PHYSIOLOGICAL AND BIOCHEMICAL STUDIES ON K$^+$ UPTAKE IN BARLEY

(*Hordeum vulgare.L*) ROOTS

by

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90

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ABSTRACT

Biophysical and kinetic studies on K^+ uptake have led to the belief that uptake of this ion by plant roots occurs via proteinacious carriers or channels depending on the availability of this ion. Without direct evidence it has frequently been postulated that the regulation of ion fluxes may be the result of changes in carrier synthesis. Such evidence should be obtained by biochemical and molecular biological studies. The work described in this thesis was undertaken with that goal in mind.

There are many observations showing that plants have a greater propensity for nutrient uptake when growing under nutrient-deficient conditions. Accordingly, barley seedlings used in this study showed increased K^+ (^{86}Rb) influx as a response to lowering of supply of this ion. This increase was discerned within a time period as short as 15 min from the onset of deprivation and was only partially inhibited by cycloheximide, an inhibitor of protein synthesis. This indicates that at least during the first hour of withholding K^+, a mechanism(s) independent of carrier synthesis and incorporation regulates K^+ influx in plant roots. However, the long-term adaptation to low K^+ supply appeared to require synthesis of new carriers. The latter part of this thesis describes the experiments undertaken to test this hypothesis.

An evaluation of the existing techniques for the isolation and characterization of membrane-enriched fractions was undertaken in order to determine the most feasible technique to be used in experiments where several treatments have to be handled simultaneously. The pros and cons of the use of available techniques in terms of obtaining a relatively high yield and considerable purity in membrane-enriched fraction in such a study are discussed.

Experiments were also performed to investigate the nature of the postulated high affinity K^+ transport system, which is suggested to be in operation under low

K^+ availability. Both *in vitro* ATPase activity and immunological cross-reactivity revealed that plasma membrane H^+ ATPase activities did not increase in parallel with K^+ influx shown by K^+ deprived plants. This evidence, although not unequivocal, indicates that a strict coupling of K^+ influx to H^+ ATPase activity is unlikely.

The studies on quantitative and qualitative changes in membrane proteins were quite rewarding. In spite of slight reduction in plasma membrane protein content as a response to K^+ deprivation, several polypeptides, unique to K^+ deprivation were expressed in the microsomal fraction. Of these a 45 kDa polypeptide was the most prominent. The expression and repression of this polypeptide were parallel with the increase and decrease of K^+ influx with K^+ deprivation and resupply, respectively. This polypeptide which was found to be membrane associated and highly labile in dissociated form, was lost when the microsomes were washed in the presence of 1 mM EDTA. This indicates the necessity of divalent cations for this polypeptide to be membrane associated. The possible role of this polypeptide in increased K^+ uptake rates is discussed.

The most convincing observation in the present study was the synthesis of a 43 kDa polypeptide within 12 h of K^+ deprivation. This polypeptide appeared to be located both on plasma membrane and tonoplast. Two more polypeptides of M_r 37 and 49 were synthesized as a response to long-term K^+ deprivation. The possibility that these polypeptides are parts of a putative K^+ carrier is discussed.

Finally, a comparison of the adaptive mechanism(s) in terms of K^+ uptake and accumulation by barley roots as a response to low-K^+ and low-temperature was made. The acclimatory changes shown as a response to low temperature can be considered as part of the general process of regulation of nutrient uptake while those shown as a response to low K^+ appeared to be quite specific.

TABLE OF CONTENTS

I. GENERAL INTRODUCTION

II. EFFECTS OF VARYING K^+ CONCENTRATIONS IN THE GROWTH
MEDIA ON K^+ UPTAKE AND TISSUE K^+ CONCENTRATIONS

III. AN EVLUATION OF METHODS USED IN ISOLATING MEMBRANE
    VESICLES IN TERMS OF PURITY AND YIELD

IV. THE HIGH AFFINITY K$^+$ TRANSPORT SYSTEM IN BARLEY: LINKAGE WITH
    THE PLASMA MEMBRANE H$^+$-ATPASE OR INVOLVEMENT OF A
    K$^+$-ATPASE SIMILAR TO THE KDP SYSTEM OF E.COLI ?

LIST OF FIGURES

ix

LIST OF TABLES

LIST OF ABBREVIATIONS

DTT   = Dithiothreitol

DEX   = Dextran

EDTA   = disodium ethylenediamine tetra acetate

EGTA   = Ethylene glycol-bis-(2 aminoethylether) N,N,N',N,,tetra acetic acid

MES   = 2-(N-Morpholini) ethane sulfonic acid.

PEG   = Polyethylene glycol

PMSF   = Phenylmethylsulfonylflouride

PVP   = Polyvinyl pyrrolidine

SDS   = Sodium dodecyl sulfate

SHAM   = Salcilhydroxamic acid

Tris   = Tris(hydroxymethyl)aminomethane

$\Delta\Psi$ = membrane potential gradient

$\Delta\mu$ = electrochemical potential gradient

ΔpH = pH gradient

To my mother

"It is hard to avoid the conclusion that further kinetic analysis of intact tissues is not the way towards an understanding of K^+ transport"

Lüttge and Clarkson (1989)

ACKNOWLEDGEMENTS

I. GENERAL INTRODUCTION

The roots of higher plants require a source of at least 16 chemical elements in order to sustain metabolism and thereby, growth. Despite wide ranges of external availabilities of these nutrients in soil solution, plants are known to maintain internal concentrations of many of these required elements within a narrow range. This condition is achieved through the operation of transport mechanisms which are capable of accumulating the required nutrients against strong electrochemical potential gradients. The mechanisms responsible for these transport processes and, more recently, their regulation have been the focus of intense study by plant physiologists.

1.1. Energetics of ion uptake

Before being taken up into the cell, most ions in the soil solution move passively into the cell wall matrix or apoplast along a "down hill" gradient of concentration or free energy by diffusion, or by bulk flow in response to the transpirational flux of water. Subsequent movement of solutes into the cell or symplast through the plasma membrane is governed by three mechanisms:

1. Diffusion; Uncharged molecules such as CO_2 and NH_3 can enter the cells traversing the lipid bilayer along their concentration gradients.

2. Facilitated diffusion; This is the accelerated movement of solutes along their electrochemical potential gradient ($\Delta\mu$) through proteinaceous channels or pores embedded in the lipid bilayer (see Stein, 1986).

3. Active transport; Early workers defined active transport as the movement of ions or solutes against a concentration gradient (chemical potential difference) through a semipermeable membrane (see Baker and Hall, 1988). Later, in the light of

membrane potential studies, this definition of active transport appeared to be inadequate. Ussing (1949), was the first to include the electrical potential differences in defining active transport. According to Ussing, active transport is the movement of a solute across a cell membrane against its electrochemical potential gradient or free energy gradient at the expense of metabolic energy. Although active transport processes impose heavy demands upon the cell's energy budget, they are essential in,

1. maintaining internal osmolarity (Cram, 1976),

2. scavenging food or nutrients (Skulachev, 1977),

3. maintaining electrical and chemical potential gradients (Poole, 1978),

4. energy storage or transduction (Mitchell, 1966) and in,

5. regulating internal pH (Raven and Smith, 1979).

An extensive discussion of active transport systems in plants in general is beyond the scope of this chapter. Moreover, a large number of such reviews on this topic are available (Poole, 1978). Instead, a brief overview on active transport in plants will be presented.

As described above, active transport is a process which requires metabolic energy. It is generally accepted that the energy source for cation transport is ATP (Hodges, 1976; Jacoby and Plessner, 1970; Leonard and Mettler, 1977; Lüttge and Ball, 1976; Lüttge and Pitman, 1976; Petraglia and Poole, 1976). Hodges (1976), Lüttge and Pitman (1976) and later Poole (1978) have emphasized the importance of the coupling between sources of metabolic energy and the transport mechanism. Higinbotham (1973b), and Poole (1978) have suggested that a major portion of the resting membrane potential across the plasma membrane is produced by an ATP-driven electrogenic H^+ pump, and that the energy conserved by this pump is utilized for the active uptake of other ions. There is now firm evidence that such H^+ carriers are located in other membranes such as the tonoplast (see Baker

and Hall, 1988; Bennett et al., 1984) and golgi membranes (Chanson et al., 1984) in addition to the plasma membrane. These electrogenic proton pumps are also important for the maintenance of the cytoplasmic pH in the range of 7.0 to 7.5 (Martin et al., 1982; Raven and Smith, 1979) by means of proton extrusion to the cell wall (through the plasma membrane) and proton transport to the vacuole (through the tonoplast).

Based upon direct or indirect coupling to the source of metabolic energy, active transport systems can be classified as primary or secondary active transport systems, respectively. In primary active transport systems, the energy required to move the solute against its electrochemical potential difference is supplied directly by a chemical reaction mediated by the transport complex. Three kinds of primary active transport systems have been identified in living organisms (see Skulachev, 1988; West, 1983):

1. ATPases and pyrophosphatases; these are membrane bound enzymes which couple transport of solutes to the hydrolysis of ATP (Pedersen and Carafoli, 1987) or pyrophosphates (Rea and Poole, 1985), respectively.

2. redox pumps; In these systems, successive oxidation reduction reactions taking place on either side of a membrane result in the transport of protons across the membranes.

3. light driven ion pumps of certain halophilic bacteria.

Although ATPases have been the most thoroughly investigated so far, Lin (1985) envisages that ATPases and redox pumps may operate in parallel to generate the necessary proton gradients across the plasma membrane. The focus of this chapter will be upon the first type of primary active transport system, ATPases, of which, the most extensively studied and characterized systems are;

1. The Na^+/K^+ ATPases of animal cells (Skou, 1965; Glynn, 1984),

2. The Ca^{2+}-ATPase of the sarcoplasmic reticulum and plasma membranes of animal cells (Guidotti, 1976; Michalak,1984),

3. H^+-translocating ATPases in fungi and higher plants (Bowman et al., 1980, 1986; Leonard and Hodges, 1973 ; Serrano, 1989).

These transporters, also known as "pumps", may transport more than one ion species, with equal or unequal stoichiometries. Pumps which transport ions of the same charge in opposite direction with equal stoichiometries may be termed "electrically silent" pumps since there is no net charge separation. In contrast, those which transport ions in opposite directions with unequal stoichiometries are considered to generate an electric field across the cell membrane (Spanswick 1981, Sze 1985). Hence such systems constitute "electrogenic pumps" (Poole, 1978; Spanswick, 1981). These pumps, e.g. the H^+ ATPase and the Na^+/K^+ ATPase, are known to be capable of maintaining an electrical potential gradient (negative interior) on the order of - 60 to - 300 mV (Poole, 1978).

Secondary active transport is driven by energy stored in gradients ultimately created by primary active transport. For example, the activity of the plasma membrane H^+-ATPase (a primary transport system) results in the generation of a pH gradient (outside acid) and an electrical gradient (inside negative) which may represent a large inwardly directed electrochemical potential difference ($\Delta\mu_H+$) for H^+. A pH gradient of 2.5 units and an electrical potential difference of -150 mV, for example, is equivalent to an inwardly directed free energy difference for H^+ of 28.5 kJ mol^{-1}. This source of energy, which is also termed "proton motive force" (pmf), can be used to transport other ions into the cell, provided there is a coupling mechanism. The high free energy requirement for anion transport (because of the negative electrical potential across the plasmalemma), for example, is thought to be provided by coupling the transport of the anion to proton influx along the proton gradient. Uniports (see Baker and Hall,

1988) such as those for K^+ (see section 1.3) or Ca^{2+} may respond to the electrical component of the pmf.

An ion such as H^+ (or Na^+ in many cases) representing the source of free energy for co-transport of the dependent ion, is sometimes referred to as the "driver ion" (Sanders, 1986). As well as these co-transport systems (symports), counter-transport systems (antiports) may also utilize the Dl_H+ by simultaneous movements of the driver ion and dependent ion in opposite directions. In animal cells, Na^+ is known to be the most common driver ion (Glynn and Ellory, 1984) while in fungi and plants, H^+ plays the equivalent role (Briskin, 1986; Leonard, 1988; Spanswick 1981). Common examples of co-transport systems in the plant kingdom are discussed in Baker and Hall, (1988). They include;

1. Cl^-/H^+ and NO_3^-/H^+ (Novaky and Lüttge, 1981) and H^+/SO_4^{2-} symports (Lass and Ulrich-Eberius, 1984),

2. The H^+/K^+ symport in *Neurospora* (Rodriguez-Navarro et al.,1986),

3. The $H^+/sucrose$ symport (Humphreys, 1988) and

4. $H^+/glucose$ symport (Slayman and Slayman, 1974).

Counter-transport or antiport systems are exemplified by the H^+/K^+ antiport in the gastric mucosa of animal cells (see West, 1983) and the Na^+/H^+ antiport in sea urchin eggs (Alberts et al., 1983), and fungi (Slayman, 1974).

The intensive efforts directed towards characterizing the plasma membrane and tonoplast primary transport systems have been highly successful. These have been purified, sequenced and cloned. By contrast there is virtually an absence of detailed information (other than physiological) for the symporters or antiporters responsible for secondary transport. The present work was undertaken to further our understanding of the mechanisms responsible for K^+ uptake by plant roots and the regulation of these fluxes. In order to place this topic in context, the

following discussions will focus on the role of K^+ in plants and on current concepts and models describing K^+ uptake and regulation.

1.2 Role of K^+ in plants

K^+ is one of 16 elements that are essential for plant growth and metabolism (Marschner, 1986). The various functions of K^+ in plants have been extensively reviewed (see Marschner and references therein, 1986), therefore only a brief overview on the role of K^+ in plants will be given in this chapter. K^+ is essential (see Mengel and Kirkby, 1982; Marschner, 1986):

1. in protein synthesis.

2. as an activator of more than 50 enzymes, including RuBP carboxylase and Pyruvate kinase.

3. as a major osmoticum and

4. as a charge balancing ion in the cytoplasm and vacuole.

1.2.1 Role of K^+ in protein synthesis

K^+ is known to be essential for protein synthesis. In cell-free translation systems, ribosomes isolated from wheat germ have been shown to require a K^+ concentration of around 130 mM K^+ for optimal activity (see Marschner, 1986). It is suggested that this ion may be involved in the translation process at several steps including the binding to and release of tRNA from ribosomes (Evans and Wildes, 1971; Wyn Jones et al., 1983). The extent of incorporation of ^{14}C-leucine into RuBP carboxylase in leaves of K^+ deficient alfalfa plants has shown the essentiality of K^+ in the synthesis of this enzyme (see Marschner, 1986). Accumulation of soluble nitrogen compounds such as amides, amino acids, amines and nitrates in K^+-deprived plants (see Marschner, 1986) parallels the decrease in protein synthesis.

1.2.2 Role of K^+ as an enzyme activator

Evans and Sorges (1966) reported that more than 50 enzymes are either completely dependent on, or are stimulated by K^+. Enzymes which require or are activated by K^+ include (see Marschner, 1986):

1. pyruvate kinase

2. starch synthase, and

3. membrane bound ATPases (Fisher and Hodges, 1969).

1.2.3 Role of K^+ as an osmoticum

All cells and tissues in plants require a low (negative) osmotic potential for water balance, expansion growth and for various types of turgor related movements. Two major requirements for cell extension are,

1. cell wall extensibility: This is probably induced by the plant hormone, indole acetic acid (IAA); and

2. accumulation of solutes, to generate the necessary turgor.

The available literature supports the view that the accumulation of K^+ within the vacuole, most often associated with organic acid anions such as malate, is a major requirement for cell extension. Haschke and Lüttge (1975) showed that in *Avena* coleoptile, IAA-induced proton efflux was stoichiometrically balanced by the influx of K^+. In the absence of external K^+, the IAA-induced elongation declined and ceased in a few hours. Other plant growth regulators such as cytokinins (Green and Müir, 1979) and gibberellic acid (GA) (la Guardia and Benlloch, 1980) have also been reported to show synergistic effects with K^+, on cell expansion and extension. *Helianthus annuus* shoots showed their highest elongation rate when K^+ and GA were applied together (la Guardia and Benlloch, 1980). Also, in expanding bean leaves supplied with K^+, turgor potential, cell size and leaf area increased to a greater extent than in leaves deficient of K^+ (Arneke, 1980).

When K^+ is unavailable, plants compensate for the deficiency by increasing the accumulation of other solutes, e.g. Ca^{2+} and Mg^{2+} (Kirkby and Mengel, 1976) or organic solutes (Pitman et al., 1971). Pitman et al. (1971) demonstrated an inverse relationship between $[K^+]$ of barley roots and reducing sugar levels.

