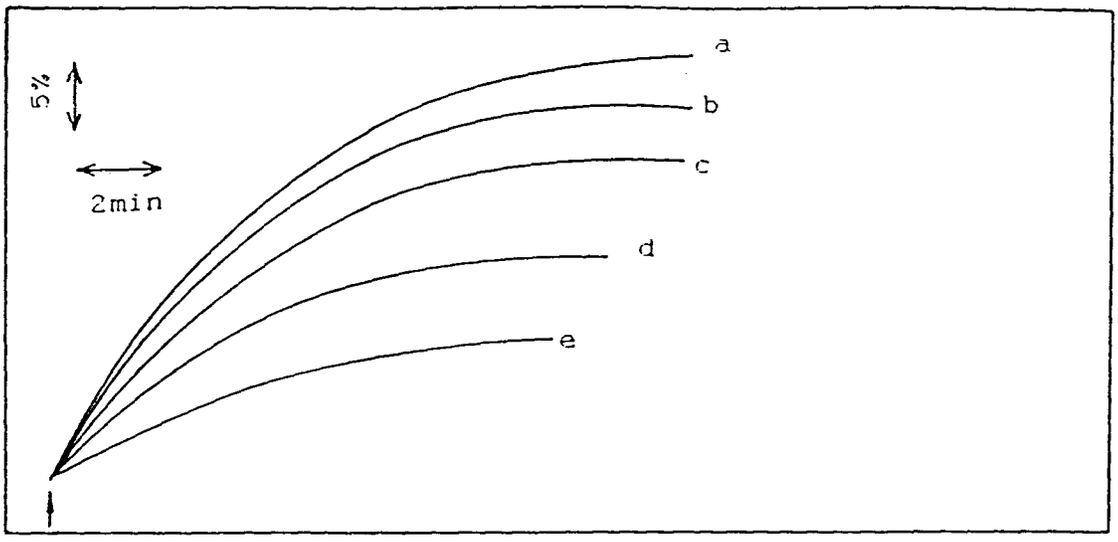


Figure 6.9. The effect of of ruthenium red on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a), with the addition of ruthenium red at final concentrations of (b) 0.25×10^{-3} mol m^{-3}, (c) 0.5×10^{-3} mol m^{-3}, (d) 2.75×10^{-3} mol m^{-3} and (e) 5×10^{-3} mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow).

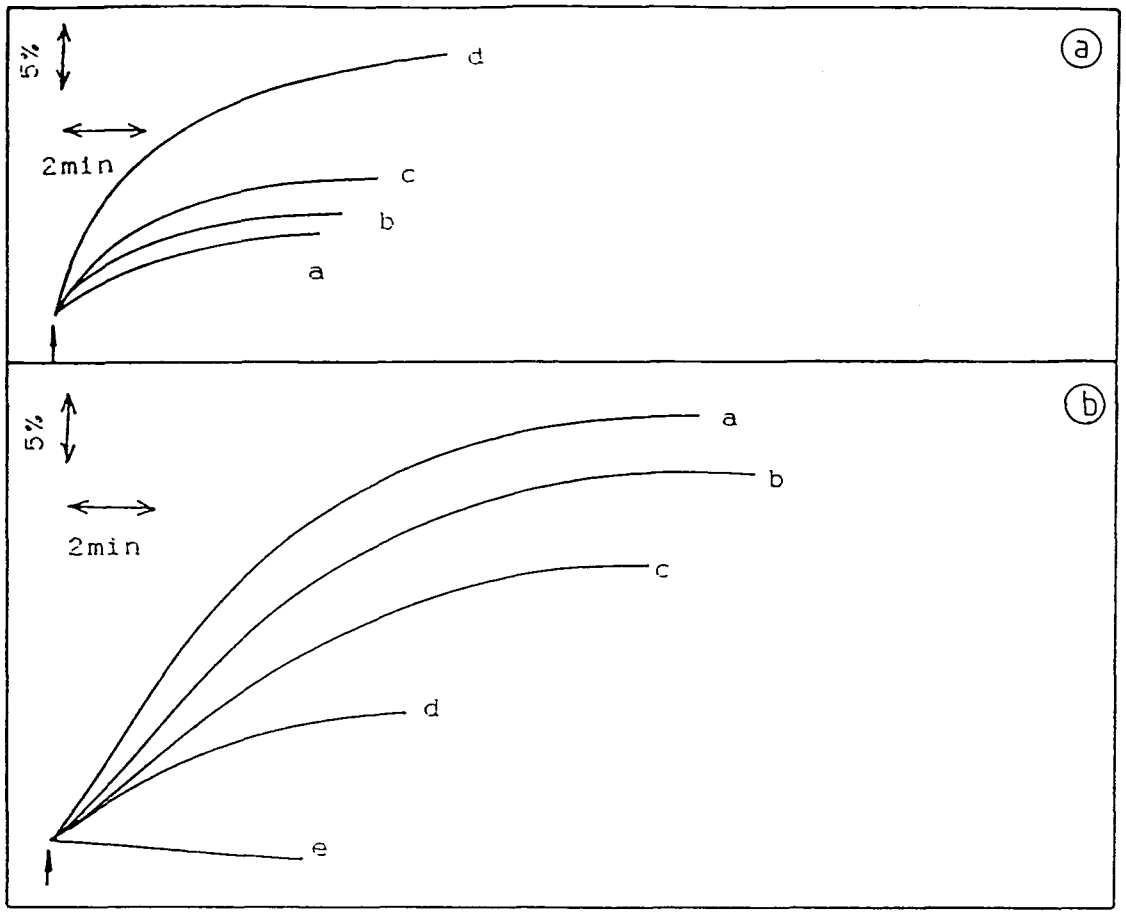
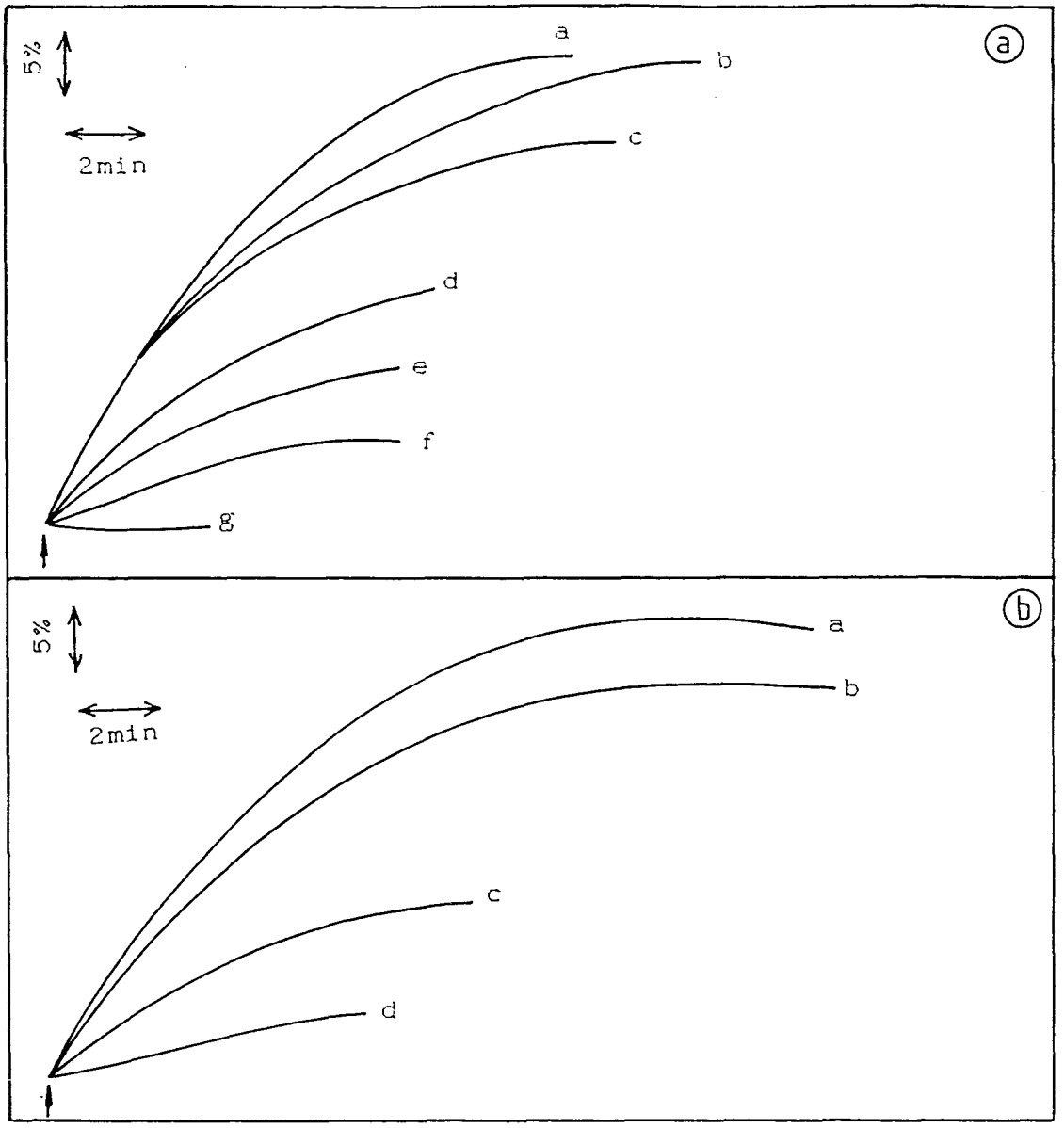


Figure 6.10a. Ca^{2+} binding of lanthanum chloride to microsomal vesicles. The standard chlorotetracycline assay mixture was used in the absence of ATP (a) and with the addition of lanthanum chloride at final concentrations of (b) $5x10^{-3}$mol m^{-3}, (c) 10^{-2}mol m^{-3} and (d) $5x10^{-2}$ mol m^{-3}. The reaction was initiated by the addition of vesicles (upward arrow)

Figure 6.10b. The effect of lanthanum chloride on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a), with the addition of lanthanum chloride at final concentrations of (b) $5x10^{-3}$mol m^{-3}, (c) $8.5x10^{-3}$mol m^{-3}, (d) 10^{-2}mol m^{-3}, and (e) $5x10^{-2}$mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow).

Figure 6.11a. The effect of CCCP prepared in DMSO on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a), with the addition of CCCP at final concentrations of (b) $2x10^{-3}$mol m^{-3}, (c) $8x10^{-3}$mol m^{-3}, (d) $12.5x10^{-3}$mol m^{-3}, (e) $17.8x10^{-3}$mol m^{-3}, (f) $25x10^{-3}$mol m^{-3} and (g) $55x10^{-3}$mol m^{-3}. Final concentration of DMSO in each assay was 1%(v/v). The reaction was initiated by the addition of ATP (upward arrow).