Many plant movements, such as stomatal opening and closure, and nyctinastic and seismonastic movements are known to depend upon turgor changes caused by movement of K^+. During stomatal opening, light-mediated H^+ efflux causes membrane hyperpolarization leading to increased K^+ uptake through inwardly directed K^+ channels. The lowering of osmotic potential and consequent influx of water causes increased turgor and opening of stomata (see MacRobbie, 1988). The reverse changes occur during closure. The plant growth regulator abscisic acid (ABA) is known to reduce transpiration by causing stomatal closure. Using intact leaves and isolated guard cells of *Commelina communis*, Mittelheuser and Van Steveninck (1971) and MacRobbie (1981) demonstrated that exogenous ABA application caused closure of stomata. This effect is considered to be exerted through the inhibitory action of ABA on the proton pump.

In leguminous plant species such as bean (Kiyosawa, 1979) and *Albizzia* (Satter et al., 1974), nyctinastic or circadian movements of leaves have been observed; i.e. the leaves open during the day and close during the night. These movements are controlled by K^+-mediated turgor changes in specialized tissue in the pulvini or motor organs. Guard cell movements and nyctinastic movements share common mechanisms since both are known to occur as a result of K^+ fluxes in response to the proton motive force. By contrast, the seismonastic movements (folding of leaflets as a response to mechanical stimuli) in *Mimosa pudica*, take place as a response to changes in the distribution of K^+ in the pulvini cells. These are correlated with changes in Ca^{2+} binding (see Marschner, 1986).

It is also well known that by increasing the total osmotic pressure, K^+ contributes to the the flow rate of photosynthate in sieve tubes, from source to sink. For example, in potato tubers, an adequate supply of K^+ increased translocation rates and the storage of photosynthate (see Humphreys, 1988 and references therein). It is also reported that K^+ deprivation decreases the transfer of photosynthate from leaves. This may be due to several factors such as,

1. a greater requirement for sugars as osmoticum in the absence of K^+

2. lower rates of sucrose synthesis,

3. lower rates of phloem loading and phloem transport.

4. lower sink demand due to reduced growth

1.2.4 Role of K^+ in maintaining charge balance

K^+ is responsible for counterbalancing the anionic charges in the cytoplasm. It commonly serves the same function in the vacuole and in xylem and phloem sap (see Marschner, 1986).

The above overview of the role of K^+ in plant growth and metabolism provides a clear explanation for the essentiality of this ion.

1.3 Energy coupling for K^+ uptake

The K^+ concentration of the cytoplasm is generally considered to be maintained around 150 mM (Leigh and Wyn Jones, 1984; also see section 1.5), regardless of variations in supply of this ion (Memon et al., 1985) Many reports (Mengel and Kirkby, 1979, 1980 and references therein) show that plants can take up K^+ at concentrations as low as 10 μM and grow without showing any deficiency symptoms (Asher, 1967; Siddiqi and Glass, 1981). Using microelectrodes the electrical potential difference across plasma membrane of root cells has commonly been reported to be around - 150 mV (see e.g. Thibaud and Grignon, 1981). If

external and internal concentrations ($[K^+]_o$ and $[K^+]_i$ respectively) are known, $\Delta\mu_{K^+}$ (the electrochemical potential difference for K^+) can be calculated using equations 1 and 2.

$$E^N = \frac{zF}{RT} \ln \frac{[K^+]_{out}}{[K^+]_{in}} \qquad (1)$$

$$\Delta\mu_{K^+} = zF (E - E^N) \qquad (2)$$

where $\Delta\mu_{K^+}$ = the electrochemical potential difference for K^+, z= charge of the ion, F = Faraday constant (96.5 kJ mol^{-1} V^{-1}), R = gas constant, T = absolute temperature, E = electrical potential difference across the membrane (in mV), E^N = equilibrium potential difference or Nernst potential (in mV).

Under the specified conditions (i.e., $[K^+]_i$ = 150 mM; $[K^+]_{out}$ = 10 μM; E = -150 mV), $\Delta\mu_{K^+}$ calculated using equations (1) and (2) was + 8.88 kJ mol^{-1}. The positive value indicates that K^+ is at a higher electrochemical potential inside the cell. The entry of K^+ into cells under such conditions must therefore be active (Cocucci & Marre 1976). However, Glass (unpublished data) has recently measured E values as low as - 300 mV in barley. Under these conditions the electrochemical potential difference (free energy) would permit passive movement of K^+ into the cell even with a concentration difference of 99.999 mM between outside ($[K^+]_o$ = 1 μM) and inside ($[K^+]_i$ = 100 mM). Under such conditions, voltage-gated channels may operate and would be the main route for inward movement of K^+ (see section 1.3.5).

If K^+ transport across the plasmalemma at low external concentration takes place against its free energy gradient, it is necessary to know the driving force for this active transport. In experiments where ATP levels have been manipulated by use of metabolic inhibitors, good correlations have been observed

between ATP levels and ion fluxes. For instance, when ATP levels in *Neurospora* were reduced by treatment with CN^- the electrogenic potential due to active H^+ efflux was correspondingly reduced (Blatt et al., 1987). Petraglia and Poole (1980) using red beet demonstrated a similar correlation between influxes of K^+ and Cl^- and tissue ATP levels. Early biochemical investigations (e.g. Fisher et al., 1970) sought to link the observed membrane ATPase activity of plant roots with a direct role in K^+ transport (see section 1.3.1). Currently it is widely held that the proton motive force (pmf) (see section 1.1) represents the direct source of energy required for "uphill" K^+ transport and that the free energy of ATP serves to transport H^+ via the plasma membrane H^+ ATPase. Evidence for an involvement of H^+ in K^+ transport is present in studies carried out as early as 1936 when Hoagland and co-workers documented the acidification of external media associated with K^+ uptake. Subsequently, Jacobsen et al., (1950) and Jackson and Adams (1963), elaborated on the K^+/H^+ exchanges in barley roots and advanced hypotheses to account for the observed stoichiometries. Hence, many treatments that affect the electrogenic H^+ pump (see Spanswick,1981) such as specific wave lengths of light, aging, pH, hormones (Lado et al., 1976), chemical modifiers such as fusicoccin and inhibitors such as DCCD (see Poole, 1978) have parallel effects on K^+ uptake. All these observations support the view that K^+ uptake is driven by pmf.

However, the nature of the coupling between the proton motive force and K^+ transport in higher plants has been a matter of considerable controversy (see reviews by Glass and Siddiqi, 1982; Sze, 1985; Lüttge and Clarkson, 1989 and Kochian et al., 1989). There appear to be three schools of thought concerning the linkage between pmf and active K^+ uptake by roots of higher plants. Two of these have been based on the nature of coupling of K^+ transport to H^+ efflux. Some researchers have advocated a direct chemical coupling, where energized H^+ efflux

and K^+ uptake occur simultaneously via the same carrier as in a K^+/H^+ antiport. Others argue that K^+ influx may be coupled indirectly to the activity of the proton pump either via responding to the electrical component as in electrophoretic coupling or by cotransport with H^+ as H^+ returns passively along the Dl_{H+} (see below).

Figure 1 provides an illustration of the major findings and suggestions for K^+ transport in the plant kingdom available to date. The possibilities illustrated in Figure 1 are discussed below. However, it should be born in mind that, despite extensive physiological studies, considerable controversy regarding the mechanisms of K^+ transport still exists.

1.3.1 Direct (K^+/H^+ exchange, Fig. 1a) or indirect coupling to H^+ ATPase (K^+/H^+ antiport, Fig. 1b)

Several researchers proposed that K^+ uptake at the plasmalemma takes place by a direct chemical coupling (or K^+/H^+ exchange) (Fig.1a) with the H^+ translocating ATPase or indirect coupling to H^+ ATPase (K^+/H^+ antiport, Fig. 1b) (Cheeseman and Hanson, 1979; Lin and Hanson, 1976; Poole, 1974). This would be analogus to the Na^+/K^+ ATPase of animals. Some experiments carried out 2 decades ago provide evidence to suggest (but not prove) that a plasmalemma bound ATPase may represent the energy transducing agent between ATP and K^+. The first experimental evidence for this hypothesis came from Fisher et al. (1970), who reported a correlation between the absorption of K^+ or Rb^+ by roots of barley, oats, wheat and maize and the K^+- or Rb^+-stimulated ATPase activities of membrane preparations from these roots. Leonard and Hodges (1973), using barley roots provided better evidence for this hypothesis by showing that the kinetics of the K^+-stimulation of ATPase and K^+ absorption by these roots were similar in their K^+-dependence. In maize roots (Leonard and Hotchkiss, 1976) and in oat roots (Sze and Hodges, 1977), the observed

similarities between the specificity of plasmalemma ATPase toward alkali cations and the specificity of their transport, also support the concept that plasmalemma ATPase activity may be involved in K^+ transport.

However, the significance of these observation has been questioned (Poole, 1978). Anions (Elzam et al., 1964; Nissen, 1971) and even sugar (Linask and Laties, 1973) show similar transport kinetics, although they are not known to activate the ATPase. Also, Glass and coworkers (1982) and Kochian et al. (1989) have raised serious reservations about these mechanisms, based on the lack of a fixed stoichiometry for K^+:H^+ exchange (Glass, 1982; Newman et al., 1987) and the pronounced electrogenicity of K^+ uptake (Newman et al., 1987).

1.3.2 Indirect chemical coupling or K^+/H^+ symport (Fig.1c)

Several other groups have advocated that K^+ transport in higher plants may occur by means of a K^+/H^+ symport as in *Neurospora* (Blatt and Slayman, 1987; Rodriguez-Navarro et al.,1986). The driving force in this instance too is considered to be the pmf (proton motive force). Newman et al. (1987), working with maize roots, showed K^+ uptake to be highly electrogenic as would be anticipated for the involvement of a K^+/H^+ co-transport system .

1.3.3 Indirect electrical coupling via a uniport (Fig.1d)

A fungal toxin fusicoccin, produced by the phytopathogenic fungus *Fusicoccum amygdali*, is known to increase H^+ efflux from plant tissues. When treated with this toxin, plant tissues demonstrated increased H^+ efflux leading to membrane hyperpolarization and increased K^+ influx (Marre et al., 1974). This provides good evidence for an electrogenic transport of K^+ along its electrochemical potential gradient (similar to b except it is not a symport). Pitman et al. (1975) proposed such an electrophoretic coupling between K^+ transport and H^+ efflux based upon studies of K^+ uptake and H^+ efflux in barley roots. Glass

and Siddiqi (1982) however, reported that in barley roots at low external K^+ concentrations (where K^+ fluxes are considered to be active), the stoichiometry for K^+ uptake : H^+ efflux could be as high as 4:1. K^+ fluxes under such conditions would be strongly depolarizing. They suggested that electrophoretic coupling was most probably limited to the high concentration range (system II) where K^+ uptake is thought to be passive.

1.3.4 Evidence for a K^+-ATPase (Fig. 1e)

Glass and Siddiqi (1982) reported that the stoichiometries for K^+/H^+ exchange do not show a strict 1:1 relationship. This was confirmed by Kochian and coworkers (1989) using ion-selective micro-electrode to measure net ion fluxes and electrophysiological responses. The latter authors reported a lack of sensitivity of the high affinity K^+ uptake system to changes in external pH. Hence they raised questions regarding directly linked K^+/H^+ antiports. In their discussion these workers warned against prematurely rejecting the concept of a K^+-ATPase of the sort (the Kdp system) found in *E.coli* (Epstein, 1985) and yeast (Gaber et al., 1988). Villalobo (1982) has reconstituted an H^+-transporting ATPase of yeast plasma membrane (Villalobo et al., 1981) in proteoliposomes and obtained results showing that the ATPase itself has K^+ transport activity. Considering the behavior of this reconstituted system, Lüttge and Clarkson (1989) suggested that the proton ATPase may have a channel-like (see section 1.3.5) structure available for K^+ uptake, when the membrane is electrically polarized. In the absence of an appropriate membrane potential the channel would be closed to K^+.

1.3.5 K^+ channels (Fig.1f)

In electrophysiology, the term "channel" has a specific connotation, related to ionic conductance and inferred from current/voltage analysis (Lüttge and

Clarkson, 1989). Changes in membrane electrical potential are known to open or close "voltage-gated" channels (Ketchum et al.,1989), whereas other channels may open or close in response to specific ions or to chemical stimulation. Tetraethylammonium (TEA) is a specific K^+ channel blocker which has been used extensively to probe the operation of channels in a variety of organisms. Available biochemical techniques may not be sensitive enough to detect channels since they occur in very small quantities in plant membranes (see Fig.1f and Clarkson, 1989). However, the use of the patch-clamp technique to study channels has provided an entirely different avenue to investigate the channel-mediated K^+ transport in different membranes under different conditions. The low-affinity K^+ transport system described below (see section 1.4) has been interpreted as an inwardly directed K^+ channel (Ketchum et.al., 1989). In *Neurospora*, the high affinity K^+ transport system (see section 1.4) has earlier been proposed to be a K^+/H^+ symport (Rodriguez-Navarro et al., 1986). Recently, Hedrich and Schroeder (1989) suggested that this K^+/H^+ symport system could be accounted for by two components; an inwardly directed ($I_{K}+$) K^+ channel and a H^+-pump. Glass (unpublished data) has recently measured electrical potential values as low as - 300 mV in barley in the absence of K+ in the medium. Under such conditions entry of K^+ even from very low concentrations into the cells could be mediated via a voltage gated channel (see section 1.3).
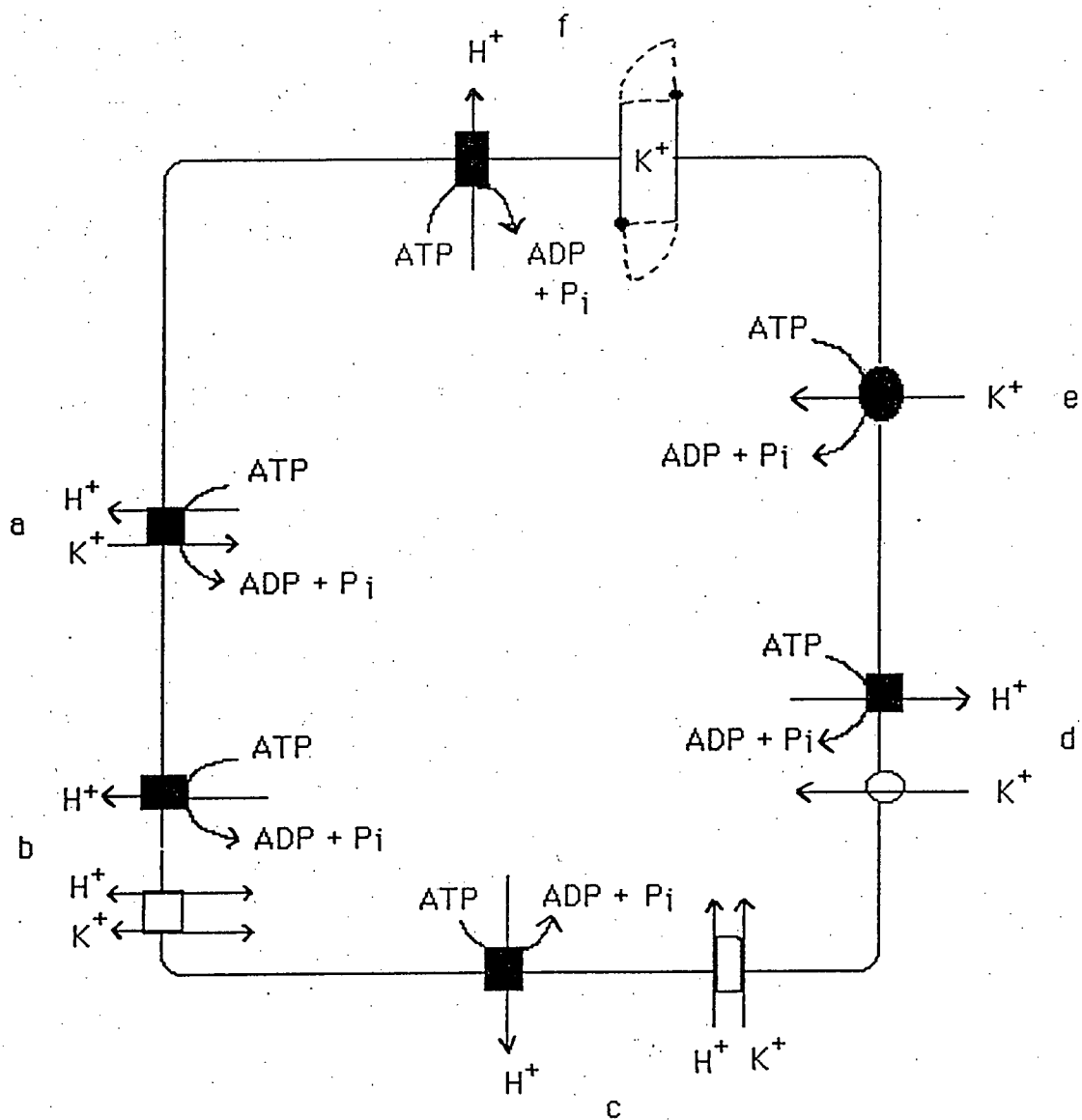
Fig. 1. Suggested mechanisms for K$^+$ transport in bacteria, fungi and higher plant cells.

1.4 Kinetics of K^+ fluxes in plant cells

Investigations of transport kinetics with intact tissues have played a dominant role in the development of hypotheses concerning mechanisms of membrane transport. As early as 1937, Van Den Honert demonstrated a hyperbolic relationship between phosphate uptake rates and external phosphate concentration in roots of sugarcane. This was the first indication of the existence of carrier-mediated transport.

One of the earliest hypotheses dealing with the mechanisms of K^+ uptake was that of Epstein and Hagen (1952). In their experiments using barley seedlings grown in very simple nutrient solutions, they reported that the uptake of K^+ (^{86}Rb) showed a typical hyperbolic relationship with external concentrations at low availability (< 1 mM). They were the first to point out that transport of an ion into a plant cell may be analogous to the binding (and release) of a substrate to (and from) its enzyme. (see model below).

$$S + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} SE \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P$$
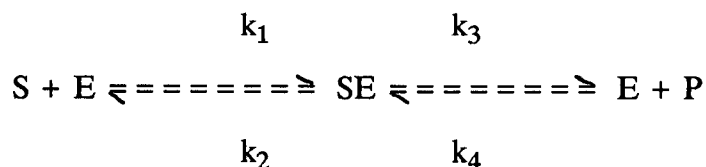
where, S = substrate, E = enzyme, SE = enzyme-substrate complex and P = product. The constant k_1 determines the rate of formation of SE while constants k_2 and k_3 govern its dissociation. k_4, the rate constant for reverse reaction leading from enzyme plus product to the enzyme substrate complex is generally considered to be virtually zero.

The ratio of these constants (k_1/k_2 + k_3), known as the Michaelis constant (K_m), is a measure of the affinity of the enzyme for its substrate. . The amount of enzyme present and its rate of turnover determines the maximum

velocity ($V_{max} = k_3[ES]$) at which the reaction takes place. Epstein and Hagen (1952) introduced this terminology of classical enzyme kinetics to describe carrier-mediated ion uptake. Many subsequent experimenters have made use of this concept as a basis for understanding ion transport in plants.

Epstein referred to the saturable transport process for K^+, occurring at low external concentrations, as "System I". Under such conditions, the electrochemical potential gradient falls short of that required for passive entry of ions (Glass, 1983). This indicates that "System I" is an active transport process. Another feature of "System I" is its selectivity towards K^+ ions. As an example it was shown that even when Na^+ concentration is 10X that of K^+, K^+ uptake was unaffected by the presence of Na^+ (see Lüttge and Higinbotham, 1979). Cation uptake by this system is independent of the nature of the anion and is sensitive to negative feedback by internal concentration of the cation (see Glass, 1990).

If the external concentration of K^+ is raised beyond the level for saturation of "System I", a second transport system becomes evident. This was referred to by Epstein (1966) as "System II". The transition level between these two systems is ~ 1 mM for K^+ uptake. In his earlier studies, Epstein (1966) showed this second transport system to be saturable, with a low affinity for K^+. (i.e. the K_m for "System II" was considerably higher than that for "System I"). Likewise the maximum velocity (V_{max}) was increased. Later, in more detailed studies of system II, Epstein and his co-workers reported that it appeared to consist of a series of hyperbolae (Elzam et al., 1964; Epstein and Rains, 1965). These two systems (system I and II) were referred to as the "dual pattern of ion absorption" or "dual isotherm of uptake". Later studies complicated the picture. For example, Nissen (1973, 1980) interpreted his own data and that of others, in terms of multiphasic transport systems located in the plasmalemma. These systems were thought to undergo concentration-dependent phase changes responsible for mediating the

observed characteristics at different external concentrations. Borstlap (1981, 1983) and Kochian and Lucas (1982) challenged this interpretation. Based on statistical basis of Nissen's analysis (Borstlap, 1981, 1983) and on the their observations of the linearity of the high concentration system and its sensitivity to a K^+ channel blocker, Kochian and Lucas (1982) proposed that transport in this system is mediated by specific K^+ channels in maize roots. Channels are known to transport ions through proteinaceous transmembrane pores along the electrochemical potential gradient and in recent years many ionic channels have been identified in plant systems. Based on random binding of solutes and driver-ion (see section 1.1) to a single transmembrane carrier, Sanders (1986) put forward another explanation for concentration-dependent ion uptake kinetics referred to as "Random ligand binding" kinetics.

Despite over thirty years of controversy concerning the details of these transport processes a clear concensus is still lacking. The arguments may, in fact, become irrelevant as biochemical and molecular approaches give every indication of resolving the very nature of the carrier systems in the near future.

1.5 Regulation of K^+ transport

1.5.1 Constancy of cytoplasmic [K$^+$]

The indispensability of K^+ for metabolic reactions (see section 1.2), provides a rationale for the universal requirement of a constant cytosolic K^+ concentration ($[K^+]_c$). It is widely reported that, despite large variations in biological and environmental factors which might influence $[K^+]_c$, the concentration of this ion is tightly maintained within a concentration range of 150 to 200 mM (Leigh and Wyn Jones, 1984). In early studies where external concentrations of K^+ were maintained by constant replenishments (Parker and

Pierre, 1928) or even in cases where there was a 5000 fold increase in the concentration of the external supply (concentrations ranging from 0.25 to 1,260 μM K^+; Williams, 1961), shoot $[K^+]$ and dry weights increased by factors of only 1.49 and 1.72, respectively. Many other studies (see Glass and Siddiqi, 1984) also provide ample evidence for the constancy of growth rate and tissue concentration over a wide range of K^+ supply. Asher and Ozanne (1969) investigated the changes in growth and tissue $[K^+]$ in 14 plant species grown at various constant K^+ levels ranging from 1 to 1000 μM. Eight of the fourteen species reached maximum yield at 24 μM. Beyond this value only small increments of tissue $[K^+]$ ($[K^+]_i$) were obtained. The half saturation constants ($C_{0.5}$) for growth and tissue $[K^+]$, either calculated from Asher's data (Glass and Siddiqi, 1984), or observed (Siddiqi and Glass, 1983a) for barley grown at 5, 10, 50 and 100 μM, were extremely low (ranging from 1.08 to 2.98 μM). However, the $[K^+]_c$ required for optimal growth may vary among plant species or even among cultivars. Memon et al., (1985), reported that three barley cultivars differed in the distribution of K^+ between vacuole and cytoplasm and suggested that, the efficiency of K^+ utilization and growth response to $[K^+]_0$, was a function of this distribution.

Vacuolar K^+ serves as a reservoir for the maintenance of a constant cytoplasmic $[K^+]$ (Leigh and Wyn Jones, 1984). By contrast to the tight regulation of cytoplasmic $[K^+]$, following withdrawal of external K^+ supply, vacuolar $[K^+]$ ($[K^+]_v$) may decline from ~ 80 μmol g^{-1} fwt to ~ 20 μmol g^{-1} fwt. However, below some critical value of $[K^+]_v$ (around 10-20 mM), $[K^+]_c$ will drop from its optimum value leading to disturbances in protein metabolism and growth. Plants accomplish such constancies in cytoplasmic concentrations of ions, including K^+, by mechanisms that operate from molecular to the tissue, organ, and even the whole plant levels of organization (Bowen et al., 1974; Kahr et al., 1977; Pitman and Cram, 1977; Raven, 1977). There can be little doubt, then, that the K^+

concentrations of tissues are regulated and that the maintenance of cytoplasmic [K^+], in particular, represents a high priority. The mechanisms responsible for this constancy are only poorly understood. Since the turn of the century (Brezeale, 1906) it has been apparent that plants respond to deprivation of particular ions by increasing their capacity for the uptake of this ion. Brezeale reported that wheat plants, deprived of K^+, showed much higher rates of K^+ uptake than plants which had been provided adequate K^+, when K^+ was resupplied. This response is highly characteristic. However, the precise signal(s) responsible for controlling the transport system so as to increase influx or the details of how the transport system, or indeed, the whole organism, responds to such signals is unknown. Ion concentration or concentration-dependent parameters such as volume, turgor or osmotic potential may be important in different systems or under different conditions. In addition, in complex multicellular organisms integration of shoot and root activities virtually demands some form of long distance signal such as hormones or ion concentrations of the xylem or phloem.

Of the external factors likely to perturb the constancy of internal ion concentration, external availability of particular ions and temperature have been extensively investigated.

1.5.2 Thermodynamic control of K^+ fluxes

From a theoretical view point, Raven (1976) has suggested that fluxes of ions through cellular membranes might be regulated through thermodynamic or kinetic controls. Thermodynamic controls are considered to be mechanisms based on changes in the electrochemical potential gradients which might influence the extent of these fluxes. Fluxes of uncharged species such as CO_2, O_2 and NH_3 occur very rapidly across cell membranes down their concentration gradients, and would be expected to respond to changes of such gradients. Likewise,

fluxes of ions such as K^+ which, under certain circumstances, move along their electrochemical potential gradients, should respond to changes of electrochemical potential gradients. For example, in guard cells, decreased (more negative) electrical potential difference, arising from increased H^+ efflux causes increased K^+ influx through inwardly directed K^+ channels (Heidrich and Schroeder, 1989) which ultimately causes stomatal opening. At high external $[K^+]$, such channel-mediated K^+ influx has been proposed to occur in corn roots (Kochian et al., 1989; Ketchum, 1989). However, under natural conditions, where external concentrations of required solutes may be quite low, uptake appears not to be determined by the electrochemical potential gradients. Cram (1973a) evaluated the situation for Cl^- influx in carrot root cells and concluded that the increase of $\Delta\mu_{Cl^-}$ associated with Cl^- loading was insufficient to account for the decrease in uptake rate of this ion. The results of Glass and Dunlop (1979) were even more conclusive. As K^+ accumulated in barley roots, the $\Delta\mu_{K^+}$ decreased, due to greater decrease of plasma membrane electrical potential difference than the decrease of chemical potential difference. Such changes more than compensated for increased tissue $[K^+]$. Hence the thermodynamic changes were opposite to those anticipated. It is therefore, unlikely that thermodynamic control is of great significance in regulating ion fluxes in roots under normal (physiologically meaningful) conditions.

1.5.3 Kinetic control of K^+ fluxes

Kinetic control is considered to be a major factor in regulating ion transport. This could be mediated via changes in velocity, and or changes in the substrate affinity of the transport system or via changes in the number of transporters (see Glass, 1975, 1976, 1983). The latter could occur by means of induction or repression of carrier synthesis. In his review, Raven (1977) referred to

the changes in number of transporters as a "coarse control" and to the regulation of pre-existing transporters as a "fine control".

Influx of ions such as Cl^- (Cram 1973), NO_3^- (Smith 1973), SO_4^{2-} (Smith 1975), Pi (Clarkson et al, 1978) and K^+ (Young and Sims, 1972; Glass, 1975, 1976, 1983; Glass and Siddiqi, 1984) have been shown to be negatively correlated with their internal concentrations. This relationship has been explained in terms of direct negative feedback regulation of K^+ influx by internal K^+ concentration (Young and Sims, 1972). For example, Glass (1976b) proposed that influx was regulated "allosterically" by direct feedback from cytoplasmic K^+ on the K^+ transporter. This proposal was based upon the following observations:

1. changes in K_m and V_{max} for K^+ influx associated with K^+ loading of low-salt barley roots,

2. the rapidity with which such changes of influx occurred following pretreatment with KCl (within 15 min after pretreatment with 50 mM KCl),

3. the nature of the relationship between influx and root $[K^+]$ and

4. The fact that the reduction of K^+ influx, associated with increased internal $[K^+]$, was independent of DNA, RNA and protein synthesis during the pretreatment period. This was concluded by the observed insensitivity of the down-regulation of K^+ influx in the presence of 5-fluorodeoxy- uridine (an inhibitor of DNA synthesis), actinomycin D (an inhibitor of RNA synthesis), cycloheximide and *p* - fluorophenylalanine (protein synthesis inhibitors) (Glass, 1976).

This type of regulation is common as a regulatory mechanism in biosynthetic pathways. Allosteric modifications usually change the affinity of an enzyme or a carrier for its substrate by means of conformational changes of the protein molecule. Conformational changes can be considered as the most rapid way of altering the affinity of an enzyme for its substrate.

Kinetic control other than by direct allosteric effects (Sanders and Hansen, 1980; Sanders, 1981) through effects upon the turnover of the putative carrier systems responsible for K^+ influx (Glass, 1975 and 1977) has also been considered as a means for regulation of K^+ uptake in long term.

By contrast, several reports have suggested that ion fluxes, including those of K^+, were regulated by "shoot demand". This hypothesis has been raised on the basis of factors such as shoot growth rate, root:shoot ratio or shoot $[K^+]$ which have been suggested to regulate the fluxes of ions to xylem (and translocation to the shoot). In turn, fluxes to the xylem may regulate fluxes at the plasmalemma of root epidermal and cortical cells (Pitman, 1972; Pitman and Cram, 1977; Drew and Saker, 1984; La Guardia et al., 1985; Siddiqi and Glass, 1987).

Drew and Saker (1984) concluded from their study using split root systems with and without external K^+, that the negative (allosteric) feedback control of K^+ influx by root $[K^+]$ was absent and suggested that shoot demand determines the rate of uptake of K^+. Siddiqi and Glass (1987) have responded to this argument, suggesting that effects of shoot demand, which may be exerted via recycling of K^+ in phloem (La Guardia et al., 1985) may well exert effects on ion uptake by the roots but that these are expressed indirectly by changing root $[K^+]$ or by changing kinetic responses of the uptake system to root $[K^+]$.

The possibility of negative feedback from cytoplasmic K^+ serving as part of a homeostatic mechanism for regulating K^+ influx has also been questioned on the basis that it is the vacuole not the cytoplasm which experiences changes of $[K^+]$ during deprivation. However, the fact that cytoplasmic $[K^+]$ is apparently constant does not mean that it is invariant. The observed constancy may strongly reflect the efficiency with which the critical $[K^+]$ is restored following minor perturbations. For example, Glass (1977) demonstrated that changes of K^+ influx following loading of K^+ occurred within several minutes. This would appear to be

too rapid to alter vacuole $[K^+]$ which has been estimated to have a $t_{1/2}$ of > 30 h (Memon et al., 1985). Also, all the experiments which have been undertaken to measure cytoplasmic $[K^+]$, e.g. compartmental analysis, require some considerable time. During this time the cytoplasmic $[K^+]$ may indeed vary more than we imagine. In other words, the constancy that is apparent may also reflect the relative time scale of the measurement, the perturbation and the adaptive response. If the measurement is slow by comparison to the perturbation and response, we will see no change.

Yet such rapid responses (the "fine control" of influx referred to by Raven, 1977) may be inadequate to adapt plants to sustained periods of K^+ deprivation. In the long- term, acclimation to such conditions may occur via repression or derepression of carrier synthesis as well as morphological changes (root:shoot ratios, development of root hairs, mycorrhizal association, etc.). The present study was undertaken with the goal of determining the existence of this type of control.

1.6 Statement of objectives

As far as I am aware, this thesis is the first reported study of the biochemical basis of adjustments in K^+ uptake rates in higher plants as a consequence of acclimation to low K^+ supply and low temperature. The ion carriers originally defined by kinetic studies can only be characterized by a combination of biophysical and biochemical methods. Preliminary reports on membrane polypeptides which may be involved in the uptake of NO_3^- (McClure et al., 1987; Dhugga et al., 1988) and K^+ (present work and Fernando et al., 1987, 1989 and 1990) have recently appeared and may be anticipated to lead ultimately to the characterization of these carriers.

In Chapter 2, plasmalemma K^+ influx and tissue $[K^+]$ were compared in barley seedlings grown under conditions of low-K^+ supply. These studies were undertaken to understand the response(s) of barley roots to short (1 h) and moderate-term (3-4 days) K^+-deprivation and to explore the mechanism(s) by which such responses are achieved. Such studies were also carried out using barley seedlings grown under differential shoot/root temperatures.