Figure 6.11b. The effect of nigericin prepared in 25% (v/v) ethanol on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used with the addition of ethanol (a), and with nigericin at final concentrations of (b) 10^{-3}mol m^{-3}, (c) $5x10^{-3}$mol m^{-3} and (d) 10^{-2}mol m^{-3}. Ethanol was present at a final concentration of 1% (v/v) in each assay. The reaction was initiated by the addition of ATP (upward arrow).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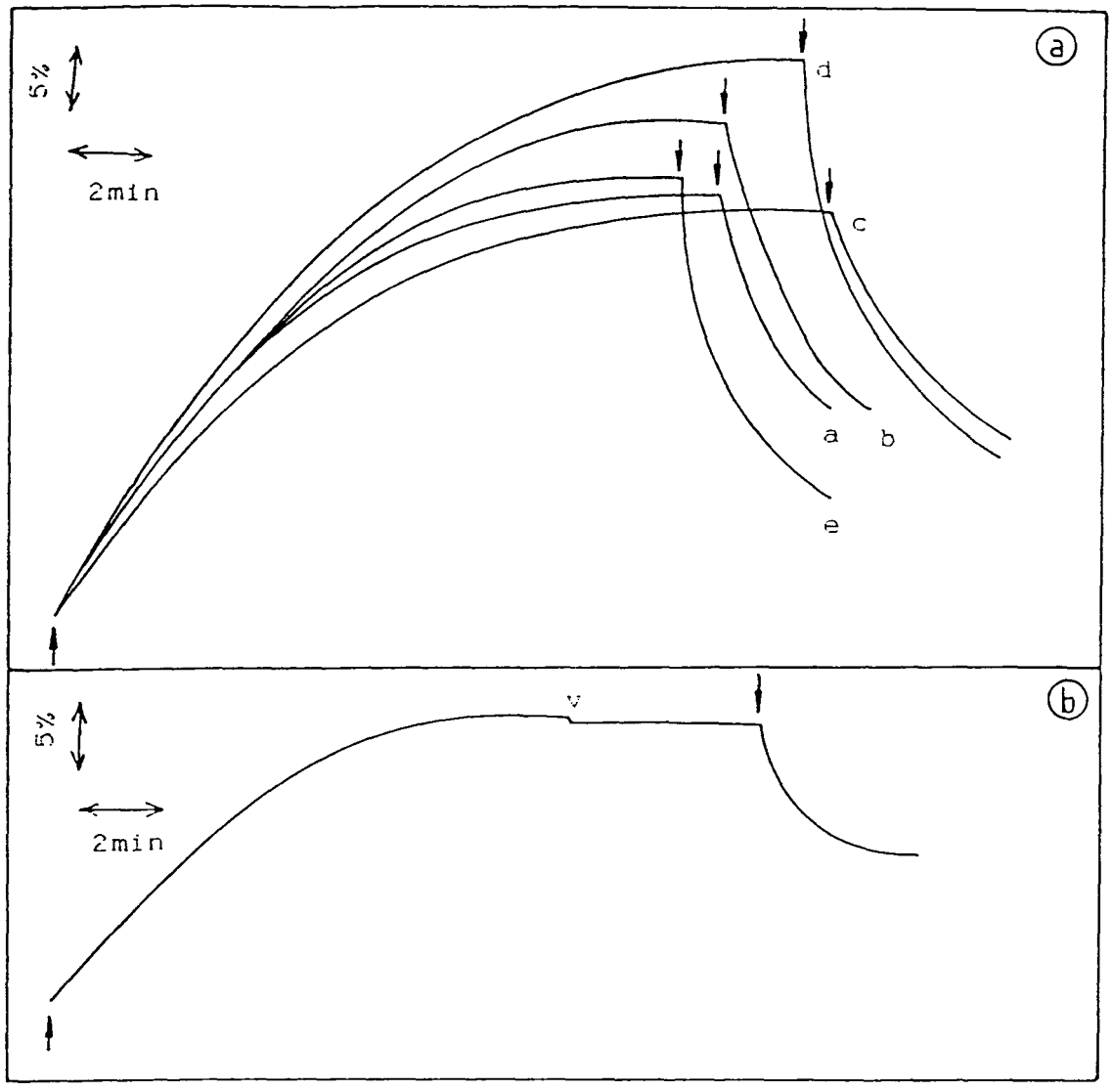


Figure 6.12a. The effect of DCCD (prepared in DMSO), on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used with the addition of DMSO (a), and with the addition of DCCD at final concentrations of (b) $\cdot 1\times$ mol m^{-3}, (c) $\cdot 2$ mol m^{-3}, (d) $\cdot 4$ mol m^{-3}, (e) $\cdot 6$ mol m^{-3}. DMSO was used at a final concentration of 1% (v/v). The reaction was initiated by the addition of ATP (upward arrow).

Figure 6.12b. The effect of verapamil on ATP-dependent Ca^{2+} transport into microsomal vesicles after equilibration with ATP. The standard chlorotetracycline assay was used and 10×10^{-3}mol m^{-3} verapamil was added as indicated (V), followed at a later stage by the ionophore A23187 (downward arrow). Verapamil was prepared in DMSO, with the latter at final concentration of 1% (v/v).

6.2.5 Effect of calmodulin, calmodulin antagonists and local anaesthetics on Ca^{2+} transport.

Inhibition of any membrane-bound calmodulin was carried out by incorporating TFP into the assay mixture, by examination of Ca^{2+} transport in microsomal vesicles (Fig.6.13). TFP caused Ca^{2+} binding to the vesicles to occur in the absence of ATP which increased with TFP concentrations. On the addition of ATP following the initial calibration, a drop in fluorescence was measured followed by Ca^{2+} transport into the vesicles which was reversed by the ionophore A23187. The effect of other phenothiazines were examined, namely chlorpromazine and phenothiazine, and both inhibited ATP-dependent Ca^{2+} transport into vesicles (Fig.6.14a,b). Addition of calmodulin to the assay mixture caused a slight increase in calcium uptake (Fig.6.15a,b) with maximal stimulation at $5\mu g cm^{-3}$. To examine if calmodulin was affecting Ca^{2+} transport *via* a hydrophobic reaction with the membrane rather than a direct effect on the calcium-calmodulin complex, the effect of the hydrophobic drugs, dibucaine and tetracaine were examined (Fig.6.16a,b). Both caused a marked inhibition of ATP-dependent transport into vesicles, with 1.5mol m^{-3} tetracaine causing complete inhibition and 0.3mol m^{-3} dibucaine causing a large reduction in transport.

6.2.6 Effect of auxin on Ca^{2+} uptake.

IAA was found to inhibit ATP-dependant calcium uptake into microsomal vesicles at concentrations of 10, 56 and $100x10^{-3}$mol m^{-3} (Fig.6.17). No effect was observed on addition of IAA to the assay when Ca^{2+} transport had reached a steady state in the presence of ATP. PAA inhibited ATP-dependent Ca^{2+} transport into microsomal vesicles as did GA3 and 2,4-D (Fig.6.17b).

Vesicles isolated from tissue that had been preincubated in IAA (10^{-2}mol m^{-3}) for 2 h were found to have a reduced calcium uptake capacity compared with vesicles that had been isolated from tissue preincubated in distilled water (Fig.6.18). A repeat of this experiment showed little difference between the two

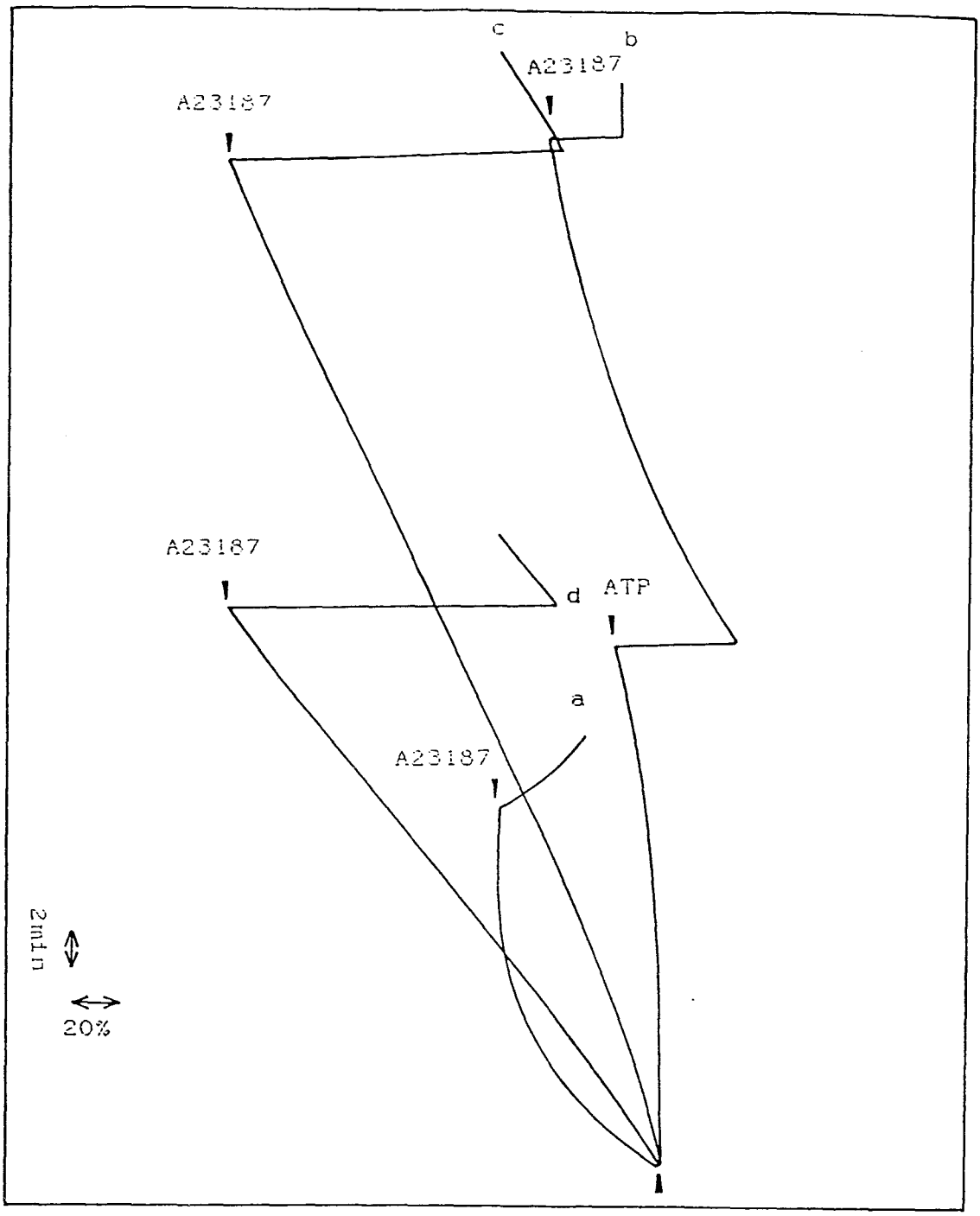


Figure 6.13. The effect of TFP on Ca^{2+} transport in the presence and absence of ATP into microsomal vesicles. The standard chlorotetracycline assay was used without ATP (a) and with the addition of ATP and TFP as indicated at final concentrations of (b) 50×10^{-3}mol m^{-3}, (c) 100×10^{-3}mol m^{-3} and (d) 200×10^{-3}mol m^{-3}.

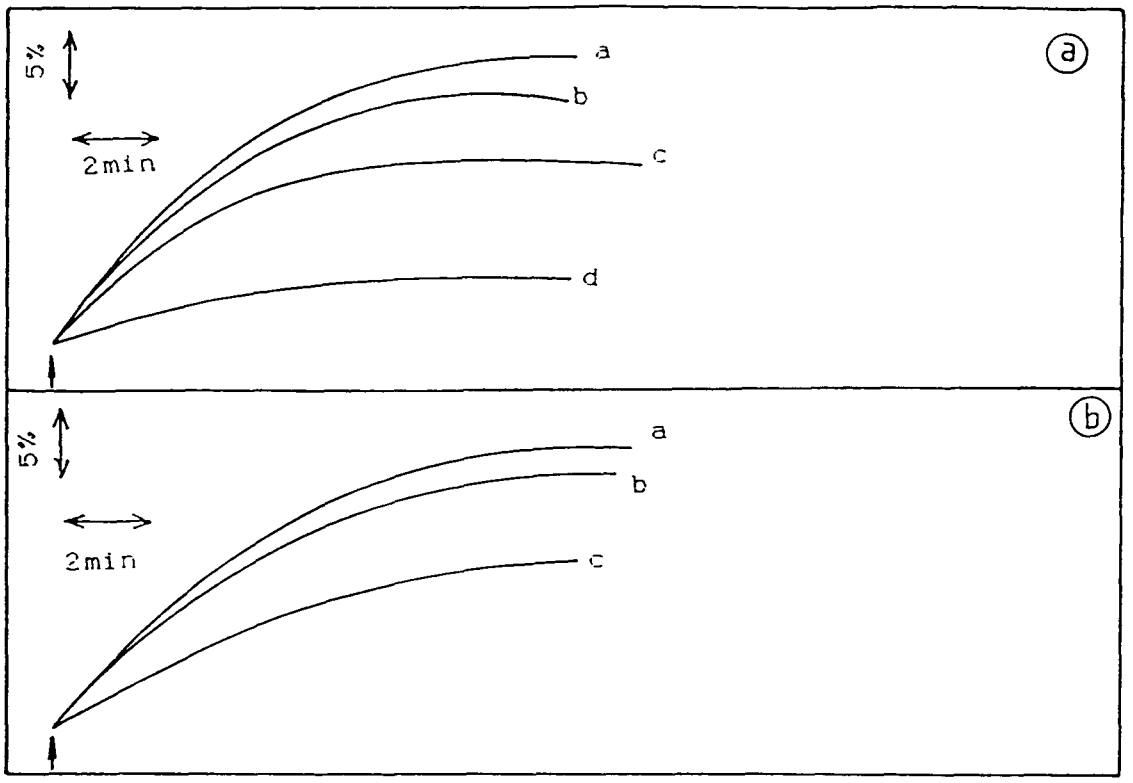
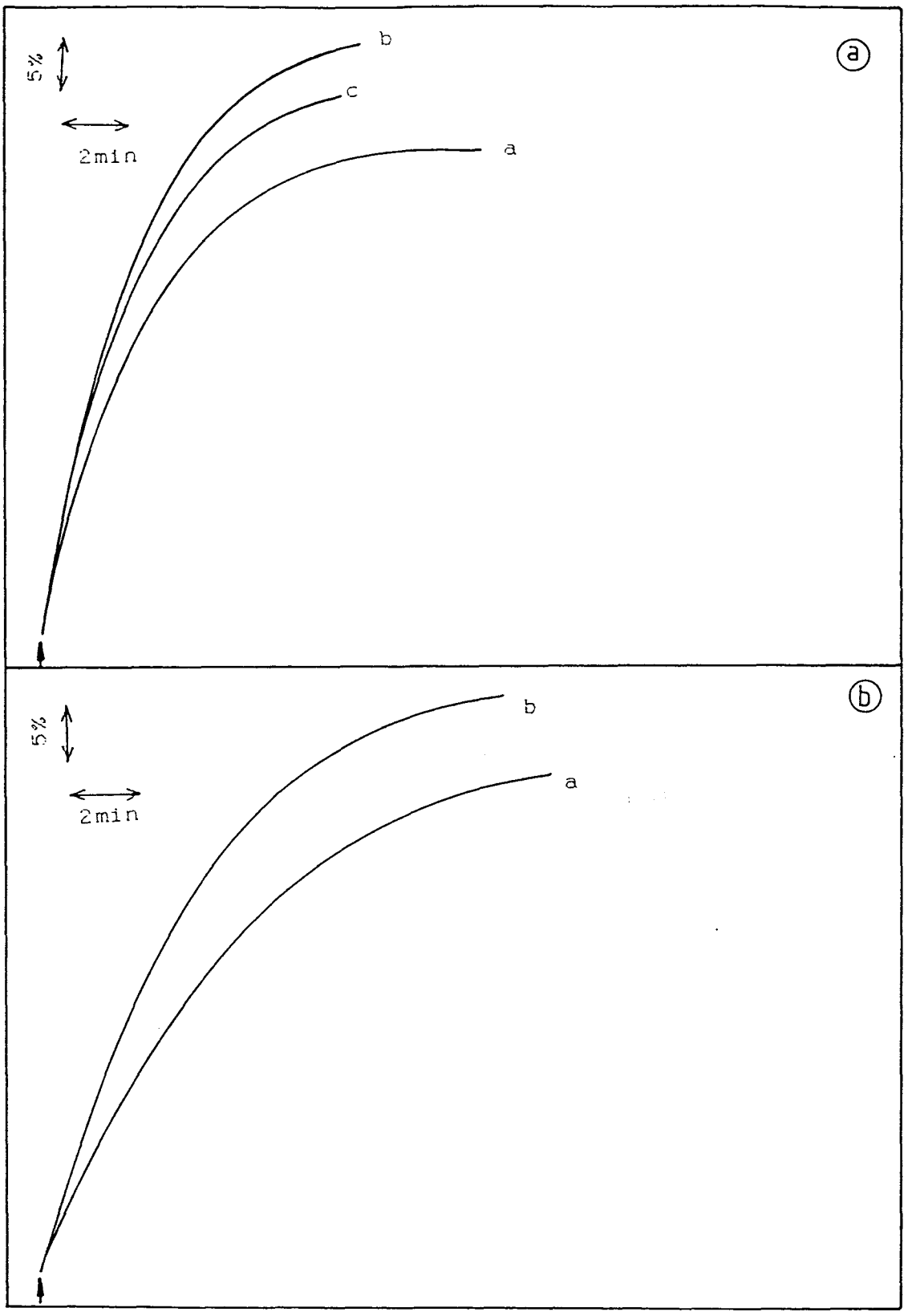


Figure 6.14a. The effect of chlorpromazine on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a), with the addition of chlorpromazine at final concentrations of (b) $5x10^{-3}$mol m^{-3}, (c) $10x10^{-3}$mol m^{-3} and (d) $100x10^{-3}$mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow).

Figure 6.14b. The effect of phenothiazine on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a), and with the addition of phenothiazine at final concentrations of (b) 10^{-2}mol m^{-3} and (c) 10^{-1}mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow).

Figure 6.15a,b. The effect of calmodulin (from bovine brain) on ATP-dependent Ca^{2+} transport into microsomal vesicles. (a) & (b) illustrate the results obtained from two different experiments. The standard chlorotetracycline assay was used (a), with the addition of calmodulin at final concentrations of (b) $5\mu gcm^{-3}$ and (c) $10\mu gcm^{-3}$.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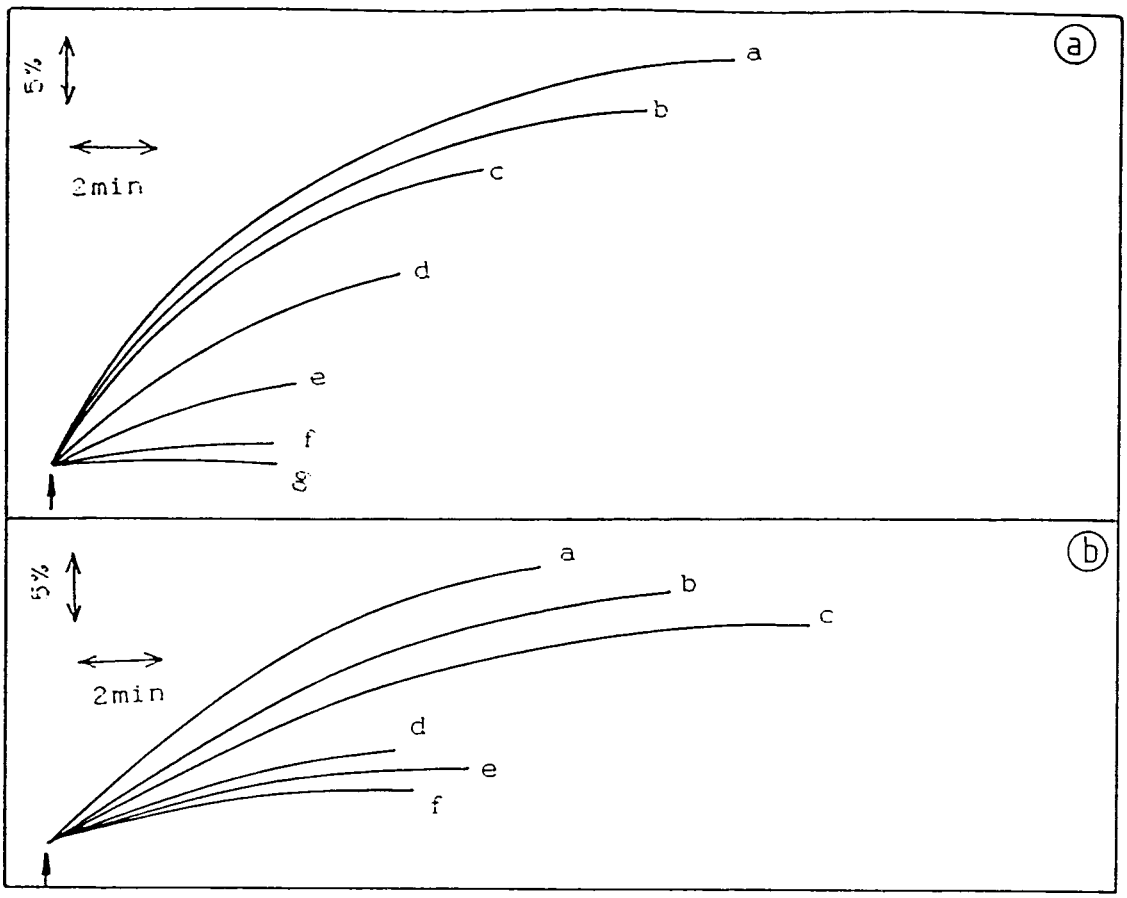


Figure 6.16a. The effect of tetracaine on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a), with the addition of tetracaine at final concentrations of (b) 4.65×10^{-3} mol m^{-3}, (c) 6.51×10^{-3}mol m^{-3}, (d) 9.3×10^{-3}mol m^{-3}, (e) 37.6×10^{-3}mol m^{-3}, (f) 150×10^{-3}mol m^{-3} and (g) 1.5mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow).

Figure 6.16b. The effect of dibucaine on ATP-dependent Ca^{2+} transport into microsomal vesicles. The standard chlorotetracycline assay was used (a) and with the addition of dibucaine at final concentrations of (b) 15×10^{-3}mol m^{-3}, (c) 30×10^{-3}mol m^{-3}, (d) 90×10^{-3}mol m^{-3}, (e) 150×10^{-3}mol m^{-3} and (f) 300×10^{-3}mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow).