Prior to undertaking any studies of membrane transport at the biochemical level, it was imperative to isolate and to characterize fractions enriched in various types of membranes. Chapter 3 describes the methods used in isolation of membrane fractions and an evaluation of conventional techniques for isolation, purification and characterization of membrane vesicles in terms of yield and purity, and protein yields in various fractions are compared. No significant difference in protein content of any fraction was observed after up to 10-12 days of K^+-deprivation. However, further exposure to low-K^+ supply drastically reduced the protein content in almost all fractions.

In order to investigate the proposed role of plasma membrane H^+-ATPase in K^+ uptake, an evaluation of *in vitro* plasma membrane ATPase activities in membrane vesicles obtained from roots of seedlings grown under low and high-K^+ conditions was made (Chapter 4). Also, studies of the extent of immunological cross reactivities of the proteins of plasma membrane and tonoplasts obtained from K^+-replete and K^+-deprived roots were made using antibodies to the plasma membrane and tonoplast H^+-ATPases generously provided by Drs. R.T Leonard and L. Taiz, respectively. Comparisons of intensities of the polypeptides in the H^+-ATPase region in SDS-PAGE were also made. None of the above comparisons revealed any significant increase of plasma membrane H^+-ATPase activity during K^+-deprivation indicating that the amount of H^+-ATPase appeared to remain constant under conditions in which K^+ supply was interrupted.

In Chapter 5, changes observed in protein profiles in relation to K^+-deprivation are detailed. Emphasis is given to membrane proteins, especially those of plasma membrane and tonoplast origin. Also, changes in *de novo* synthesis of proteins (both soluble and membrane), in relation to the duration of K^+-deprivation are discussed. In order to determine the specificity of observed responses to K^+ deprivation, protein profiles of membranes from seedling roots deprived of NO_3^- and Pi were also studied.

The observed correlations between increased K^+ influx and the increased expression of certain membrane polypeptides is discussed in terms of the possible involvement of these polypeptides as components of the high-affinity K^+ transport system.

Studies were also undertaken to investigate the biochemical changes in membrane fractions resulting from exposure to low-temperature and to compare these changes to those invoked by low-K^+ conditions. Chapter 6 describes the results obtained for K^+ (^{86}Rb) influx and tissue K^+ concentration in relation to low temperature (5°C) acclimation. The H^+ translocating ATPase activities of the seedlings grown under the same conditions are also discussed. These observations are compared to those resulting from K^+-deprivation.

Chapter 7 provides an overall summary of findings and future goals.

II. EFFECTS OF VARYING K^+ CONCENTRATIONS IN THE GROWTH
MEDIA ON K^+ UPTAKE AND TISSUE K^+ CONCENTRATIONS

INTRODUCTION

Rates of ion uptake vary according to several factors, both intrinsic and extrinsic. These include such important determinants as growth rates and environmental factors such as temperature and external concentration (see review by Glass, 1990).

Experiments were performed to investigate the effects of inhibitors of protein synthesis on K^+ influx after short-term K^+ deprivation. Because of the proteinaceous nature of the carriers and the whole metabolic machinery of the plants, the inhibition of protein synthesis would inevitably affect K^+ uptake in the long-term. However, to determine whether the observed increased uptake rates after short-term K^+ deprivation (Fig. 2 a-b) depend on protein synthesis, it was necessary to use specific inhibitors of protein synthesis during short-term exposures. It was imperative to determine the effectiveness of the selected inhibitors, in significantly decreasing protein synthesis during these short exposures. Two inhibitors of protein synthesis, cycloheximide and puromycin were used in the present study. Cycloheximide is known to inhibit the peptidyl transferase activity of the 60S ribosomal subunit in eukaryotes and is considered to be useful in studies on the inhibition of cytoplasmic protein synthesis. This inhibitor does not affect protein synthesis in organelles such as mitochondria and chloroplasts (see Stryer, 1988). Puromycin is known to cause premature chain termination by acting as an analog of aminoacyl-tRNA, both in prokaryotes and eukaryotes. Administration of this inhibitor to roots should result in release of nascent polypeptide chains. However, neither of these inhibitors should affect existing proteins.

Previous workers have reported different effects of cycloheximide upon membrane transport of different ions and solutes (see Murphy, 1988). Läuchli et

al. (1973) demonstrated an inhibition of K^+ transport from stelar cells into xylem while no inhibition of the uptake of this ion was detected. Schaefer et al. (1975) failed to observe any effect of cycloheximide on K^+ uptake but reported a decrease in translocation to the shoot and suggested that the carriers involved in ion accumulation are longer-lived compared to those involved in ion translocation. By contrast, Glass (1975, 1976) demonstrated that, when roots were pretreated with 10 μg ml^{-1} (~ 36 μM) cycloheximide for 2 h, K^+ uptake of high-salt (0.5 mM $CaSO_4$ + 5 mM KCl) roots, from an external concentration of 5 mM KCl, was reduced by 26 % while influx from 0.05 mM KCl solutions was reduced by 58%. Incorporation of [^{14}C]leucine into barley roots was reduced by 58% by cycloheximide (10 μg ml^{-1}) (Glass, 1977). These experiments indicate that uptake by both system I and system II are affected when protein synthesis is inhibited. However, these plants were grown in very simple solutions initially (0.5 mM $CaSO_4$) for 5-6 days. Such conditions would have almost certainly reduced rates of protein synthesis. In the present work, 7-10 day old seedlings grown in 0.1X Johnson's solutions ([K+] = 600 μM) were used throughout. This is the first report which demonstrates the effects of inhibitors of protein synthesis on K^+ influx in seedlings deprived of K^+ for as short a period as 1 h.

Prior to undertaking investigations on the molecular mechanisms of K^+ uptake (as the principal objective of this thesis), it was necessary to determine the rates of ion uptake under the conditions which were to be used for the biochemical studies. This chapter therefore describes the variation in K^+ influx and internal concentration in relation to short-term and long-term deprivation of K^+. The results agree well with those reported earlier from this laboratory (Glass, 1975; Siddiqi and Glass, 1983; Glass and Siddiqi, 1984).

2. MATERIALS AND METHODS

2.1 Plant material and growth conditions

Seeds of barley (*Hordeum vulgare L.* cv Halcyon) were surface-sterilized with 1% hypochlorite, washed several times with distilled water and then germinated on Plexiglas discs with a plastic mesh in sterilized sand, moistened with distilled water . After 3 days of germination in darkness in a temperature- controlled growth room (temperature 20°C), sand was removed from roots and discs by gently washing in tap water. Discs and plants were then transfered to hydroponic tanks containing full or 0.1X strength Johnson's modified inorganic medium depending on the experiment (Siddiqi and Glass 1987). Root and shoot temperatures were held at 20°C and a 16 h light/8 h dark photoperiod was maintained; irradiance at plant level was ~300 μE m^{-2} s^{-1}. Concentrations of K$^+$ (either 6 mM or 600 μM) in growth media were monitored daily and maintained by constant infusion from concentrated stock solutions by means of a peristaltic pump (Fluid metering Inc.). As plant demand for nutrients increased with time, infusion rates were correspondingly increased. For long-term studies influx was measured after plants had been grown under low-K$^+$ (0 to 10 μM K$^+$) conditions for 4-6 days, while influx in short-term studies was carried out using plants which had been deprived of K$^+$ for 1 - 18 h. After these pretreatments, seedlings were used for growth studies, K$^+$ (^{86}Rb) influx determinations and analysis of tissue [K$^+$]. The age of seedlings at harvest was 10-14 days.

2.2 Cycloheximide and puromycin treatment

These experiment were undertaken to evaluate the importance of protein synthesis in the short-term increase of influx resulting from withholding K$^+$. In order to properly design this experiment, it was necessary to determine minimum

duration of exposure to the selected inhibitors required to inhibit protein synthesis. In subsequent experiments, the duration of exposures to these inhibitors determined to be effective in inhibiting protein synthesis was used to evaluate the effects of these inhibitors on K^+ influx (see below).

2.2.1 Effects of inhibitors on incorporation of ^{35}S-methionine into proteins

For each treatment two sets of seedlings were used. Each set included 6 replicates. Each experiment was repeated twice. Seedlings were transferred to control solutions (0.1 strength Johnson's media with 600 μM K^+) or to -K^+ solutions (0.1 strength Johnson's solution without added K^+), labeled with ^{35}S-methionine (1 mCi l^{-1}) in the presence or absence of inhibitor (either 100 μM cycloheximide or 400 μg ml^{-1} of puromycin) for 0, 30, 60 and 120 min. At the end of these periods 5 g roots were excised and ground using a mortar and pestle in buffer containing 20 mM Tris-Mes (pH 7.6) and 4% SDS. Homogenates were spun at 1000xg for 15 min in an Eppendorf microcentrifuge. To a 0.5 ml aliquot of each of the resulting supernatants, an equal volume of 20% TCA was added, after which samples were left on ice for 1 h. The precipitate was filtered through Whatman GF1 glass fibre filter paper, washed twice with 5 ml of 10% TCA, followed by a wash with 5 ml of 95% ethanol. Filter papers were air dried, transferred to scintillation vials and counted in 5 ml of ACS (aqueous counting scintillant). All TCA precipitations were carried out in duplicate.

2.2.2 Effects of inhibitors on K^+ influx

Conditions used for influx experiments were identical to those described in section 2.3. Seedlings were transferred from high K^+ (600 μM) solutions to fresh high K^+ solutions (1 L each) containing either 100 μM cycloheximide or 400 μg/ml puromycin and treated for 1 h. Controls were transferred to fresh high K^+

solutions without inhibitors. This control was undertaken to check for changes other than those due to inhibitors (pH, solution composition etc.) resulting from transfer to fresh solutions. After 1 h, one set of inhibitor treated seedlings was again transferred to solutions lacking K^+ but containing the inhibitor, while the other set was transferred to high K^+ solutions containing inhibitor. The controls were transferred to high- (600 μM) or low-K^+ (minus K^+) solutions which contained no inhibitors. After another hour in these solutions, roots were gently washed and used for determination of ^{86}Rb influx (section 2.2).

2.3 Influx determinations

Typically, plant roots were equilibrated by two 5 min exposures to unlabeled influx medium (0.1 strength Johnson's solution with K^+ at 100 μM). This treatment served to remove excess K^+ bound to cell walls which might otherwise have caused dilution of the specific activity of the ^{86}Rb-labeled influx solution. After equilibration, roots of intact plants were transferred to identical medium labeled with ^{86}Rb. Measurements of plasmalemma influx were obtained by means of a 10 min influx period, under the same conditions as applied during the growth periods. This was followed by a short (5 min) desorption in unlabeled medium, identical to the equilibration solutions, in order to remove adsorbed isotope. Roots were then excised, spun for 30 s in a basket centrifuge to remove excess equilibration solution, weighed into glass scintillation vials, ashed at 450°C for 12 h and the ashes dissolved in 10 ml of distilled water. Radioactivities and K^+ concentrations of these samples were determined by Cerenkov counting and flame photometry, respectively, as described previously (Siddiqi and Glass 1983). In experiments involving transfer of intact plants to low-K^+ solutions, discrepancies between controls and treatment which might have been caused by transfer to fresh medium or by injury arising from handling the plants, were evaluated in the short-term study (0 - 1 h), by transferring

control plants back to appropriate control solutions at the same time intervals as for the treatments. Each experiment, which included 6 replicates, was repeated three times.

3 RESULTS AND DISCUSSION

3.1. Effects of inhibitors of protein synthesis on K^+ (^{86}Rb) influx

Both cycloheximide and puromycin treatments resulted in pronounced inhibition of protein synthesis estimated by the % incorporation of ^{35}S-methionine into TCA precipitable fraction (Table 1). During the first 30 min of pretreatment, inhibition by cycloheximide was very low. By contrast, puromycin showed its highest inhibition during this time period. However, puromycin treatment increased incorporation of label during further incubation. This may be due to the ability of puromycin to incorporate radiolabel, releasing nascent polypeptide which is still TCA precipitable (Pesatka, 1971). Nevertheless, with even longer (2 h) incubation, incorporation seemed to decline. Therefore, 30 min pretreatment seemed to be the best exposure for the use of puromycin as an inhibitor of protein synthesis under the conditions employed herein using barley roots.

Exposure of seedlings for 2 h to cycloheximide caused 32% and 38 % reduction in ^{86}Rb influx (from a solution containing 0.1 mM K^+) in controls and in low-K^+ (1 h) seedlings, respectively (Table 2). Glass (1976) reported 58% reduction of influx from a solution containing 0.05 mM KCl and 26% reduction from a solution containing 5 mM KCl in high-salt roots which are comparable to our controls. As expected, deprivation of K^+ for 1 h caused an increase (45%) of influx. In the -K^+ seedlings, in which the high affinity system is suggested to be induced or derepressed by K^+ deprivation, cycloheximide caused only a very small reduction (12% of the untreated seedlings) of influx. This indicates only a partial dependence on de novo protein synthesis for induction or derepression of the latter transport system. Therefore, the operation of a mechanism(s) independent of protein synthesis (Glass,1976), may be responsible for the observed increase of

Table 1 Effect of duration of pretreatment (h) with protein synthesis inhibitors on the incorporation of ^{35}S-methionine into TCA precipitable fractions.

Duration of of exposure to inhibitor (h)	% of control	
	Puromycin	Cycloheximide
0.5	60	19
1.0	120	53
2.0	112	45

Results are expressed as percent of controls (no inhibitors) and represent average from two independent experiments.

Table 2. Effects of cycloheximide on K^+ *(^{86}Rb) influx*

Treatment		^{86}Rb Influx	% reduction/increase from control level	
$[K^+]$ μM	CHX	$\mu mol.g^{-1} h^{-1}$	K^+	CHX
600	--	1.07 ±.005	--	--
600	+	0.73 ±.041	--	32
0	--	1.55 ±.035	145	--
0	+	0.96 ±.082	133	38

Cycloheximide (CHX) treatment was given for 2 h during which the roots were exposed to low-K^+ treatment in the last hour. After this treatment ^{86}Rb influx was measured as described in the text. Values represent mean of triplicates. Experiment was repeated twice.

influx in roots deprived of K^+ during the initial time periods.

Puromycin is known to terminate binding of aminoacyl t-RNA carrying the amino acid to the m-RNA on polysomes since it is structurally similar to the t-RNAs. However, since it does not carry an amino acid, a nascent but acid precipitable, polypeptide chain is released (Pestka, 1971; Leninger, 1982; Stryer, 1988). This can be considered as competitive inhibition. Therefore, the concentration of puromycin at the site of protein synthesis should be a critical factor which determines the degree of inhibition. Its availability in the cytoplasm is mainly determined by the rate of uptake and accumulation.

In contrast to what was expected, we observed that K^+ influx was increased during the first hour in the presence of puromycin (Fig. 2). This may indicate that puromycin exerts some effect on the membrane permeability which is independent of its effect on protein synthesis.

3.2. K^+ (^{86}Rb) influx after short-term K^+ deprivation

Effects of K^+ deprivation on its influx have been extensively investigated (see Chapter 1). The amount of information, however, on K^+ uptake after very short term K^+ deprivation is sparse. Such information would be helpful in predicting the nature of the transport system(s). Bearing this in mind the experiments were undertaken.