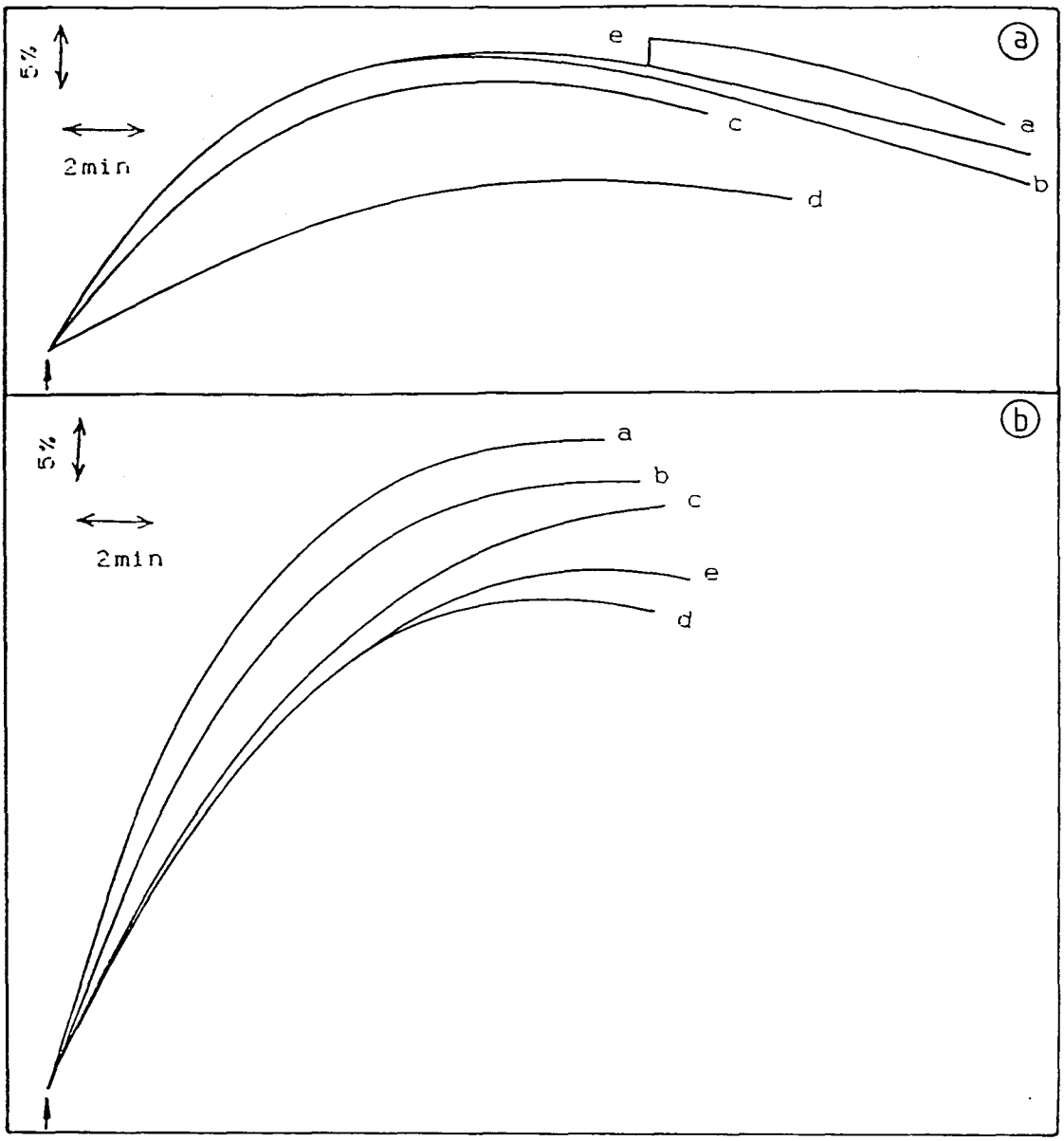


Figure 6.17a. The effect of IAA on ATP-dependent Ca^{2+} transport into vesicles derived from a microsomal fraction. The standard chlorotetracycline assay was used (a), with the addition of IAA to final concentrations of (b) 10^{-2}mol m^{-3}, (c) $5.5x10^{-2}$mol m^{-3} and (d) 10^{-1}mol m^{-3}. IAA was added to a control at the steady state at a concentration of $5.5x10^{-2}$mol m^{-3} (e). The reaction was initiated by the addition of ATP (upward arrow).

Figure 6.17b. Effect of PAA, GA3 and 2,4-D on ATP-dependent Ca^{2+} transport into vesicles derived from a microsomal fraction. The standard (a) chlorotetracycline assay was used, with plant hormones/regulators at final concentrations of (b) 10^{-2}mol m^{-3} PAA, (c) $5x10^{-1}$mol m^{-3} GA3, (d) 10^{-3}mol m^{-3} GA3 and (e) 10^{-2}mol m^{-3} 2,4-D. The reaction was initiated by the addition of ATP (upward arrow).

treatments on ATP-dependent Ca^{2+} transport. On addition of IAA *in vitro* to these two vesicle types, the IAA-depleted vesicles showed a marked reduction in ATP-dependent Ca^{2+} uptake (Fig.6.19a) over that of vesicles from non-auxin depleted tissue (Fig 6.19b). Protein levels used per assay in each case were identical.

6.2.7 Ca^{2+} stimulation of ATPase activity.

Mg^{2+}.K^{+}-dependent ATPase activity was examined in the presence of Ca^{2+} in purified membrane fractions from a sucrose gradient. Marked inhibition of activity (80-90%) was found at all Ca^{2+} concentrations (Fig.6.20a). In the presence of Mg^{2+} and Ca^{2+} (Fig.6.20b), inhibition was reduced (50-75), but there was no indication of a stimulatory effect by calcium.

EGTA was found to stimulate ATPase activity in microsomal preparations (Fig.6.21). Ca^{2+}, when added to the assay at low concentrations (10^{-3}mol m^{-3}), was found to cause only slight inhibition of ATPase activity, indicating the possible existence of a Ca^{2+}-ATPase. ATPase sensitivity to EGTA was examined in all the gradient interfaces (Fig.6.22). Varying stimulation of ATPase activity occurred indicating that following differential and sucrose gradient centrifugation, membrane associated Ca^{2+} remained and at varying concentrations in each interface. When a ratio of 4 cm^{3} homogenization medium to 1 g fresh weight tissue was used, no stimulation by 1mol m^{-3} EGTA was observed. Ca^{2+} may therefore be removed from the membranes during the homogenization stage with this procedure.

Ca^{2+}-stimulated ATPase activity was examined in membrane-associated preparations that had been prepared with a homogenization medium containing 13mol m^{-3} EGTA. Calcium *in vitro* at a concentration of 10^{-3}mol m^{-3} was slightly stimulatory to ATPase activity in the presence of Mg^{2+} and K^{+} (Fig.6.23). At higher Ca^{2+} concentrations, ATPase was inhibited. When the EGTA concentration in the homogenization medium was increased to 30mol m^{-3} stimulation of the ATPase by calcium occurred over a higher concentration range 1-3x10^{-3}mol m^{-3} (Fig.6.24). At 3x10^{-3}mol m^{-3} Ca^{2+} concentrations, ATPase activity was inhibited over that of the control.

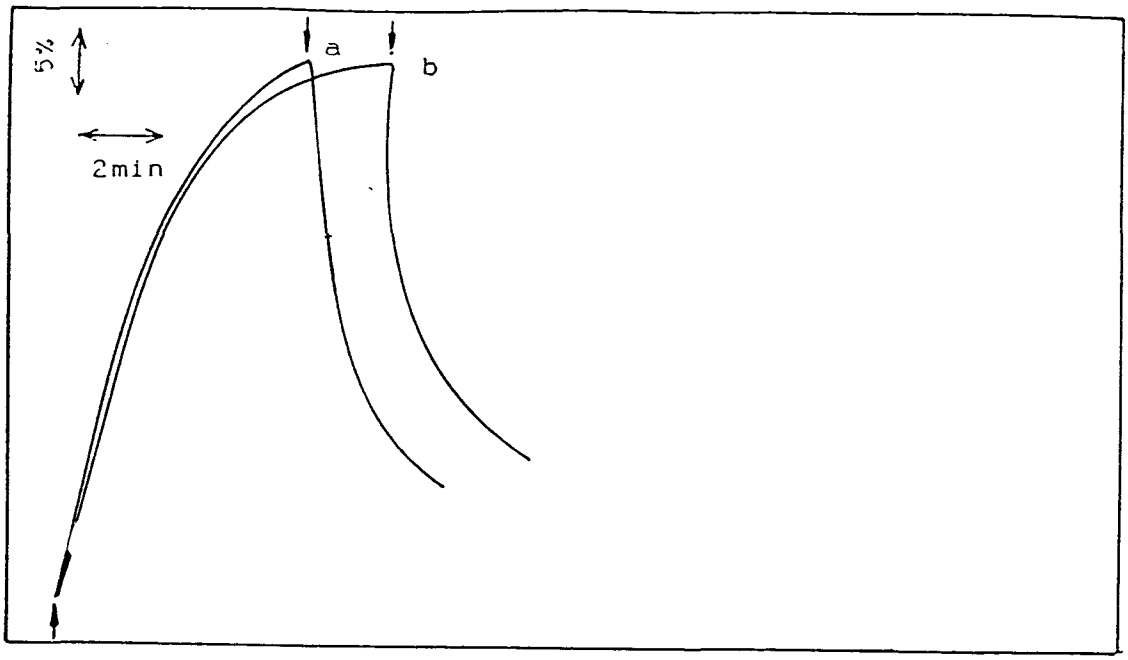
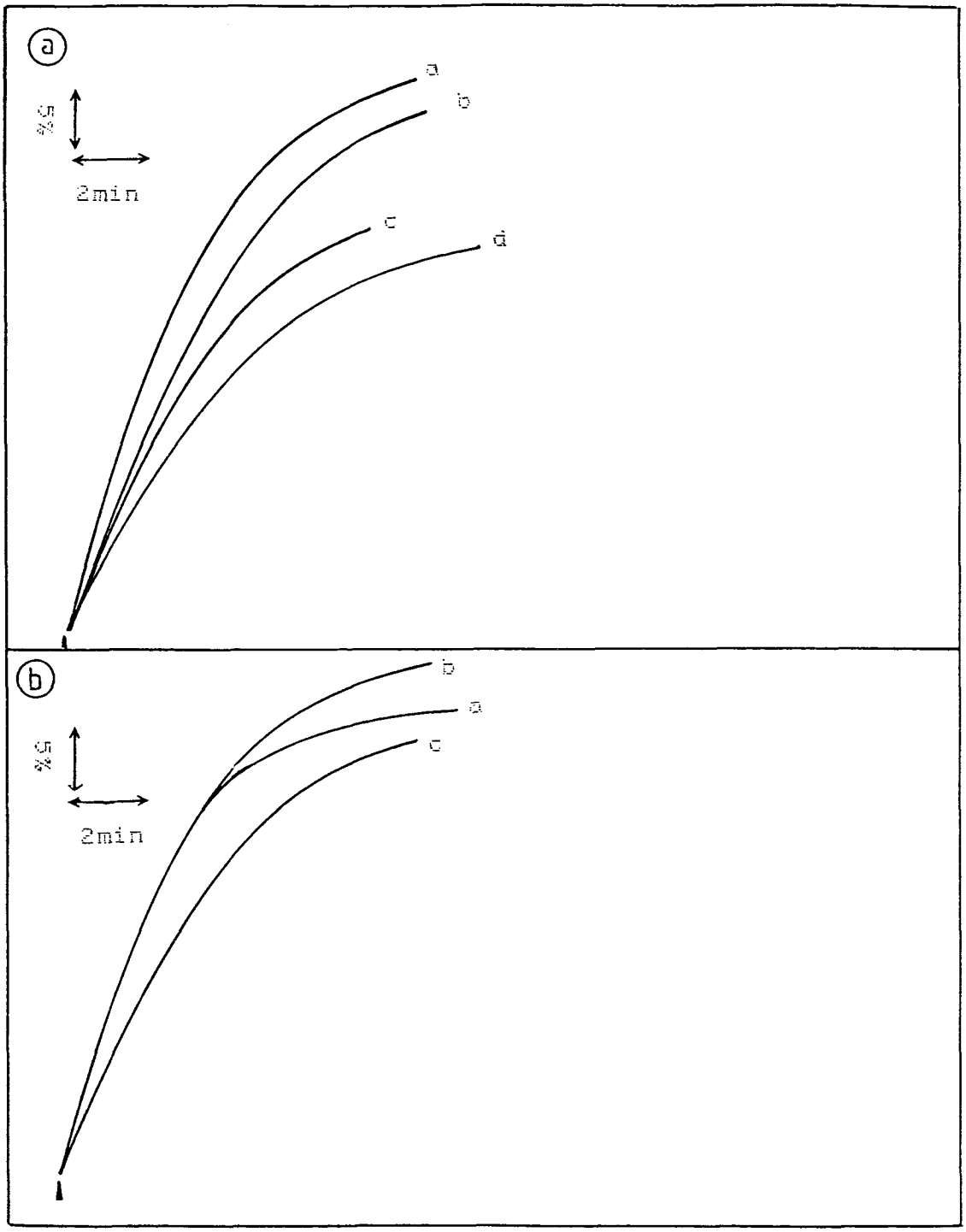