K^+ influx almost doubled (Fig. 3a) by 1 hour after K^+ deprivation and increased gradually during the next 17 h of K^+ deprivation (Fig. 3b). Experiments carried out to safeguard against extraneous effects on influx arising from handling the plants on transfer to fresh solutions, revealed that the increased influxes which developed during the first hour of K^+ deprivation were not evident in plants
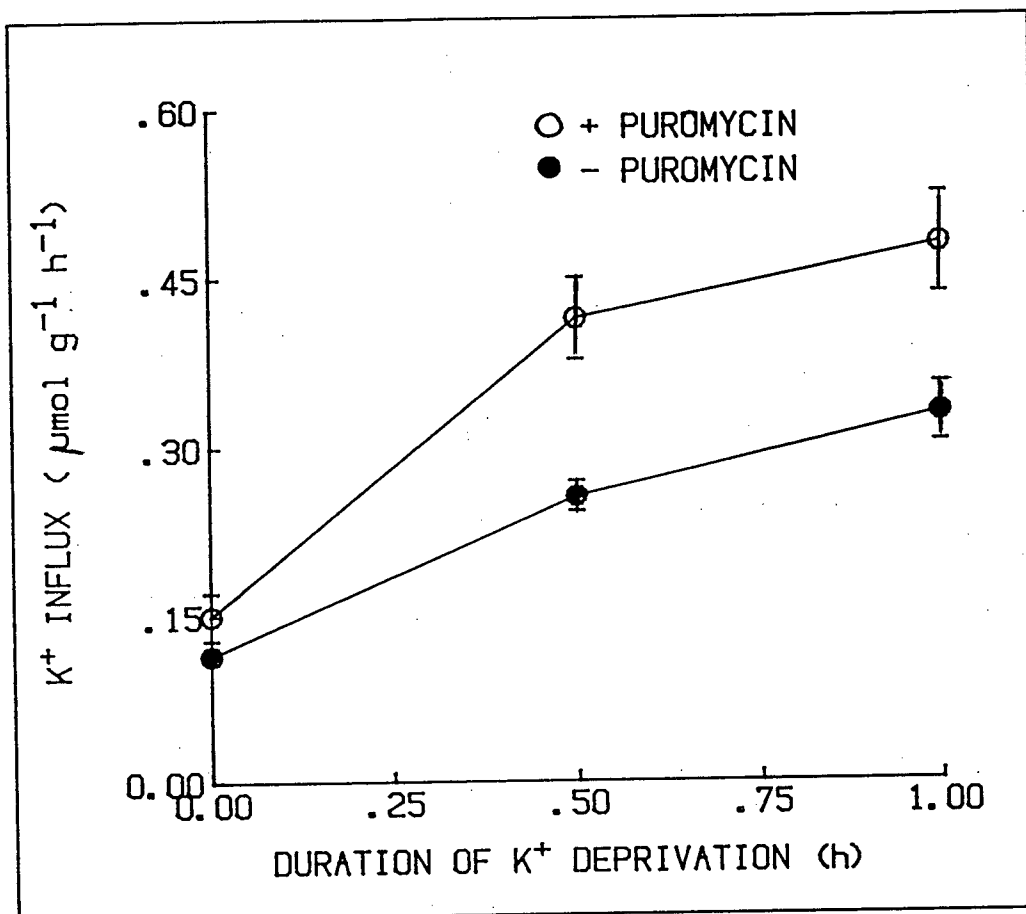
Fig.2   Effect of pretreatment of seedlings with puromycin on K$^+$ *influx*

[86]Rb influx in seedlings deprived of K$^+$ for short time periods (0-1 h) either pretreated or not with 400 μg/ml puromycin. Bars represent $\pm$ S.E. (n = 3). Experiment was repeated twice.

transferred to high K^+ solutions. This showed that the seedlings subject to low-K^+ conditions were not just responding to handling.

3.3. Effects of changes in K^+ supply on tissue K^+ concentration and K^+ influx after longer term K^+ deprivation

Withdrawing K^+ from full strength nutrient medium supporting the growth of barley seedlings caused root $[K^+]$ to decline from 125 to 62 μmol. g^{-1}, a 50% reduction by 24 h (Fig. 4A). Three days without K^+ reduced root K^+ to 40 μmol g^{-1} (a 68% reduction). Shoot K^+, by comparison, declined much more slowly. Compared to control plants maintained in solutions containing 6 mM K^+, withdrawing K^+ caused only slight changes in growth rates; fresh weight of roots of plants deprived of K^+ for 3 days had increased by only 4% of K^+-sufficient plants. Restoration of the K^+ supply brought ambient K^+ concentration to its original value of 6 mM, which caused both shoot and root K^+ concentrations to increase to values equivalent to those prior to withdrawing K^+ (Fig. 4B)

Plasma membrane K^+ (^{86}Rb) influxes responded to these changes in plant K^+ status in characteristic fashion (Glass 1975, 1976; Glass and Dunlop,1978; Ülrich-Eberius et al. 1981, Siddiqi and Glass 1983 and Kochian and Lucas 1988). Typically fluxes rose as root $[K^+]$ fell following interruption of the K^+ supply (Fig. 4A) and declined as root $[K^+]$ increased following resupply of K^+ (Fig. 4B). Responses to altered plant K^+ status can occur quite rapidly following resupply of K^+ to K^+-starved barley plants (Glass 1978) whereas increased K^+ influx following removal of the K^+ source may occur more slowly because reduction of internal reserves depends upon dilution by growth (Glass 1975, Siddiqi and Glass 1987). Such rapidly induced changes, which have been demonstrated to influence the kinetic parameters K_m and V_{max} for K^+ influx have been interpreted as evidence for direct allosteric effects (Glass 1976, 1983 and Ülrich-Eberius et al.,

1981). Results obtained for experiments using inhibitors of protein synthesis appear to support the hypothesis of an allosteric control of K^+ influx (Glass, 1976), at least for the very short-term K^+ deprivation.
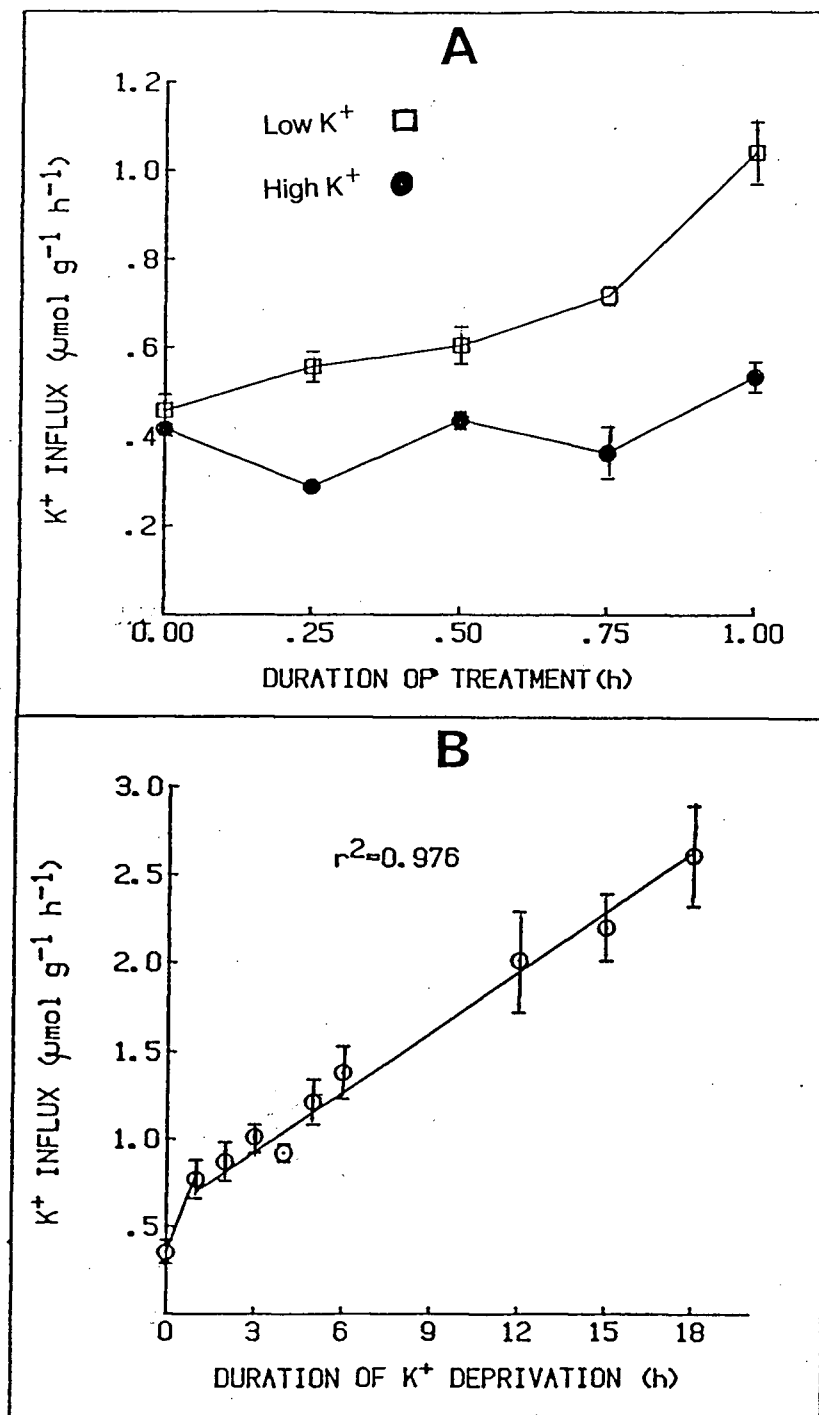
Fig. 3 Effect of short term K^+ *deprivation on* K^+ *influx.*

K^+, (^{86}Rb) influx from 100 μM K^+ solutions in barley roots after 1 hour (A) and 18 h (B) of K^+ deprivation □,○ indicate influx of seedlings transferred to minus K^+ solutions and ● indicates influx of seedlings transferred to plus K^+ solutions at the given time periods. Bars represent the standard deviation of the mean of 3 observations.
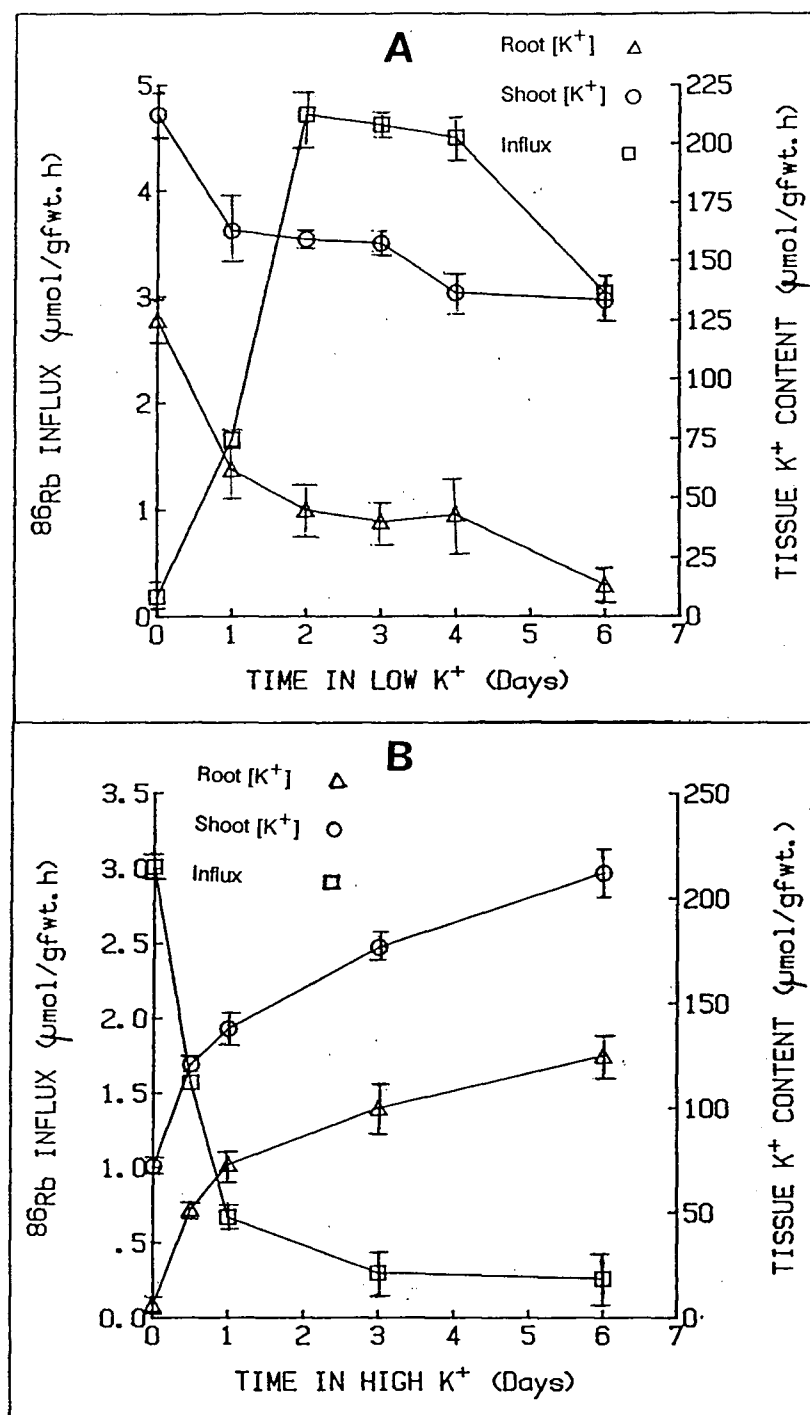
Fig 4 Effect of withdrawal of K^+ *from (A) and resupply to (B) the growth medium, respectively, for several days on its influx and tissue concentrations.*

Root [K+] (△), shoot [K+] (○) and K^+ (^{86}Rb) influx (□) into intact roots of barley. Treatments were staggered so that plants subject to 0 - 6 days of K^+ deprivation (A) or resupply (B) were of the same chronological age. Bars represent the standard deviation of the mean of 3 observations.

III. AN EVALUATION OF METHODS USED IN ISOLATING MEMBRANE

VESICLES IN TERMS OF PURITY AND YIELD

INTRODUCTION

A major requirement for biochemical characterization of membrane proteins is to obtain cellular membranes with considerable purity. Several techniques of subcellular fractionation of plant cells are available (Quail, 1979) and have been used in a multitude of experiments in various disciplines. Of these, differential centrifugation (which use the size and mass of particles in separation), gradient centrifugations (which are based on the bouyant densities of membrane vesicles or even macromolecules such as proteins) and partitioning of membrane vesicles according to their surface properties such as hydrophobicity and surface charges in a two polymer phase system (Albertsson, et al., 1982) appear to be the most widely used techniques.

Although methods available are vast in number and have been improved over the last decade, work on plant membranes still lags behind that on animal cell membranes. The reasons for this may include;

1. The difficulty in isolating membranes without cross contamination by other membranes (Kjelbom & Larsson, 1984).

2. Interference of the cell wall especially in plasma membrane isolation (see Goldberg, 1985; Nagahashi, 1985)

3. The release of large numbers of hydrolytic enzymes from the vacuole. This leads to the degradation of many membrane proteins.

4. The presence of inhibitory phenolic compounds released upon cellular disruption. However, recently techniques have been developed to overcome most of these limitations (see Sandelius and Morre,1990).

This chapter describes the methods used in isolating membrane fractions enriched with various types of cellular membranes in terms of yield and

purity. Emphasis is given to the practicality of these methods when handling several treatments such as those used in the present study, at one time.

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

The methods used for growth of the plant material in these studies are as described in chapter II. The duration of K^+ deprivation and the periods of ^{35}S methionine-labeling varied according to the goals of the experiments and are given for individual experiments with the Figure and Table legends. K^+ deprivation was usually initiated after 3 - 4 days of high-K^+ conditions (high-K^+ conditions are defined as full strength, half strength or 1/10 x Johnson's solutions, see Table 3 for composition). The salts used were varied according to required conditions. For the control growth solutions the following salts were chosen; $Ca(NO_3)_2$ as the source for Ca^{2+} and NO_3^-; $NH_4H_2PO_4$ for NH_4^+ and PO_4^{3-}; $MgSO_4$ for Mg^{2+} and SO_4^{2-}; K_2SO_4 for K^+ and SO_4^{2-}. For other treatments slight variations in the composition of the media were introduced, however, care was taken not to significantly diminish or to supply in excess ions other than those under investigation. The compositions of the growth solutions were maintained both in the controls and in the low-K^+ treatment tanks (Table 3) by constant infusion of the appropriate stock solution at a speed of 2 liters per 24 h using a peristaltic pump. Before transfer to the low-K^+ tanks, seedlings were routinely washed in distilled water for a few minutes and left in well aerated solutions having a composition identical to low-K^+ growth solutions for approximately an hour. This was carried out in order to remove excess K^+ from the root surface and to minimize the introduction of additional K^+ to the low-K^+ tank by K^+ efflux. Generally, $[K^+]$ in the external solution increased to about 20 μM (0.01 -0.02 % of root concentration) during the first hour. Further increase in $[K^+]_o$ was negligible when roots were held in the same solution for longer time periods.

Table 3   Concentrations of inorganic ions in growth media

Concentrations (mM) of the macronutrients in different growth media (full strength Johnson's). All the micronutrients were supplied according to the strength of the Johnson's medium used in each experiments.