Figure 6.18. The effect on ATP-dependent Ca^{2+} transport into microsomal vesicles prepared from auxin depleted tissue. Tissue was incubated for 2 h (a) in distilled water, or (b) 10^{-2}mol m^{-3} IAA. The standard chlorotetracycline assay was used and the reaction was initiated by the addition of ATP (upward arrow) and reversed by the addition of A23187 (downward arrow).

Figure 6.19a. The effect of IAA on ATP-dependent Ca^{2+} transport into microsomal vesicles prepared from auxin-depleted tissue. The standard (a) chlorotetracycline assay was used (a), with (b) the addition of IAA at final concentrations of 10^{-3}mol m^{-3}, (c) 10^{-2}mol m^{-3} and (d) 10^{-1}mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow).

Figure 6.19b. The effect of IAA on ATP-dependent Ca^{2+} transport into vesicles derived from a microsomal fraction prepared from auxin incubated tissue. The standard chlorotetracycline assay was used (a) with the addition of IAA at final concentrations of (b) 10^{-3}mol m^{-3} and (c) 10^{-1}mol m^{-3}. The reaction was initiated by the addition of ATP (upward arrow).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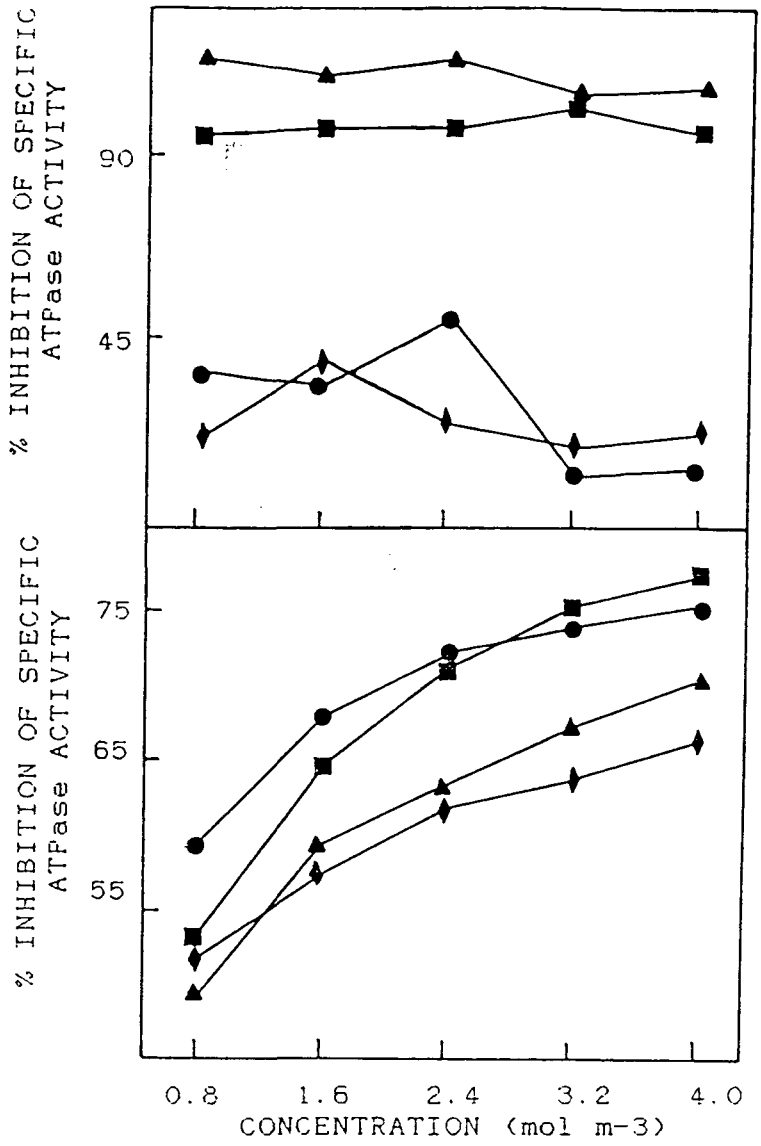


Figure 6.20a. Percentage inhibition of specific ATPase activity caused by the replacement of Mg^{2+} in the standard ATPase assay by Ca^{2+} at various concentrations in interface 2 (■), interface 3 (▲), interface 4 (●), interface 5 (◆) at varying calcium concentrations. ATPase assay was carried out as outlined in "Materials and Methods" using the standard assay.

Figure 6.20b. Percentage inhibition of ATPase activity caused by the addition of Ca^{2+} at the concentrations indicated to the standard ATPase assay in interface 2 (■), interface 3 (▲), interface 4 (●), interface 5 (◆). The standard ATPase assay was used.

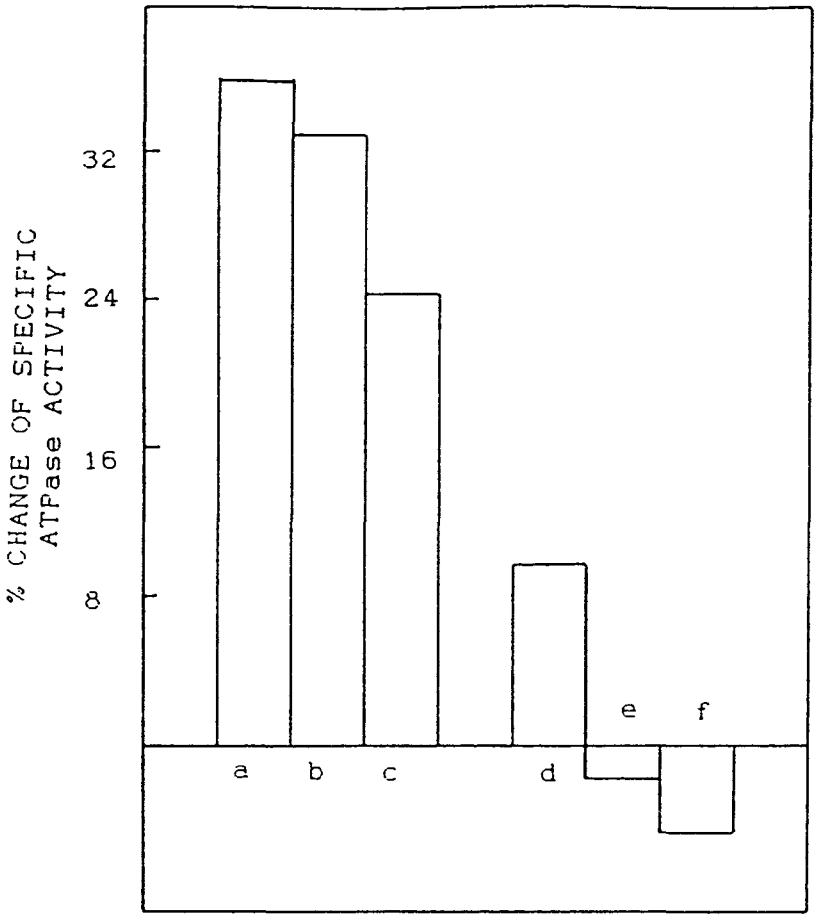


Figure 6.21. Percentage stimulation/inhibition of ATPase activity in a microsomal fraction by EGTA and Ca^{2+}. All homogenization, resuspension and assay solutions were made up in deionized water and the resuspension medium contained no EGTA. The standard assay was used (a) with the addition of EGTA at a final concentration of 1 mol m^{-3} in the presence of molybdate, (b) in the presence of EGTA, molybdate and Mg^{2+}, (c) and EGTA, molybdate, Mg^{2+} and K^{+}. Alternatively the standard assay contained (d) Ca^{2+} at a final concentration of 10^{-3}mol m^{-3}, (e) with the addition of molybdate Mg^{2+} and (f) molybdate, Mg^{2+} and K^{+}.