Nutrient	Treatment			
	Control	$-K^+$	$-N$	$-P$
K^+	6	--	6	6
NH_4^+	2	2	--	2
Mg^{2+}	1	1	1	1
Ca^{2+}	4	7	4	4
NO_3^-	14	14	--	14
PO_4^{3-}	2	2	2	--
SO_4^{2-}	4	1	7	2

2.2 ^{35}S-Methionine labeling

Control and K$^+$-deprived seedlings were transferred to 400 ml of well-aerated solutions labeled with ^{35}S-methionine 6-12 h before harvesting. The temperature of these solutions and the inorganic composition were identical to that of the medium in which plants had been grown. Labeling was carried out for a 6- 12 h period in solutions with approximately 1 - 1.5 μCi ml^{-1} in either 0.8 or 1 L plastic beakers. These were aerated through needles inserted into the beakers. Solutions were replaced after the first 6 h to prevent significant depletion of inorganic nutrients. After the labeling periods, roots were washed in cold distilled water to remove isotope from the Water Free Space. They were then blotted dry with paper towels, weighed and used for isolation of membranes or frozen in liquid nitrogen and stored in the -80°C deep freezer until used for membrane preparations.

2.3. Isolation of membrane fractions

Root tissue (~ 40 g) was briefly washed in ice cold distilled water, finely cut with scissors and ground in an ice cold homogenizing buffer (80 ml) with a mortar and pestle. Homogenizing buffer consisted of 0.25 M sucrose, 5 mM EGTA, 0.2 mM PMSF, 2 mM SHAM, 1 mM DTT, 1.5% soluble PVP and 25 mM Tris-Mes, pH 7.6. All isolation procedures were conducted at 4°C. The homogenate was strained through 4 layers of cheesecloth and then centrifuged at 1000 x g$_{avg}$ for 20 min in a GSA rotor. The pellet (1K pellet) was collected in 5 ml of resuspension buffer consisting of 0.25 M sucrose, 1 mM EDTA, 10-150 mM KCl, 0.2-2 mM PMSF (depending on the experiment), 1 mM DTT and 5 mM Tris-Mes at pH 7.3. This pellet consists mainly of cell wall debris, unbroken cells and tissues and may also contain plasma membrane. The supernatant was then centrifuged at 10,000 x g$_{avg}$ for 20 min in a Sorvall SS 34 rotor to remove mitochondria and nuclei. The resulting pellet was collected in 5 ml of resuspension buffer (10 K pellet). A microsomal

pellet was obtained by centrifuging the 10,000 x g_{avg} supernatant at 80,000 x g_{avg} for 45 min in a Beckman SW 27 rotor. The pelleted microsomes (80K pellet) were then resuspended using a camel hair brush in 4 ml of resuspension buffer and spun at the same speed for the same time period. An aliquot was left for further analyses and/or fractionated as described in the following paragraphs. Compositions of the resuspension media used for washing of microsomes were varied considerably during the course of these experiments in order to examine the effect of chelating compounds such as EDTA, or increased concentrations and types of proteolytic inhibitors on the protein profiles. Specific conditions will be described in details in the Figure and Table legends.

The following methods were employed for the isolation of membrane fractions enriched in particular types of membrane from crude microsomal fractions:

1. Linear sucrose gradient

2. Sucrose step gradient

3. Aqueous two polymer phase partitioning

4 Sucrose step gradient followed by phase partitioning

5. Partitioning of membrane proteins in Triton-X-114

2.3.1. Linear sucrose gradient

Linear sucrose gradients were used at the outset to determine the sucrose concentrations where plasma membranes and tonoplast equilibrate. Linear gradients were made manually by the following procedure. First, a step gradient was prepared by overlaying the 15-22-34-45 % sucrose in buffer (composition was similar to that of resuspension buffer without sucrose) and left at 4°C over night allowing the layers to diffuse. After carefully layering 1-2 ml of microsomes, the gradients were centrifuged at 80,000 x g_{avg} for 2h. Fractions of 0.5 ml were collected

either from the top or the bottom of the gradient by means of a Gilson fraction collecter. To collect fractions from the bottom of the tube, a capillary tube was carefully inserted into the gradient. Aliquots of these fractions were used to estimate the protein content (by determining absorbance at 280 nm in a Pye Unicam spectrophotometer and to determine refractive indexes (refractrometer). The latter technique allows estimation of the sucrose density (or the percent sucrose in each fraction) by reference to Tables (see Griffith, 1986) . Since this method was not efficient for routine fractionation of membranes, step gradients (Dupont et al., 1988) were used with slight modifications in subsequent experiments.

2.3.2 Sucrose step gradient fractionation

Microsomes washed in resuspension buffer were used for further fractionation by means of sucrose step gradients. Solutions having various concentrations (15%, 30% 34% and 45%) of sucrose were prepared by diluting a 66% (w/w) sucrose stock (see Griffith, 1986) in sucrose gradient buffer having 1 mM Tris-Mes (pH 7.3), 1 mM EDTA, 2mM PMSF, 1 mM DTT and 10 mM KCl. Eight ml of 45% sucrose was carefully overlayed with 9 ml each of 38%, 30% and 15% respectively. 1-2 ml of each sample were then layered over the 15% layer. The gradients were centrifuged at 80,000 x g_{avg} in a Beckman SW 27 rotor for 2 h. Slightly yellowish bands which were well formed at each interphase were very carefully collected with a Pasteur pipette. According to Reid and Williamson (1974) the 15-30% interphase represent the tonoplast enriched fraction while that at 30-34% represents the endoplasmic reticulum and golgi enriched fraction. The plasma membrane enriched fraction was collected at the 34-45 % interphase (Hodges and Leonard 1974) (confirmed by marker enzyme assays, see page 55). All fractions were subsequently diluted at least 3 times with sucrose gradient buffer and spun at 100,000 x g_{avg} for 45 min in a Beckman Ti 50 or Ti 70 rotor. The

resulting pellets were resuspended in 0.5-1 ml of resuspension buffer, frozen in liquid nitrogen and stored at -70 $^\circ$C until used.

2.3.3 Aqueous polymer two-phase partitioning.

Plasma membrane was isolated from the 80K pellet using an aqueous two polymer phase system as described by Lundborg et al. (1983) and Yoshida and Uemura (1984). In brief, after a wash in resuspension buffer, the 80K pellet was loaded into a two-phase system consisting of 6.3% (w/w) of PEG 4000 and Dextran T 500 in a final concentration of 0.25 M sucrose and 5 mM potassium phosphate (pH 7.8) and 30 mM NaCl. The phase mixture was thoroughly mixed by several inversions (20-30 times) and centrifuged at 400 x g_{avg} for 3 min to hasten the phase setting. All procedures were performed at 4°C. After phase settling, the top phase was removed and subjected to repartition by mixing with a new lower phase. The top phase (indicated as PP1 in legends) after the second partitioning (identified as PP2 in legends) has been shown to be highly enriched in plasma membrane (Yoshida and Uemura 1984). The lower phase which contains the endo membranes was repartitioned with a new upper phase. The resulting phases, top phase (PP2) and lower phase (LP2) were diluted in resuspension buffer and pelleted at 100,000 x g_{avg} in a Beckman Ti 70 rotor. The resulting plasma membrane pellets were resuspended in 0.5-1 ml of resuspension buffer, and frozen at -70°C until analysed further.

2.3.4. Sucrose step gradient followed by phase partitioning

This was essential since the yield of plasma membrane in the upper layer from phase partitioning was too low (see results section of this chapter) and it was assumed that most of the plasma membrane had been left behind in the interphase and had been collected along with the lower phase. To investigate this

possibility, the lower phase was diluted in resuspension buffer and spun at 100,000 x g_{avg}. The resulting pellet was loaded on to a sucrose step gradient. A well resolved band at the 34-45 % interphase (plasma membrane enriched band) was obtained indicating that this assumption might be correct. Therefore, if not properly collected there is a strong possibility of losing plasma membrane in the phase partitioning procedure. On the other hand, if the interphase is collected, the probability of obtaining a fraction contaminated by other membrane vesicles is high; e.g. I found mitochondria to be the most notorious contaminant as shown by cytochrome C oxidase assay. Therefore, depending on the priorities, i.e. purity vs yield, either phase partitioning procedure or separation in sucrose gradients alone were carried out in later preparations. For instance, when larger amounts of proteins were required (for assays etc,), only sucrose gradient was used for separation of membrane vesicles. Also in experiments using ^{35}S-methionine, the latter procedure was employed to ensure that sufficient radiolabelled protein was obtained for further analyses.

2.4. Marker enzyme assays

A widely applied method to measure the purity of subcellular fractions is to use "marker enzyme assays". (see Hodges and Leonard, 1974; Nagahashi, 1985; Widell and Larsson, 1990). These permit the determination of purity or extent of enrichment of a particular membrane within a fraction. During the preliminary experiments, the following enzyme assays were routinely carried out to assess the purity of the membrane fractions.

1. Vanadate-inhibitable, K^+-stimulated-Mg ATPase activity for the plasma membrane vesicles (Hodges & Leonard, 1974; Nagahashi, 1985).

2. NO_3^--sensitive ATPase activity as a marker for the tonoplast (Sze, 1985).

3. Cytochrome *c* oxidase and antimycin A insensitive NADH-cytochrome *c* reductase to determine the contamination by mitochondria (Applemans et al., 1955) and endoplasmic reticulum (Omura et al., 1967) respectively.

2.4.1 *ATPase assay for plasma membrane*

Vanadate sensitive, K^+ stimulated Mg-ATPase activity (Hodges and Leonard, 1974) was determined by measuring the release of inorganic P according to Ames (1966) with modifications. Reaction mixtures contained; 3 mM ATP (Tris form), 3 mM $MgSO_4$, 5 mM sodium azide (to inhibit mitochondrial ATPase activity), 1 mM sodium molybdate (to inhibit non-specific phosphatase activity), 50 mM potassium nitrate (to inhibit tonoplast ATPase activity); and 250 mM sucrose in 30 mM Tris-Mes buffer (pH 6.5). In the appropriate instances, 50 mM KCl, 0.5 mM sodium orthovanadate, .02% Triton-X-100 and 2 mM EDTA were present in a final volume of 0.45 ml. To this, 10-30 ug of membrane protein were added to start the reaction and the incubation was conducted at 26°C for 30 min. After incubation the tubes were transferred to ice, 0.5 ml of ice-cold TCA-Perchloric acid (10% TCA w/v in 4% perchloric, v/v) mixture was added to stop the reaction and samples were incubated on ice for a further 30 min. The precipitate formed was then pelleted by centrifugation in a microfuge at 10,000 x g for 3 min. 0.5 ml samples of the supernatant were removed to clean test tubes, 1 ml of Ames reagent (0.42% ammonium molybdate in H_2SO_4) was added and tubes were allowed to stand at room temperature for 45 min. The absorbance was then measured at 800 nm. Standards were prepared using 10 mM KH_2PO_4 in appropriate reaction mix to account for the effect of interfering substances in absorbance measurements.

2.4.2. NO₃⁻ sensitive ATPase activity

Tonoplast ATPase activity was measured according to Sze (1985). The method was similar to that for the determination of plasma membrane ATPase activity except for the pH optimum (pH 8.0 for tonoplast ATPase activity compared to pH 6.5 for plasma membrane activity). The tonoplast vesicles isolated from barley in the present study did not show sufficient NO_3^- sensitivity. This may have been due to the presence of vanadate in the assay medium, which has now been shown to inhibit tonoplast ATPase activity of isolated vacuoles from *Acer pseudoplatanus* (Montrichard et al., 1989). However, the method appears to work satisfactorily for tonoplast vesicles obtained from other plants (e.g. red beet, Parry et al., 1989).

2.4.3 Cytochrome c oxidase and NADH-cytochrome c reductase activities

Cytochrome c oxidase and reductase assays were carried out according to Applemans et al. (1955). Briefly, for oxidase activity, rate of oxidation of cytochrome c, (reduced by adding a few crystals of sodium dithionite, $Na_2S_2O_4$, immediately before use), was measured as the decrease in absorption at 550 nm. To the cuvette, 1.1 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 0.08% Triton-X-100 and 0.1 ml of membrane suspension (~ 10-15 μg of protein) were added. To start the reaction, 100 μl of 0.6 mM reduced cytochrome c was added to the cuvette and the decrease of absorbance at 550 nm was recorded.

To determine the reductase activity, 1.1 ml of 50 mM potassium phosphate, 50 μl of 50 mM NaCN (to inhibit oxidase activity), 50 μl of 0.6 mM cytochrome c and 100 ul of membrane suspension were added to the cuvette. To start the reaction, 100 μl of 3 mM NADH or NADPH were added and the increase of absorption at 550 nm was recorded.

2.5 Protein determination

Several methods (Bradford, 1976; Lowry et al., 1951: Markwell et al., 1978 and Peterson, 1977) were used to determine the protein content in subcellular fractions. Most of the methods available had limitations due to the presence of various chemicals in the buffers which interfered with the assay. The degree of interference was greater in the membrane protein assays, due to the presence of detergents such as SDS which were used to solubilize the membrane proteins. The method which gave the most consistent results was that of Markwell et al. (1978), which is a modified Lowry protein assay (1951). Bovine serum albumin was used to obtain the standard curve. Briefly, 100 μl of sample undiluted or diluted in resuspension buffer was made to 500 μl with distilled deionized water. To each set of standards (0, 20, 40, 80 and 100 ug protein taken from 1 mg/ml BSA stock), 100 μl of resuspension buffer was added and made up to 500 μl with appropriate amounts of distilled deionized water. To these, 1.5 ml of reagent C (2% Na_2CO_3, 0.16% Na-tartarate, 0.4% NaOH, 1% SDS and 0.04% $CuSO_4$) were added and incubated at 37^oC for 30 min. This treatment allows the solubilization of membrane proteins which are therefore available to complex with Cu-tartarate and react with the 150 μl of Folin-Ciacoltau reagent. Samples were then incubated at 37^oC for 45 min. The absorbance of the blue colour product was measured at 660 nm.

2.6 Estimation of the amount of ^{35}S-Methionine taken up and incorporated into various fractions

In order to compare the uptake and incorporation of ^{35}S-Methionine, known volumes from labelling solutions before and after transfer of roots were counted in 10 ml of ACS (Aqueous counting scintillant, Amersham). Also, known weights of roots (washed in cold water) and shoots were reacted in a mixture of 5% Na_2CO_3 and 4% NaOH for 24 h at 50^oC in a shaking water bath to obtain a

homogeneous digest. A known volume of this digest was counted in the presence of ACS. In order to determine the quenching effects of the digesting solution, amounts identical to those taken to count the digest were counted in ACS and corrected for quenching. Also a disc of Whatman GF/F glass fiber filter paper was inserted in all of the scitillation vials (see below for details). All the membrane fractions were spotted on Whatman GF/F filter paper prior to counting. This was carried out in order to treat all the samples in the same manner as those treated with TCA. This latter procedure was undertaken to determine the incorporation of radiolabel into the soluble protein fractions. Briefly, 100 μl of soluble protein fraction was added to 900 μl of 10% ice cold TCA in microfuge tubes. The tubes were left on ice for 30 min. The resulting precipitate was suction filtered through GF/F filters and the filters were washed 3X with 5 ml each of 5% ice cold TCA followed by 2X with 5 ml of 95% ethanol. Filter papers were then air dried and counted in ACS.

3 RESULTS AND DISCUSSION

3.1 Characterization of subcellular fractions

Conventional techniques such as homogenization followed by differential centrifugation were used to obtain microsomal fractions (Materials and Methods). Generally, microsomes are considered to consist of plasma membrane and endomembranes and should be devoid of organellar membranes (e.g. mitochondria and nuclei). However, based on the method of homogenization, microsomes may get contaminated by mitochondrial and plastid membranes (Nagahashi, 1985). The degree of contaminations by such membranes can be detected using marker enzyme assays (see below). In general, the yield and/or purity of a particular membrane fractions depend upon the procedures used for cell disruption and postmicrosomal fractionation (Nagahashi, 1985). The cell disruption technique used was chopping with scissors and grinding by mortar and pestle. These are known as low and medium shear forces respectively (Nagahashi, 1985). In the next section, the techniques used in postmicrosomal fractionation are evaluated.

3.1.1 Evaluation of sucrose linear gradients and aqueous polymer two phase system.

While density gradient centrifugation separates membrane vesicles according to their density or bouyant characteristics (Quail, 1979), partitioning of membrane vesicles in an aqueous polymer two phase system separates them on the basis of the hydrophobic and electrostatic properties of their outer surfaces (Albertsson 1970, Albertsson et al., 1982). By combining the two methods, a two dimensional-membrane separation can be obtained, separating according to size and density in the first dimension and surface properties in the second. Berczi and Moller (1986) have performed such cross purifications. However, they first

partitioned microsomes in a sucrose gradient then repartitioned the bands obtained from sucrose gradient on a two-phase system.