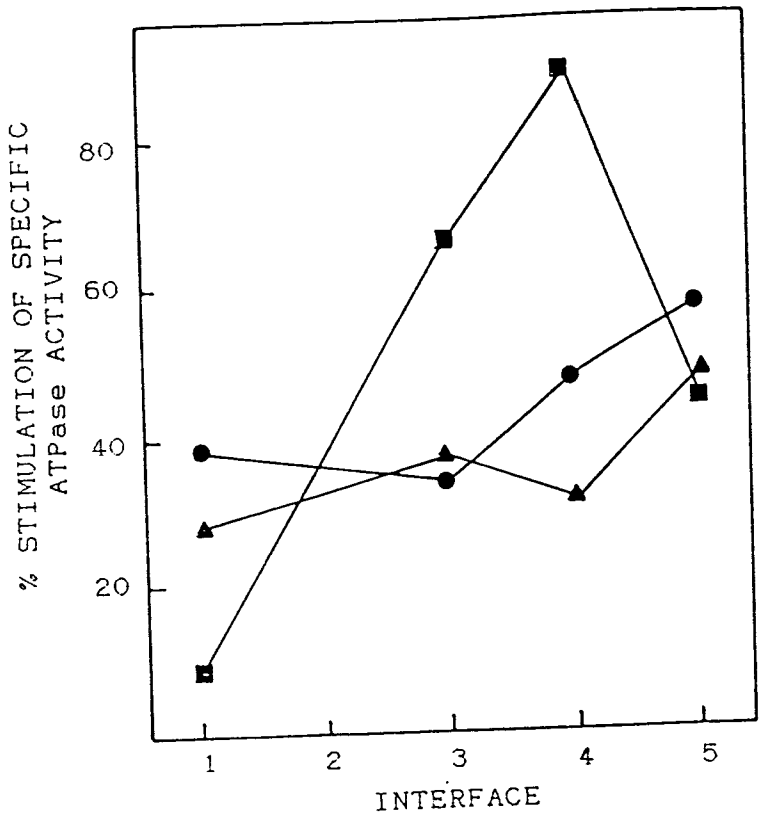
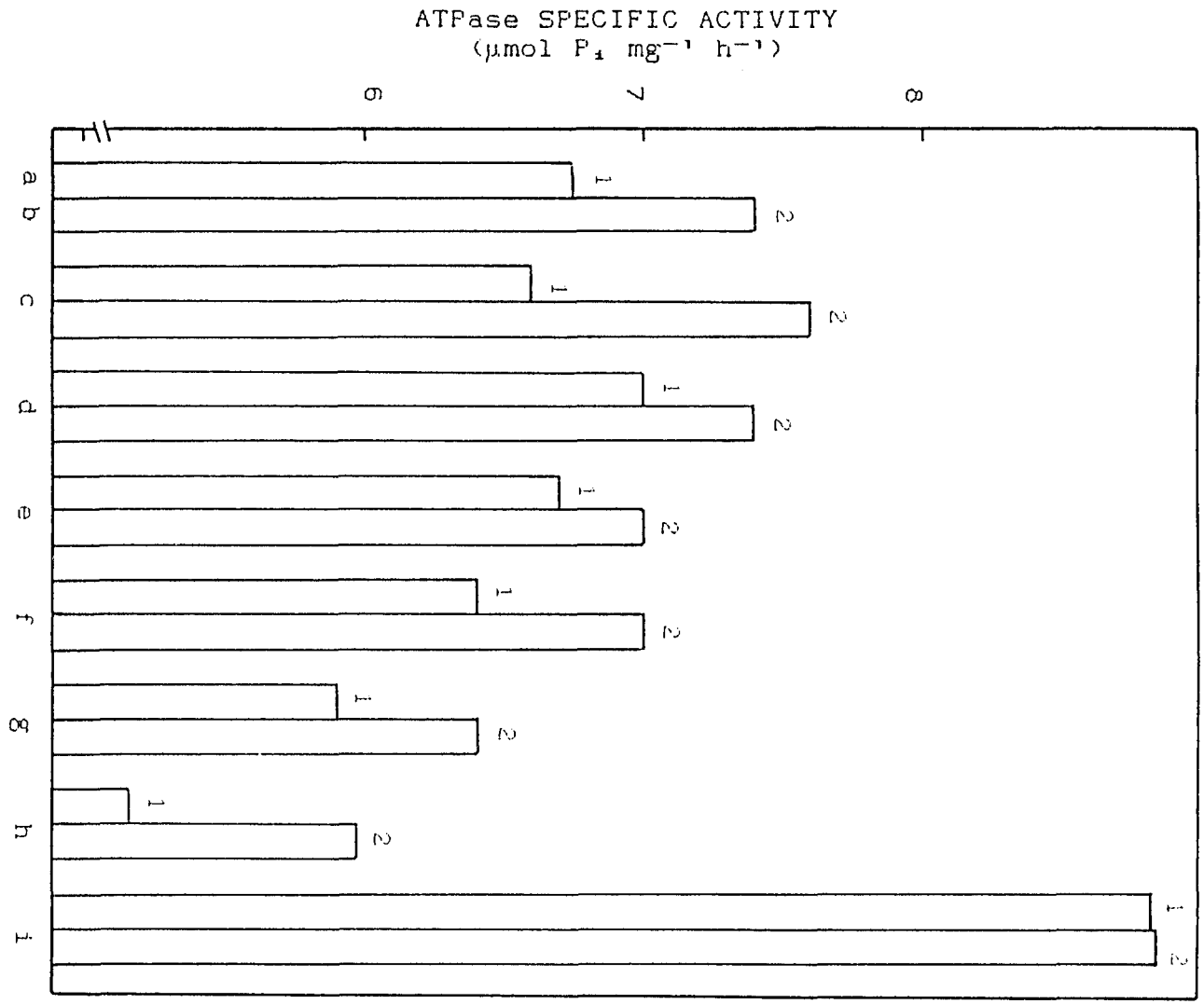


Figure 6.22. Percentage stimulation of ATPase activity by 1mol m^{-3} EGTA in the five interfaces from a sucrose gradient. The standard assay was used in the absence of magnesium and potassium (■), in the absence of potassium (●) and in the presence of magnesium and potassium (▲).

Figure 6.23. Effect of Ca^{2+} on specific ATPase activity. All solutions were made up in deionized water. Tissue was ground in the standard homogenization medium with the addition of 13mol m^{-3} EGTA and resuspended in the standard medium containing no EGTA. Each bar pair represents (1) the standard assay medium in the absence of potassium and (2) in its presence. The standard assay was used (a) in the absence of potassium and (b) in its presence, and with the addition of calcium chloride at final concentrations of (c) 10^{-3}mol m^{-3}, (d) $5x10^{-3}$mol m^{-3}, (e) $10x10^{-3}$mol m^{-3}, (f) $20x10^{-3}$mol m^{-3}, (g) $50x10^{-3}$mol m^{-3}, (h) $100x10^{-3}$ mol m^{-3}, and (i) EGTA at a final concentration of 10mol m^{-3}.


ATPase SPECIFIC ACTIVITY
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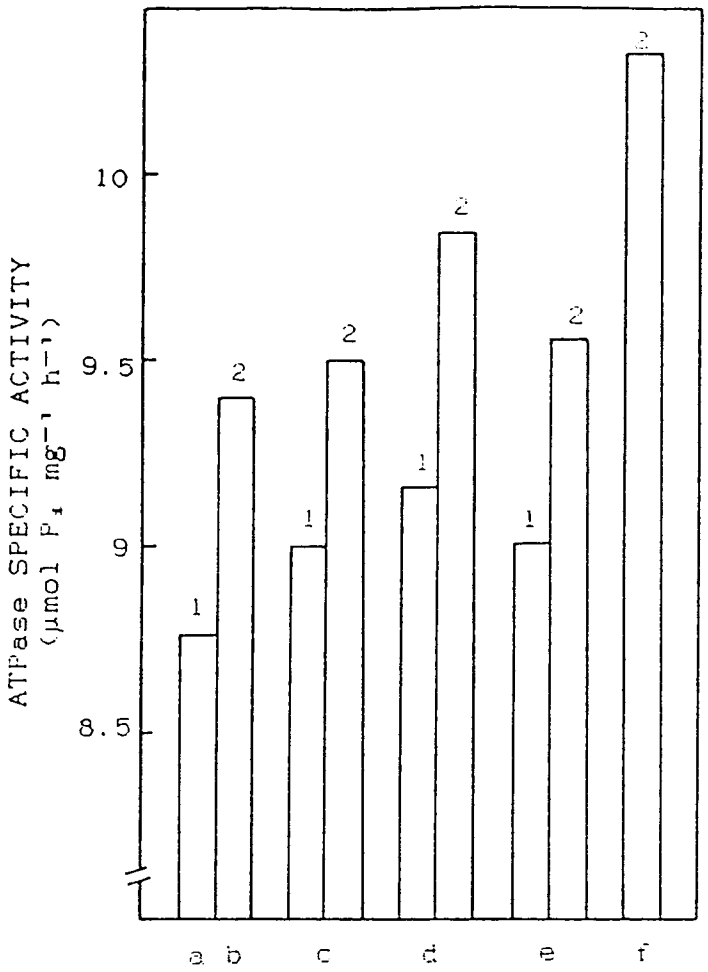

Figure 6.24. The effect of Ca^{2+} on ATPase specific activity in a microsomal fraction. All homogenization, resuspension and assay solutions were made up in deionized water. Tissue was ground in the standard homogenization medium with the addition of 30 mol m^{-3} EGTA and resuspended in the standard medium containing no EGTA. Each bar pair represents (1) the standard assay medium in the absence of potassium and (2) in its presence. The standard assay was used (a) in the absence of potassium, (b) in the presence of potassium and with the addition of calcium at final concentrations of (c) 10^{-3}mol m^{-3}, (d) $3x10^{-3}$mol m^{-3} (e) $5x10^{-3}$mol m^{-3} and EGTA at a final concentration of (f) 10^{-3}mol m^{-3} .

6.3 Discussion.

The variable accumulation of calcium within the hypocotyl was found to depend on the concentration of the cation in the external bathing solution. This was in agreement with the findings of Ingestad (1974), who found little regulation to occur by *Cucumis* species of calcium uptake from the external medium, but sequestration took place within the plant. In this species little variation in the Ca^{2+} distribution occurred in the young hypocotyl and the cotyledons were found not to act as calcium sinks but were maintained at a lower calcium status than the hypocotyl. Geographical distribution of this species is therefore not limited by the calcium status of the soil and these plants are able to establish themselves over a wide calcium regime.

The inhibitory action of Ca^{2+} on membrane associated ATPases has been much reported (e.g. Memon *et al.* 1986). In this tissue considerable amounts of Ca^{2+} was found to remain associated with the membranes. Initial chelation of cations was therefore found necessary to detect ATP-dependent Ca^{2+} transport into membrane vesicles. With substantial chelation of Ca^{2+} in the homogenization medium, a calcium-stimulated ATPase was also detected and found to be associated with microsomal membranes.

The ATP-dependent Ca^{2+} transport into microsomal vesicles which was reversible by the Ca^{2+} ionophore, A23187, indicated that an active transport mechanism was operative. The finding that vanadate and CCCP both reduced Ca^{2+} transport indicated that two Ca^{2+} transport mechanisms may be operative in this system. The CCCP-sensitivity suggests that one of the mechanisms of Ca^{2+} accumulation is dependent on the proton electrochemical gradient. Similarly, the sensitivity of transport to the proton channel blocker DCCD at the higher concentrations and nigericin (K^+/H^+ ionophore) is supportive of Ca^{2+} transport occurring *via* a proton gradient.

In chapter 3 it was shown that pyrophosphate was utilized by microsomal vesicles to establish a H^+ gradient. However only a small amount of Ca^{2+} transport took place when pyrophosphate was supplied as substrate, this indicates that Ca^{2+} transport via a Ca^{2+}/H^+ antiport may

only play a minor role in the isolated vesicles.