Figure 5 shows the main features of the sucrose linear gradients and the distribution pattern of membrane proteins in the gradients. Fractions were collected from the bottom of the gradient by inserting a capillary tube. Collection of the fractions from the bottom of the tube is less subjective and thereby gave consistent results. However, inserting the capillary tube might have disturbed the gradient. Samples shown in Figure 5A were those previously fractionated into the PEG upper layer by two polymer phase partitioning. It clearly indicates that almost all the proteins in this fractionation are in the region of 30-45 % sucrose indicating that this fraction was almost exclusively composed of plasma membranes. Partitioning in the PEG/DEX polymer system is known to result in highly pure plasma membrane fraction (Larsson, 1985). Fractionation of membranes recovered from the dextran lower layer in the sucrose linear gradient is shown in Figure 5B. The protein distribution in this separation reveals a somewhat broader peak indicating a more heterogeneous nature of the membranes from the dextran lower layer of the two polymer phase.

After determining the sucrose concentrations at which various membrane vesicles equilibrate, in the latter experiments, sucrose step gradients instead of the linear gradients were used. Most of the vesicles appeared to equilibrate between the 10-40% region. Plasma membrane vesicles are known to equilibrate around a density of 1.15 -1.17 $g.cm^{-3}$ (~ 40 -44 % sucrose) (Hodges and Leonard 1974). As indicated by protein assays plasma membrane in this preparation represents about 5% of the microsomal protein. However, interpretations of results obtained by means of sucrose gradients must be made with caution, since it has been reported that the apparent density of plasma membrane vesicles on sucrose gradients varies considerably even between growing and

dormant material (Poole et al., 1984). This observation has technical importance and must be considered when carrying out developmental studies. Also, there seem to be differences in the purities of membrane preparations depending on the origin of the tissues. For instance, Leonard and Hodges (1974) reported that the vesicles at the 34-45% interphase in a discontinuous sucrose gradient of a microsomal fraction from oat roots contained purified plasma membrane vesicles. Berczi and Moller (1986) using dark grown wheat roots, showed that plasma membrane collected at the 34-45 % interphase was less pure than that collected at 33-41 %. Therefore, preliminary experiments must be carried out to determine the conditions suitable for a particular system.

In the present work, several methods were tested in fractionating sub-microsomal membranes. In addition to sucrose gradient and phase partitioning alone, microsomes were first fractionated on two polymer phase system and the resulting pellets from the polyethylene glycol (PEG) upper layer and the dextran (DEX) lower layer were further purified on a sucrose linear gradient (PPSG). This additional purification step was carried out to improve yield while achieving maximum purity in plasma membrane fractions.

Figures 5 illustrate the distribution of proteins resulting from this fractionation procedure. Most of the protein (as indicated by A_{280} nm) from the PEG pellet equilibrated in the 34-45% region corresponding to a density of 1.15 - 1.2 $g.cm^{-3}$ The small amounts of protein at the other densities in this gradient indicate that this gradient has only plasma membrane vesicles. This agrees well with the findings of others, who stated that the aqueous polymer two phase partitioning gives about 95% pure plasma membrane vesicles (Widell and Larsson 1981, Lundborg et al., 1981, Uemura and Yoshida 1983, Kjelbom and Larsson 1984 and a review by Larsson 1985, Berczi and Moller 1986). However, separation by two phase partitioning appears to be less efficient in terms of yield especially in studies which

have several treatments to test. For instance, the amount of protein in the plasma membrane enriched fraction obtained by separation of the DEX pellet on a sucrose gradient is 15 fold higher (Fig 5B) at the same sucrose density (1.15-1.2 g cm^{-3}), than that obtained by separation of the PEG pellet in a sucrose gradient (Fig. 5A). This indicated that most of the plasma membrane partitioned into the upper phase had not been collected. The presence of a broad yellowish interphase between the two phases caused difficulties in determining the exact margin between the upper and lower phase. In order to avoid contamination from other membranes vesicles, interphases were usually not collected along with the upper layer. Since the underlying feature of phase separation is the surface charges of the vesicles (electrostatic properties), this method may be of limited use, especially in instances where there are differences in net surface charge and charge densities between treatments. Such situations may arise in studies using tissues subjected to different nutritional levels. For example, the surface charge densities of vesicles isolated from roots grown in high-K$^+$ may be different from those of roots grown in low-K$^+$ or low-NO$_3^-$. This may not drastically influence the quality of the vesicles partitioned in to the upper layer but may result in quantitative differences. Therefore, although phase partitioning is the best of the available methods in terms of purity, it results in significant reduction in yield unless large amounts of original material are used (Berczi and Moller, 1986; Widell et al., 1982).

3.1.2 "Marker" enzyme assays

It is imperative to characterize the membrane fractions obtained, before using them for further studies. Generally, specific enzymic properties characteristic of particular membranes, (now known as intrinsic markers) are used to identify the origin of a particular membrane fraction and to determine its purity. According to Quail (1979), a marker should ideally be confined to, but uniformly distributed in, a single membrane. In other words, a membrane will be present in direct proportion to its marker. However, none of the membranes, especially those of plants, studied to date have ideally fulfilled these criteria. Cytochrome c oxidase in the inner mitochondrial membrane (Appelmans et al., 1955) and chlorophyll for chloroplast thylakoids (see review by Widell and Larsson, 1990) are the only markers close to the definition of an ideal marker. Nevertheless, the use of markers to identify membrane enriched fractions has been widely practiced (Leonard and Hodges, 1974; see review by Widell and Larsson, 1990). To compensate for the lack of ideal markers, more than one marker is usually used to characterize membrane fractions. These enable one to either verify the enrichment of a fraction with a particular membrane type or to rule out the presence of possible contaminants. Another factor which should be taken into consideration but is often overlooked is the latency of the vesicles, which relates to their sidedness (Widell and Larsson, 1990). For instance, although the vanadate-sensitive, K^+ stimulated Mg^{2+}-ATPase activity is suggested as a marker for the plant plasma membrane (Leonard and Hodges 1974), if the membrane vesicles obtained are sealed and right-side-out, the accessibility to ATP and other substances necessary for the activity of this enzyme is obstructed. Therefore, use of a detergent such as Triton-X-100 to partially solubilize the vesicles is routinely practiced (Larsson et al., 1984). However, it should be noted that the presence of Triton-X-100 in the assay medium generally causes a turbidity which also has an absorption maximum around 650 -700 nm (Eibel, 1969).

Therefore, adjusments such as the inclusion of this detergent in solutions used for the preparation of the standard curves or the use of stronger detergents such as SDS (Bloomwald personal communication, Eibel, 1969) are necessary. However, ultrapure SDS must be used since even analytical grade SDS generates a very dark aquamarine color with Ames reagent indicating the interference of even minute amount of impurities in the reaction.

Characterization of barley root membrane fractions obtained from a linear sucrose gradient (as in Fig.5), using vanadate-sensitive ATPase activity is given in Figure 6A. Vanadate sensitive ATPase activity was taken as a marker for plasma membranes, since vanadate is known to inhibit plasma membrane ATPase in beet (Briskin and Poole, 1984), *Neurospora* (Bowman and Slayman 1979), in corn roots (Gallagher and Leonard 1982) and in many other tissues. The presence of 0.5 mM sodium vanadate in the assay medium caused a 50% reduction of K^+-stimulated, Mg^{2+} ATPase activity in fraction 9 which overlaps the sucrose density of 1.15 g. cm$^-$ ^{3}. The difference between the activity with and without vanadate, represent the plasma membrane H^+ translocating ATPase activity. According to Fig. 6A there are two peaks of vanadate sensitivity; one in fractions 6 - 7 and the other in fraction 9. The densities of sucrose in fractions 6 - 7 are 1.05 - 1.12 g.cm^{-3} (20-22 %). Tonoplast and golgi vesicles are known to equilibrate at these densities. DuPont et al. (1982) and Chanson et al.(1984), have also found multiple peaks of K^+-stimulated, Mg^{2+} ATPase activity overlapping the golgi vesicles on a linear sucrose gradient. Therefore, the validity of this enzyme assay alone as a marker for plasma membranes is questionable.
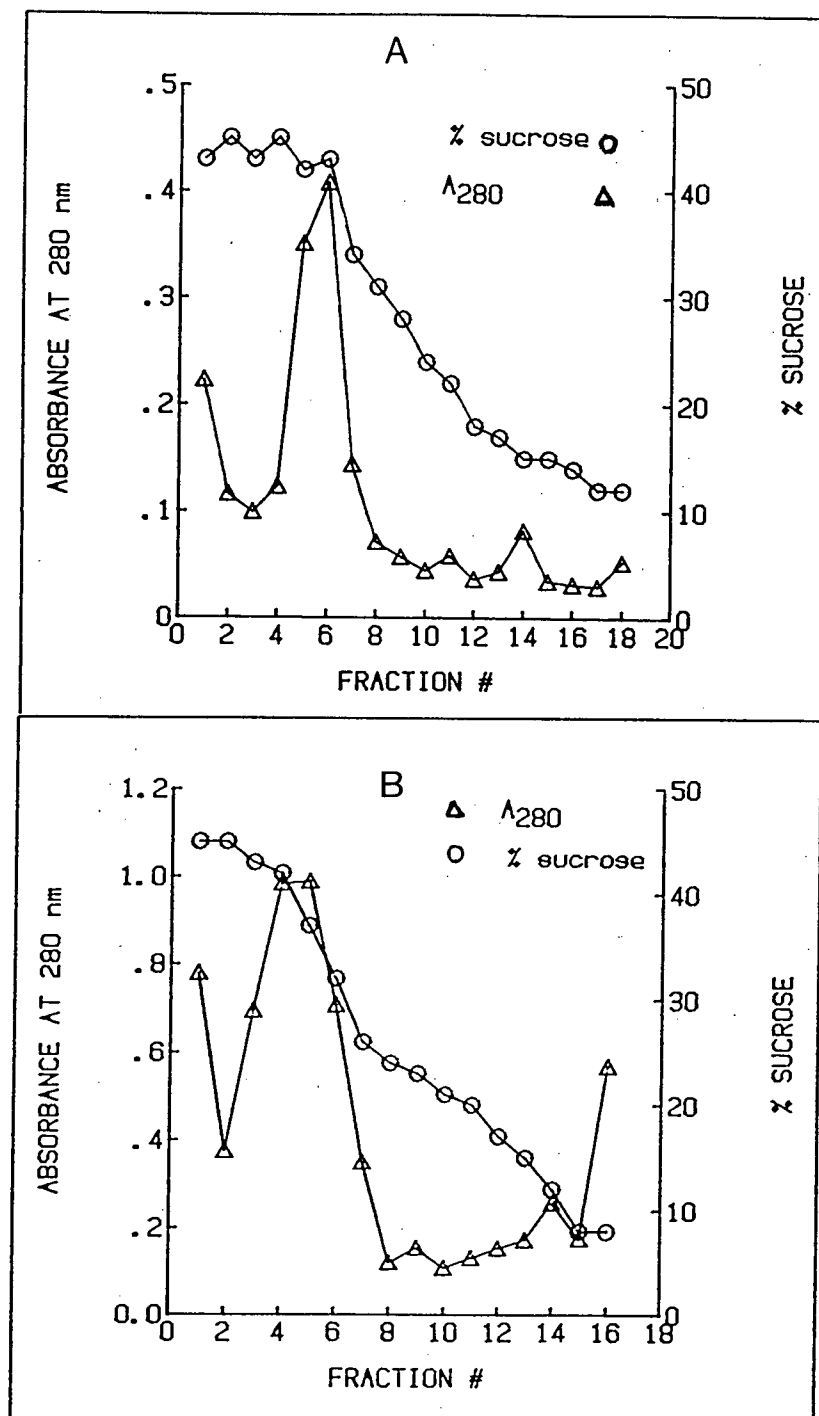
*Fig 5 Cross purification of plasma membranes(A) and endo membranes (B)
obtained from phase partitioning on a sucrose linear gradient*

Distribution of plasma membranes obtained from PEG upper layer (A) and
dextran lower layer of phase partitioning (B). Absorbance at 280 nm (Δ) and %
sucrose (O) in fractions collected from a 15-45 % linear gradient. Fractions were
collected from the bottom of the gradient.

Cytochrome c oxidase and NADH-cytochrome c reductase assays were used to determine the degree of contamination by mitochondria and ER. From the results of cytochrome oxidase assays (Fig. 6b), it is obvious that inner mitochondrial membrane tended to contaminate a broader range of the gradient. Disruption of organelles is known to occur if abrasives are used and if the tissue is ground instead of macerated during homogenization (see Sandelius and Moore, 1990). Although little consideration was given to these factors during the preliminary experiments, abrasives (acid washed sand) were not used and "macerating" instead of "grinding" of roots in buffer was routinely carried out in the subsequent preparations. In fraction 9, at which plasma membrane equilibrates, there is a negligible amount of cytochrome c oxidase activity indicating that contamination of the plasma membrane fraction with mitochondrial membranes is not significant, if at all. Membrane vesicles isolated by phase partitioning once (PP1) and from the lower phase of phase partitioning twice (PP2 LP) had the highest cytochrome c oxidase activity (Table 4). In fact, PP1 plasma membrane fraction appeared to be highly enriched in mitochondria. This indicates that separation in a single operation of the two phase system is inadequate to obtain a pure plasma membrane fraction. Further separation of a PP1 plasma membrane-enriched fraction on sucrose gradient (PPSG PM) resulted in a higher level of mitochondrial activity (Table 5) indicating that, vesicles originated from inner mitochondrial membrane also can equilibrate at the 34-45 % interphase of a sucrose gradient. This agrees with findings of Berczi and Moller (1986). Plasma membrane obtained from sucrose gradient followed by phase partitioning (SGPP PM) seem to result in membranes, largely devoid of mitochondrial or ER contamination (Table 4). However, this fraction had a very low yield of protein (Table 6).
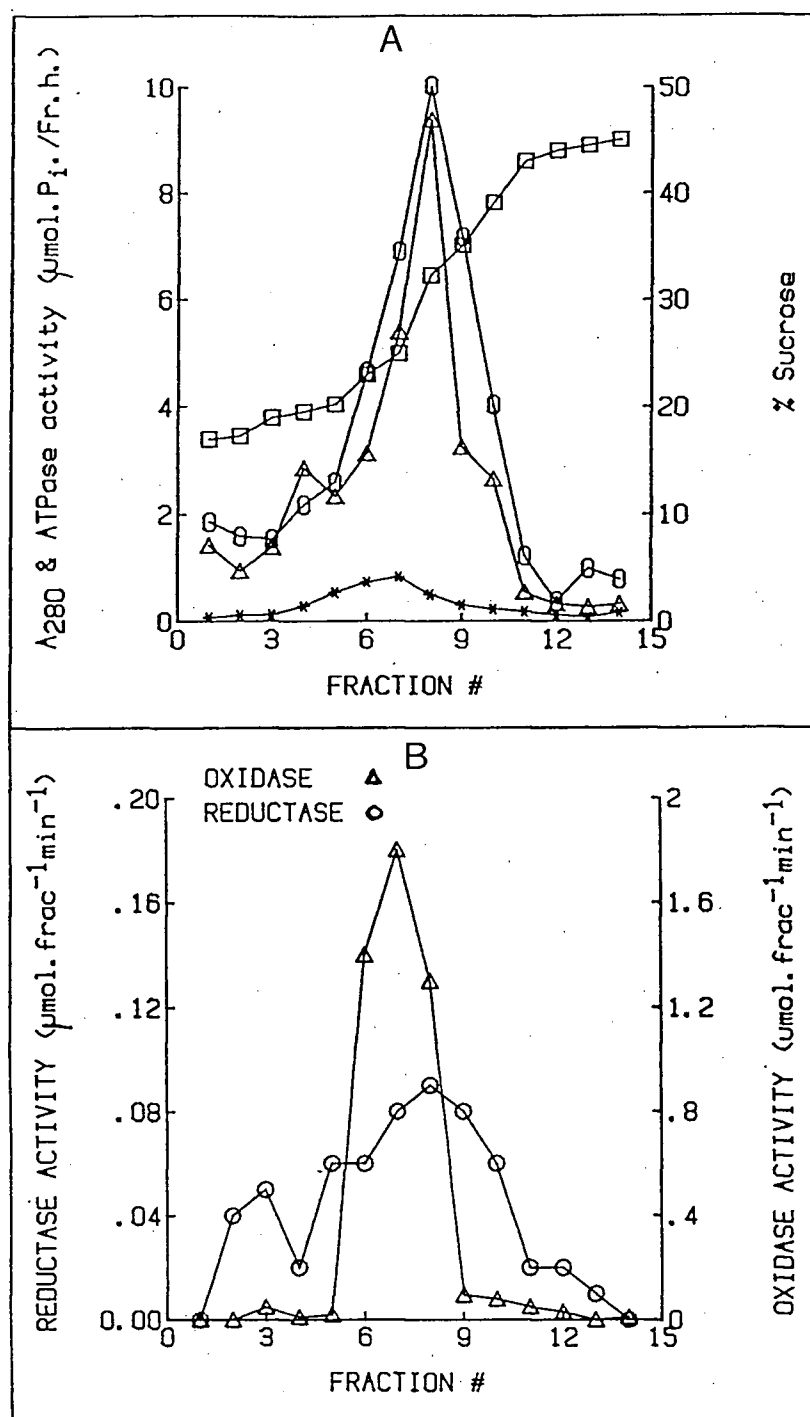
Fig. 6. Distribution of proteins, ATPase activity (A) and cytochrome c oxidase and reductase activities (B) of microsomal membranes on a linear sucrose gradient

6A. Protein (A_{280} nm, ✳), % sucrose concentration (□), ATPase activity in the absence (○) and in the presence (△) of 0.5 mM sodium orthovanadate.
6B. Cytochrome c oxidase (△) and cytochrome c reductase (○) activities of the microsomal fractions collected from a linear sucrose gradient.