Bush & Sze (1986) similarly found Ca^{2+} transport to be sensitive to CCCP and suggested that such a mechanism may be associated with tonoplast-derived vesicles. Their associated ATPase was found to be sensitive to nitrate, which would suggest that the proton electrochemical gradient is generated by the nitrate-sensitive ATPase associated with the tonoplast. However the gradient data described here may not be in agreement, since this CCCP-sensitive system was associated with a membrane having a wide range of densities. However, the cation chelation that was applied to the membranes, may have considerably affected membrane distribution within the gradient and therefore further characterization should of been made. The large reduction of Ca^{2+} transport that took place in the presence of nitrate or CCCP indicates that this antiport is of widespread occurrence in this tissue and may have an important role in maintaining the calcium-stat of the cytoplasm.

The second component of ATP-dependent Ca^{2+} transport was found to be sensitive to vanadate and similarly distributed throughout the gradient. From the ATPase inhibitor data in Chapter 3, it may be suggested that these vesicles are derived from the endoplasmic reticulum and not the Golgi apparatus or plasma membrane, although the latter may contain a similar enzyme. The results suggest there to be a direct coupling of Ca^{2+} transport to ATP hydrolysis, involving a Ca^{2+}, Mg^{2+}-dependent ATPase (Bush & Sze 1986). No inhibition of calcium transport occurred in the presence of azide, ruling out any contribution from mitochondria. Isolation of two distinct Ca^{2+} transport systems has been reported previously in pea stem derived vesicles (Rasi-Caldogno *et al.* 1982) and corn roots (Zocchi & Hanson 1983) and following characterization, both attributed a Ca^{2+}/H^{+} antiport to the tonoplast and a direct Ca^{2+} Mg^{2+}-ATPase predominantly to the endoplasmic reticulum. In sharp contrast, Fukumoto & Venis (1986) provide evidence for a direct tonoplast associated Ca^{2+}-ATPase, that is insensitive to ionophores but sensitive to vanadate. No markers for endoplasmic reticulum were included in their experiments, and may

therefore have been a contaminant in their vacuole preparations. The pH optimum of pH 7.5 for the active transport of Ca^{2+} found in this tissue was in agreement with the results of other workers (Lew *et al.* 1986) and is near the cytoplasmic pH which has been reported as slightly alkaline (Brummell & Hall 1987).

The endoplasmic reticulum has a high affinity for Ca^{2+} of the order necessary to regulate basal Ca^{2+} levels in the cell (Macklon 1984, Bush & Sze 1986). This report supports the recent suggestion that the endoplasmic reticulum may be an important regulator of cytoplasmic Ca^{2+} levels in eukaryotic cells (Somlyo 1984).

There was no evidence for Ca^{2+} movement out of vesicles in the presence of verapamil, following the steady state after ATP addition. Efflux typically occurred following active transport, and its prevention might suggest that a voltage gated channel is in operation.

The Ca^{2+} binding that occurred initially in the absence of ATP varied enormously between preparations as did the extent of Ca^{2+} transport that occurred in the presence of ATP. Chlorotetracycline measures only the calcium associated with membranes, and gives no indication of movement into the lumen of the vesicle. The binding of Ca^{2+}-CTC is not to specific sites, as indicated by the increase in Ca^{2+} transport that occurred in the presence of oxalate. One may speculate that the initial variation of fluorescence that occurred before ATP was added may be due to a variable initial Ca^{2+} status of the vesicles and the latter to be due to leakiness of the vesicles.

The stimulation of Ca^{2+} transport by DCCD is in agreement with the finding of Lew *et al.* (1986), although other workers have found the chemical to be inhibitory (Bush & Sze 1986). This hydrophobic carboxyl reagent may interact with the membrane vesicles and cause the increased Ca^{2+} transport observed.

Ruthenium red has been used to raise the cytoplasmic Ca^{2+} concentration, by inhibiting uptake into mitochondria (Picton & Steer 1985). Our data suggests that ruthenium red may also inhibit ATP-dependent Ca^{2+} transport into vesicles of endoplasmic reticulum and tonoplast. La^{3+} is

reported to be a competitive inhibitor of the calcium-binding process due to its similar ionic radius to Ca^{2+} and higher charge density (Martin & Richardson 1979). The increased Ca^{2+} binding that took place in the presence of lanthanum in the absence of ATP suggests that either La^{3+} is increasing the number of unspecific binding sites or that chlorotetracycline is binding competitively to lanthanum which binds to the vesicle membranes with a consequent rise in fluorescence.

Calmodulin caused stimulation of ATP-dependent Ca^{2+} transport *in vitro* and this would suggest that either calmodulin does not have a regulatory role in Ca^{2+} uptake in these vesicles, or that calmodulin remains bound during the isolation procedure, and is present at optimal concentrations in the assay. Buckhout (1984) tested the latter possibility by resuspending the membranes in a high concentration of Ca^{2+}, EGTA and fluphenazine and incubating for 20 min at 4°C to remove any membrane bound calmodulin. With this treatment, Ca^{2+} uptake was still unaffected by calmodulin *in vitro*, suggesting that no regulatory role is played by calmodulin in these vesicles. Addition of the calmodulin inhibitor, phenothiazine, however reduced ATP-dependent Ca^{2+} uptake. These local anaesthetics interact hydrophobically with membranes (Schatzman *et al.* 1981) and the inhibition observed may therefore be the result of a non-specific hydrophobic interaction with the membranes.

The inhibition of ATP-dependent Ca^{2+} transport by IAA, 2,4-D and gibberellic acid in vitro, differs from the report of Kubowicz *et al.* (1982) who found IAA *in vitro* to be without any effect on ATP-dependent Ca^{2+} transport. Differences may result from the use of radioactive $^{45}Ca^{2+}$ probes. These workers reported that ATP-dependent Ca^{2+} transport was increased in vesicles from expanding hypocotyl tissue following an IAA incubation *in vivo*. Ca^{2+} transport in vesicles prepared from this tissue were found to be inhibited by this treatment. The differential effect of IAA *in vitro* and *in vivo* suggests that the action of Ca^{2+}-ATPase is not directly affected by IAA, but may involve a subsidiary step. Auxin has been reported to inhibit cell growth in the meristematic zone and to be

promotive in the region of elongation and in mature tissues of soybean (Key & Hanson 1961). A differential response of ATP-dependent Ca^{2+} transport in regions of the soybean hypocotyl has been reported (Kubowicz *et al.* 1982). A positive correlation between ATP-dependent Ca^{2+} transport in vesicles prepared from these regions following an auxin pretreatment *in vivo* was found. Thus ATP-dependent Ca^{2+} uptake into vesicles derived from the meristematic zone was found to be inhibited by IAA pretreatment. The microsomal vesicles prepared in our system were from the complete hypocotyl and unless meristematic cells only formed tightly sealed vesicles, no correlation between this report and Kubowicz *et al.* (1982) is immediately apparent.

6.4 Conclusions.

ATP-dependent Ca^{2+} transport was demonstrated in vesicles isolated from *Cucumis* hypocotyls. Ca^{2+} transport in this mixed vesicle population was *via* two mechanisms, a direct coupling of Ca^{2+} *via* a Mg^{2+} dependent ATPase and transport by a proton gradient antiport generated by a Mg^{2+} dependent ATPase. Gradient separation of these two transport systems was not possible, but with the use of inhibitors and markers, it was surmised that the former system was associated with the tonoplast and the latter with the endoplasmic reticulum or plasma membrane. Following chelation of Ca^{2+} in a microsomal preparation, evidence was found of a Ca^{2+} stimulated ATPase

CHAPTER SEVEN.

General Discussion.

7.0 The initial aim of this thesis was to prepare fractions enriched in specific membranes from hypocotyl tissue that is responsive to a number of physiological stimuli *in vivo*. Plant hormones and red and far red light treatments both affect hypocotyl development *in vivo* and it was planned to study their effect *in vitro* on the plasma membrane associated ATPase and H^+-pumping activities. Initially an attempt was made to isolate purified membrane fractions from protoplasts prepared from hypocotyls of *Cucumis sativus* using the charged silica bead method (Polenko & Maclachlan 1984). However considerable contamination with mitochondrial membranes persisted in all the preparations and the more conventional technique of differential and sucrose gradient centrifugation was used. Isolation and characterization of membrane-enriched fractions were made at each of the gradient interfaces for marker activity in order to determine the location of cellular membranes. Conventional markers were used and a plasma membrane-enriched fraction was indicated by its associated ATPase sensitivity to orthovanadate (Gallagher & Leonard 1982) and a tonoplast fraction by its associated ATPase sensitivity to nitrate (O'Neill *et al.* 1983). The plasma membrane enriched fraction was located in a region of the gradient which had a density of $1.17 gcm^{-3}$ and the tonoplast fraction at a density of $1.08 gcm^{-3}$. Assuming that the majority of these vesicles are leaky, their densities are dependent solely on the membrane composition and agree with the reported density range for the plasma membrane of $1.13 - 1.17 gcm^{-3}$ and tonoplast $1.10 - 1.13 gcm^{-3}$ (Hodges & Leonard 1974, Quail 1979, Bennett *et al.* 1984, Poole *et al.* 1984).

The identification of plant membranes by their associated enzyme activity can only be as good as the uniqueness of the enzyme. The plasma membrane contains no specific enzyme and consequently many identifications have been made on the differential inhibition of the associated

ATPase. In *in vivo* studies it is also important that these inhibitors are capable of penetrating the intact tissue. The lack of a specific inhibitor of plasma membrane ATPase has limited the studies on the correlation between ATPase activity and ion transport in plant materials. DES, DCCD and vanadate have been shown to inhibit the activity of plasma membrane associated ATPase (Gallagher & Leonard 1982, Briskin & Poole 1983). However, none of these inhibitors appear to be fully satisfactory. DCCD is known to inhibit the ATPase of mitochondria and chloroplasts (Maloney 1982) and DES, apyrase activity (Tognoli & Marrè 1981). DCCD and DES also inhibit the nitrate-sensitive ATPase of the tonoplast (Bennett & Spanswick 1983, Tognoli 1985). Their use in *in vivo* studies is also limited since both cause inhibition of oxidative phosphorylation (Balke & Hodges 1979). Vanadate has received criticisms both from its use *in vivo* due to poor penetration (Saftner *et al.* 1983, Williams & Hall 1987) while *in vitro* studies have indicated that ATPases associated with the endoplasmic reticulum and Golgi may also be sensitive to vanadate (Quail 1979). Consequently evidence has been presented that the vanadate-sensitive ATPase activity as well as being associated with the plasma membrane is also associated with secretory vesicles (Binari & Racusen 1983).

Thus potential new markers and inhibitors for the plasma membrane and associated ATPase are of great interest. Erythrosin B has been reported to be a specific inhibitor of the plasma membrane and tonoplast ATPase (Cocucci 1986) and SW26 as a specific inhibitor of the plasma membrane associated ATPase (Blein *et al.* 1986). With this tissue and with *Ricinus* cotyledons erythrosin B appeared to markedly inhibit the plasma membrane associated ATPase (Ball *et al.* 1987). In addition, it was found to inhibit medium acidification by abraded cucumber hypocotyl segments, hypocotyl growth and to inhibit sucrose uptake in *Ricinus* cotyledons, suggesting that this chemical readily penetrated intact tissues (Ball *et al.* 1987). SW26 gave a proportionately higher increase in the sensitivity of the plasma membrane-enriched ATPase than vanadate in both of these systems, which might indicate

that at the concentrations used, SW26 may be specific for the plasma membrane ATPase. However, inhibition of ATPase activity by SW26 was found to depend markedly on the protein concentration used in the assay which may result from of an irreversible binding of SW26 to specific sites on the membrane.

The cytochemical study indicated that ATPase activity was associated with the plasma membrane, with little associated with the tonoplast. Staining was notably observed in the vascular tissues, whereas none was typically associated with the cortical tissues. However staining was observed in the epidermal cells. The outer epidermal cell layers have been reported to play a crucial role in the auxin growth response. When the outer hypocotyl layers are removed by peeling, the inner tissues show little capacity for auxin-stimulated growth (Brummell & Hall 1980). The epidermal-associated ATPase may well play a role in auxin stimulated growth. A similar lack of tonoplast associated ATPase staining has been reported in other tissues (e.g.Sossountzov *et al.* 1985). In this tissue tonoplast associated ATPase activity was notably inhibited by the fixative glutaraldehyde and an alternative fixation procedure without the incorporation of any potential inhibitors would be of interest.

The acid growth theory first proposed by Rayle & Cleland (1970) suggested that the fast acid-inducible phase of cell growth is mediated by an active extrusion of protons across the plasma membrane into the wall space. This movement of protons was considered to be brought about by an active transport process and much evidence has been presented illustrating that a plasma membrane ATPase does actively transport protons from the cytoplasm into the wall space (e.g. Sze 1984). Vanderhoef and Stahl (1975) were the first to report that the response of soybean hypocotyl segments to auxin consists of two overlapping responses, a short-term acid inducible phase, which probably results from wall loosening, and a longer-term phase thought to result from an activation of gene transcription (Vanderhoef & Dute 1981). Using isolated hypocotyl segments, medium acidification was found to be stimulated by IAA as were the two phases of auxin-

stimulated growth. Although a trans-membrane redox system may be operative at the plasma membrane of some plants that could provide the energy for electrogenic proton extrusion (Lin 1982), it is widely accepted that the H^+ extrusion by most cells *in vivo* in response to IAA is due mainly to the activity of H^+ transporting ATPases. This is confirmed by the observation that vanadate almost completely inhibits acidification of the medium as demonstrated with segments of pea stem (Jacobs & Taiz 1980), and cucumber hypocotyl (Brummell *et al.* 1986). Vanadate similarly has been shown to inhibit growth of cucumber hypocotyl segments (Brummell *et al.* 1986). Erythrosin B inhibited plasma membrane associated ATPase and the initial phase of auxin stimulated growth in this tissue. This may indicate that a plasma membrane associated H^+ extrusion is involved in the initial growth response. As wall acidification has been demonstrated to be inhibited by vanadate, and that growth occurs in the presence of an acidic buffer (Brummell *et al.* 1986), rather than H^+ extrusion being the factor controlling growth, an acidified cell wall may be merely required. However, it has been found that at an acidic pH, auxin-promoted growth occurs above that due to the acid alone (Kutschera & Schopfer 1985) suggesting that auxin affects processes other than cell wall acidification. Acid and the auxin-stimulated wall loosening processes are likely to occur by separable and distinct mechanisms.

ATPase activity associated with a plasma membrane enriched fraction was examined in relation to its response to IAA. The addition of this growth regulator *in vitro* had no effect on ATPase specific activity associated with this membrane fraction. However, if the tissue was incubated with IAA *in vivo*, the ATPase specific activity associated with the plasma membrane was stimulated. These results suggest that the fast initial phase of growth is not brought about *via* a direct stimulation of the membrane bound ATPase, but may be involved in the second phase of growth.

However, Scherer (1981) reported that 10^{-3} mol m^{-3} IAA stimulated ATPase activity in gradient fractions that had received no preincubation, but this was at low ATP

concentrations.

ATP-dependent H^+ transport was found to be stimulated by IAA, but only at micromolar ATP concentrations although estimates of the cytoplasmic ATP concentration lie in the range of 0.6 - 1.0-mol m^{-3} (e.g. Petraglia & Poole 1980). Similar difficulties were encountered by other workers in obtaining significant and repeatable auxin promotion of ATPase activity *in vitro* (Venis 1985). More recently with zucchini hypocotyl tissue, 2,4-D has been shown to have a stimulatory effect on ATP-driven proton pumping, but again only at ATP concentrations in the micromolar range (Scherer 1984a). Subsequently using pea root microsomes, it has been reported that ATP-dependent H^+ transport was stimulated by IAA at an ATP concentration of 0.1mol m^{-3}; the anti-auxin p-chlorophenoxyacetic, benzoic acid and FC were found to be without effect (Gabathuler & Cleland 1985). The latter data suggests that plant hormones may be involved in specifically stimulating ATP-dependent H^+ transport, at non-physiological ATP concentrations. However, local variations in ATP concentration may well occur in the vicinity of the plasma membrane.

If the reports that IAA has a direct effect on ATPase activity are confirmed, a close relationship must exist between the auxin receptor and the ATPase. However, auxin binding sites have been found to lack any ATPase activity (Cross *et al.* 1978), although Cross (1985) subsequently pointed out that the detergents used in the extraction procedure may have removed lipids essential for ATPase activity. A close association between the auxin receptor and ATPase is indicated by the experiments of Thompson *et al.* (1983) who incorporated the solubilized auxin-binding protein from maize into an artificial lipid bilayer membrane, and in the presence of ATP found an auxin-dependent increase in transmembrane current.

ATPase activity associated with dividing, elongating and differentiating zones of the hypocotyl were examined to determine if a relationship exists between the maturation state of the tissue and the associated ATPase specific activity. Both tonoplast- and plasma membrane-associated ATPase specific activity were found to increase basipetally in the hypocotyl. Activity when expressed on

total ATPase activity per FW basis in each region of the hypocotyl, a higher activity was found to be associated with the meristematic region of the hypocotyl. Similarly, the meristematic region of the roots has been reported to possess a higher ATPase and ATP-dependent proton pumping activity than non-meristematic regions (Dupont et al. 1982b) suggesting a relationship may exist. An alternative approach would have been to study ATPase activity associated with the epidermis with the use of immunofluorescence techniques. Although at present antibodies are not readily available specific to the plasma membrane associated ATPase, it is envisaged that with this procedure the distribution of ATPase associated with specific cell types could be precisely calculated. It is also assumed that the ATPase associated with the plasma membrane is of one distinct class; however there may be sub-populations located within the membrane, and again this might be clarified with the use of antibodies.

The cellular changes that must occur for the second growth phase to take place are likely to involve the interaction of auxin with the genome (Matthysse & Phillips 1969). Using indirect immunofluorescent labelling, an auxin-binding protein has been localized at the plasma membrane facing outwards in the outer epidermal cells of maize (Löbler & Klämbt 1985a,b). Following this binding, although the receptor may then transmit a message itself to the metabolic machinery, some sort of soluble rapid amplification is to be expected and calcium is emerging as a likely candidate. An increase in cytosolic Ca^{2+} levels upon auxin treatment has been proposed by several authors (Morris & Northcote 1977, Brummer & Parish 1983, Hertel *et al.* 1983). ATP-dependent Ca^{2+} transport in microsomal vesicles was found to be inhibited by IAA in this tissue. If these were of endoplasmic reticulum origin, Ca^{2+} would have been pumped into the interior to maintain the gradient. An inhibition of this process would have resulted similarly in an increase in the cytosolic Ca^{2+} concentration. Several mechanisms for an auxin-induced increase in cytosolic Ca^{2+} levels have been suggested. Ca^{2+} may be displaced from membranes as auxin has been reported to release Ca^{2+} from soybean microsomal membranes

in vitro (Buckhout *et al.* 1981). Ca^{2+} may alternatively enter the cytosol from the vacuole (Hertel *et al.* 1983) or endoplasmic reticulum (Brummer & Parish 1983) or, if Ca^{2+} channels are opened in the plasma membrane, from the cell wall. Auxin was not found to cause a release of Ca^{2+} from this tissue. On the contrary, an incubation *in vivo* in IAA was found to reduce ATP-dependent Ca^{2+} uptake suggesting that IAA causes a sequestration of the cation.

Evidence was presented in this tissue for the involvement of calmodulin in regulating the activity of an ATP-dependent Ca^{2+}-transport enzyme. Several enzymes are promoted either directly by calmodulin, which then become active upon binding Ca^{2+} (e.g.Dieter 1984, Hepler & Wayne 1985). The involvement of calmodulin in the initiation of auxin-stimulated growth has not been established, but calmodulin inhibitors when applied have been found to inhibit auxin stimulated growth. For example, in wheat coleoptiles, trifluoperazine (Elliot *et al.* 1983) and in oat, chlorpromazine (Raghothama *et al.* 1985), both inhibited growth. Auxin stimulated growth and medium acidification by hypocotyl segments have both been reported to inhibited by trifluoperazine (Morrell 1987). Similarly, in this tissue, calmodulin inhibitors were found to inhibit ATP-dependent Ca^{2+} uptake. This may be evidence for the intimate involvement of calcium in the auxin induced growth response. However, as these inhibitors are hydrophobic, they may influence the membrane conformation rather than the enzyme. Hydrophobic reagents that are not calmodulin inhibitors were found to inhibit ATP-dependent Ca^{2+} transport, suggesting that a hydrophobic action may be present. Additionally it must be considered that if calmodulin binds to Ca^{2+}, the Ca^{2+} concentration in the assay medium will be reduced and may thus affect enzyme activity.

Haupt & Weisenseel (1976) were the first to propose that some phytochrome effects on cellular enzyme activities, cell growth and development could be mediated through Ca^{2+} and Ca^{2+}-dependent regulator proteins. The hypothesis would predict that photoactivation of phytochrome rapidly leads to an increase in the Ca^{2+} concentration in certain subcellular compartments which

activates calmodulin. There are no direct reports that the cytoplasmic Ca^{2+} concentration is regulated by R or FR, but indirect evidence would indicate that it is intimately involved. For example, R and FR have been shown to affect medium acidification by hypocotyl segments from this tissue (e.g. Roth-Bejerano & Hall 1986b) and ATPase activity associated with a microsomal fraction was found to be markedly influenced by R or FR treatments (Roth-Bejerano & Hall 1986a). No such marked effect was found by R and FR on ATPase activity and this can only be ascribed to a different seed batch. Indeed when seed from a different country was used, no effect of R or FR on ATPase activity was found. The effect of R and FR on ATP-dependent Ca^{2+} transport would have been of great interest to determine if phytochrome mediates a primary affect ATP-dependent Ca^{2+} transport.

7.1 Conclusions.

Plasma membrane and tonoplast enriched fractions were prepared from the hypocotyl of *Cucumis sativus* and the ATPase activities associated with these fractions were found to be distinct. ATP-dependent H^+ transport was also demonstrated in these two fractions. IAA was found to stimulate the ATPase activity associated with the plasma membrane *in vitro* providing the tissue received an *in vivo* incubation in IAA prior to isolation. ATP-dependent H^+ transport was stimulated by IAA at low non-physiological concentrations of ATP. R was found to stimulate ATPase activity to a small extent, but no affect was seen on ATP-dependent H^+ transport. ATP-dependent Ca^{2+} transport was demonstrated in microsomal vesicles, but no clear origin could be assigned to the vesicles.

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