Table 4 Comparison of Cytochrome c oxidase and NADH-cytochrome c reductase activities in membrane fractions

Cytochrome c oxidase and NADH-cytochrome c reductase activities (markers for mitochondria and e.r. respectively) expressed in nmol. mg^{-1} protein min^{-1} ± S.E. in membrane fractions obtained from various isolation procedures. These activities are representative of those of 3 separate preparations having 3 replicates in each assay.

Fraction	Specific Activity	
	Oxidase	Reductase
Microsomes	200 ± 10	21 ± 1
PP1 PM	600 ± 50	510 ± 20
PP1 LP	190 ± 10	440 ± 10
PP2 PM	120 ± 11	253 ± 11
PP2 LP	820 ± 60	334 ± 20
SG PM	210 ± 10	360 ± 20
SGRP	230 ± 20	250 ± 40
SGPP PM	30 ± 2	Trace
SGPP LP	310 ± 10	230 ± 10

Abbreviations:

PM: Plasma membrane enriched fraction
PP1 : Phase partitioning once in two phase system
PP2 : Phase partitioning twice in two phase system
SG: Sucrose step gradient fractionation
SGPP: Sucrose gradient followed by phase partioning once in two phase
       system
LP: Lower phase (Dextran) of phase partition
SGRP: Pellet obtained from spinning the remaining sucrose after collecting
       the PM and TP fractions

Table 5 Purity of membrane vesicles obtained from a time course study

Cytochrome *c* oxidase activities ($nmol.mg^{-1}.protein\ min^{-1}$) ± 1 S.E. in membrane fractions obtained from roots of control (HK), and from roots deprived of K^+ for 6 and 12 days (Other abbreviations are similar to those in Table 4.) ND, not detected. These activities are representative of those of 3 separate preparations having 3 replicates in each assay

	Days of K^+ deprivation		
Fraction	0	6	12
MS	138±12	115±11	88±11
PP2 PM	trace	trace	trace
PPSG PM	141±6	139±10	58±4
TP	45±2.1	69±5.6	ND

Abbreviations:

MS: microsomes, PPSG PM: Plasma membrane obtained from further separation of pellet from DEX lower layer of phase system on a sucrose gradient. This is different from the SGPP PM fraction given in table 4).

3.2. Comparisons of protein yield in various fractions

3.2.1 Influence of preparation techniques

Loss of protein can occur during subcellular fractionation due to degradation by proteolytic enzymes, mechanical force and by changes in the lipid environment. Generally, protease and lipase inhibitors, anti-oxidants to inactivate oxygenases, chelators to prevent the activation of hydrolytic enzymes by divalant cations, buffers having pH values unsuitable for the pH optima of degradative enzymes, protective substrates for proteases, chemicals to bind polyphenols and sulphydryl group protectants are all routinely included in the homogenization and resuspension buffers (Nagahashi, 1985). Also, all isolations are carried out at low temperature (0 - 4°C) to minimize protein degradation. However, there is no perfect recipe for complete protection of protein from degradation. Even after protecting protein from degradation, losses can occur due to the methods of collection of membrane fractions, speeds at which membranes are pelleted (see below), and inadequate dilutions of the phase systems (see below) before centrifuging to obtain pellets. A comparison of methods used in fractionation of microsomal membrane, in terms of protein yield, is given in Table 6. It shows that partitioning once in aqueous two polymer phase system gives the highest yield in the upper phase. However, marker enzyme assays (Table 4) show that this fraction has more than 50% of other types of membrane. Although phase partitioning carried out twice consecutively (PP2) gave a very highly pure (~95 %) plasma membrane fraction (see Larsson and Moller, 1990), the amount recovered is about 12% of the PP1. Most of the membrane vesicles partitioned into the upper phase were not recovered. This is shown by the amount of protein in the supernatant "PMSN", after pelleting plasma membrane (57% of the membranes partitioned to the upper phase). It is also apparent that only 43% of the membranes partitioned to the upper phase had been

recovered. This seem to be due to the incomplete pelleting of the membrane vesicles, most probably caused by inadequate dilution of the very viscous polyethylene glycol upper phase. Later it was found that at least 10X dilution of the upper phase was required for complete recovery of vesicles. These findings (1985, unpublished) agree well with those reported later by Berci and Moller (1986). Further partitioning in a fresh phase system appears to result in purer plasma membranes (Berci and Moller, 1986). However, to obtaining reasonable amount of proteins using 3 sequential partitionings, it is necessary to use very large amount (200- 300 g) of tissue. In experiments with several treatments to compare, such as those carried out in the present work, handling such large amounts at one time was technically impossible. Pelleting of membrane from the lower phase also had similar shortcomings. Therefore, separation on sucrose step gradient appeared to be the best compromise method suitable to obtain a reasonably pure (75%) fraction, "enriched" with either plasma membrane or tonoplast, together with satisfactory yields from modest (30 - 50 g) amounts of original material. These results agree with many other studies (Leonard and Hodges, 1973; see also Hodges, 1976; Hodges and Mills, 1986).

3.2.2 Effects of nutrient deprivation on protein content

At the commencement of this study there were no reports on changes of protein content or profiles of particular proteins, especially on those of membranes in relation to nutrient deprivation. In Table 7 effects of K^+ and NO_3^- deprivation on protein contents in various sub-cellular fractions are shown. These plants were grown under the conditions specified (see Table 3) for 10 -12 days in 0.1 strength Johnson growth media. K^+ deprivation decreased root K^+ concentration by 90% and NO_3^- concentration by 47%. NO_3^- deprivation reduced root NO_3^- concentration by 88% and root K^+ concentration by 42%.

*Table 6 Comparison of two postmicrosomal membrane isolation procedures
in terms of protein yield*

Protein contents (mg g^{-1}fwt) ± 1 S.E. in sub-microsomal fractions obtained from two different membrane isolation procedures. Equal amounts of microsomal protein (1.2 mg.g^{-1} fwt) were loaded to the two phase system and the sucrose gradient. (SE of the mean for n = 3).

Fraction	Procedure			
	PP1	PP2	SG	SGPP
PM	.285 ± .002	.037 ± .002	.123 ± .002	.02 ± .004
PMSN	.352 ± .009	Trace	na	Trace
LP	.400 ± .002	.123 ± .001	na	.063 ± .001
LPSN	.328 ± .023	.120 ± .017	na	.219 ± .011
TP	na	na	.109 ± .001	na
TPSN	na	na	.040 ± .002	na
SGP	na	na	.045 ± .001	na
SGRP	na	na	.089 ± .002	na

Abbreviations:

PP1:   Phase partitioning once in two phase system
PP2:   Phase partitioning twice in two phase system
SG: Sucrose step gradient fractionation
SGPP: Sucrose gradient followed by phase partioning once in two phase system
PM: Plasma membrane-enriched fraction
PMSN: Supernatent from wash of the PM fraction
LP: Lower phase (Dextran) of phase partition
LPSN: Supernatent from a wash of the dextran fraction
TP: Tonoplast enriched fraction from sucrose gradient
TPSN: Supernatent from a wash of the above fraction
SGP: A pellet found at the bottom of sucrose gradient
        fractionation (assumed to be mitochondria)
SGRP: Pellet obtained from spinning the remaining sucrose   after collecting the PM and TP fractions
na: not applicable

Therefore changes in the supply of these two nutrients seem to interact in such a manner as to interfere with the accumulation of the other ion. Any observed effects on protein synthesis and content may therefore, reflect the combined effect of changes in concentration of both of these nutrients. As expected, NO_3^- deprivation resulted in a lowering of protein content in almost all the fractions. In low-K^+ roots, except for the tonoplast and other endomembranes, no significant differences were observed in the protein content compared to control values. SGP, the sucrose gradient pellet, represents the membranes, pelleted at the bottom of the sucrose gradient. These may be of mitochondrial origin, since it is known that mitochondrial vesicles can take up sucrose and sink to the bottom of the gradient. This may therefore, imply a lowering of mitochondrial protein content under low-K^+ conditions.

3.2.3 Time course of changes in protein content and synthesis with K^+ deprivation

No significant differences in protein content were observed in pre-microsomal and microsomal fractions during the experimental time periods (Table 7). However, it should be noted that these values are given on a fresh weight basis. It is known that K^+ deprivation results in reduced water content of the tissues (Marschner, 1986). Therefore, the values for high-K^+ (control seedlings) could have been underestimated when expressed on a fresh weight basis compared to a unit dry weight basis. For example, if the dry matter per unit fresh weight accounts for 10%, in seedlings grown under favourable conditions (controls) and 30% in those grown under K^+ deprived conditions, then the values for microsomal proteins in controls and roots deprived of K^+ for 1 day would be 0.071 mg g^{-1} dwt. and 0.023 mg g^{-1} dwt., respectively.

Changes in protein contents of the sub-cellular fractions as a response to duration of K^+ deprivation is given in Table 8. Except for microsomes, other

fractions revealed a gradual increase of protein content up to 6 days of deprivation. obtained by two separation methods are shown in Table 8. The amount of protein in plasma membrane and tonoplast represent 3% and 4%, respectively, of that of microsomal membranes. Protein content in plasma membranes obtained from both methods showed a slight reduction with prolonged K^+ deprivation. Tonoplasts, on the other hand revealed no significant differences. However, as previously shown, these differences might have been significant if calculated on a dry weight basis.

Comparisons of protein synthesis were also made on the basis of the amounts of ^{35}S-methionine incorporated into each fraction. Tables 9 and 10 show the percent distribution of label in sub-cellular fractions of roots. Most of the labeling was found in the soluble fraction. The cell wall debris (1K pellet) and the nuclei and mitochondria (10K pellet) showed a gradual increase, while microsomes showed no significant difference in the incorporated counts with increased duration of K^+ deprivation. Solubles on the other hand, showed a decrease in incorporation of counts. Most of the post-microsomal counts were in the mitochondrial fraction (pellet at the bottom of the sucrose gradient). The counts in plasma membrane and endo-membrane (tonoplast, Golgi and ER) increased gradually during the first 2 days while the counts in mitochondrial fraction decreased with low K^+ treatment. Significant amounts of label were present in the supernatants after centrifugation of PEG upper layer (PMSN) and dextran lower layers (LPSN) to obtain plasma membranes and endomembranes, respectively (data not shown), indicating incomplete pelleting. This indicates inadequate dilution of the upper and lower phases of the two phase partition before centrifugation to obtain the pellets. In subsequent preparations these layers were diluted ~ 10X in the resuspension buffer before centrifugation.

Table 7 Relationship between root [K$^+$], [NO$_3^-$] and protein content in sub-microsomal fractions

Root [K$^+$], [NO$_3^-$] (μmol g^{-1}fwt) ± S.E. and protein content (mg gfwt^{-1}) ± 1 S.E. in sub-cellular fractions obtained from HK, LK and LN (high-K$^+$, low-K$^+$ and low-NO$_3^-$ roots, respectively) seedlings (11 days old). Values represent 3 preparations and 2 protein determinations from each preparation. 1K pellet: 1000 x g pellet, 10K pellet: 10,000 xg pellet, MS: microsomal pellet, PM: plasma membrane; TP: tonoplast obtained from fractionation of endo-membranes from the lower layer of phase partitioning, SGP: pellet at the bottom of sucrose gradient, [ ]: concentration. Figures marked by asterics are significantly different from the control (HK) values.

[ ] or fraction	Treatment		
	HK	LK	LN
[K$^+$](μmol g^{-1}fwt)	91.70 ±.20	8.60 ±.65*	53.81 ±.50*
[NO$_3^-$] "	81.80 ± 2.3	43.70 ±.84*	9.9 ± .65*
Protein (mg g^{-1}fwt)			
1K pellet	0.74 ±.016	0.760 ±.031	0.67 ±.005
10K pellet "	0.060 ±.004	0.047 ±.009*	0.053±.01*
solubles "	5.34 ±.040	5.17 ±.010	4.20±.030*
Microsomal "	0.54 ±.014	0.49 ±.014	0.26±.004*
TP "	0.119 ±.006	0.062 ±.023*	0.06±.003*
PM "	0.026 ±.006	0.018 ±.014	0.012±.011
SGP "	0.108 ±.013	0.083 ±.018*	0.064±.02*

Table 8: Changes in protein contents of sub-cellular fractions as a response to duration of K^+ deprivation.

Protein content (mg g^{-1}fwt) ± 1 S.E. (n=3) of sub-cellular fractions obtained from roots of seedlings grown for 12 days in 0.1 strength Johnson's solutions (600 μM K^+) and transferred to 0.1 strength Johnson's -K^+ (low-K^+) media for the number of days indicated. Other nutrients were supplied and maintained at a constant value.

Days in Low-K^+	Fraction			
	1K pellet	MS	Solubles	Total
0 (HK)	1.35 ±.02	0.71 ±.04	10.70 ±.21	12.8
1 (LK)	1.55 ±.01	0.69 ±.01	10.28 ±.16	12.6
3 (LK)	1.65 ±.004	0.76 ±.15	11.32 ±.65	13.7
4 (LK)	1.67 ±.07	0.57 ±.04	9.90 ±.26	12.1
6 (LK)	1.56 ±.03	0.58 ±.02	14.4 ±1.5	16.6

*Table 9 Changes in protein contents of sub-microsomal fractions as a response
to duration of K^+ deprivation.*

Protein content (μg g^{-1}fwt) $\pm$ 1 S.E. (n=3) of sub microsomal fractions obtained from phase partitioning (2X) of the microsomes from the above experimental system. Lower phase (LP) was applied to a sucrose step gradient for further fractionation (PPSG). Legend is as given in Table 6. ND, not determined.

Days in Low-K$^+$	Phase partitioning		PPSG	
	PM	LP(DEX)	PM	TP
0 (HK)	18±.01	250±.001	2±.002	4±.001
1 (LK)	16±.03	230±.001	20±.001	47±.002
3 (LK)	12±.002	ND	ND	ND
4 (LK)	14±.20	270±.005	14±.001	49±.002
6 (LK)	19±.001	257±.004	ND	ND
12 (LK)	7±.001	126±.008	ND	ND

The increased ^{35}S-methionine labeling in sub-microsomal fractions after 1 day of K^+-deprivation inspite of no significant change in labeling microsomes (Table 10) with prolonged exposure to K^+ deprived conditions was intriguing (Table 11). It correlates well with the increased activity of certain polypeptides within a similar time scale. This provides some idea of the rates of synthesis of the proteins, especially those of membrane origin. There was a significant loss of counts which might have been due to incomplete pelleting of all these fractions caused by inadequate dilution of bands collected from sucrose gradients.

In this chapter the techniques used in isolation of membrane fractions have been evaluated. It is clear that no one method is better than another. The selection of a particular technique must be made with caution and depends on the goals of a particular experiment. For comparative studies, sucrose step gradient appeared to represent a useful compromise, while the PEG/DEX two phase system appeared to be better for the isolation of plasma membrane proteins for definitive characterization.

Table 10 The percent distribution of ^{35}S-methionine labeling in sub-cellular fractions

Counts (as a percent of total radioactivity taken up by roots) of various fractions obtained by differential centrifugation of root homogenate. 1K pellet, cell wall and cellular debris. 10K pellet, nuclei & mitochondria.

Fraction	# of days in low-K^+			
	0	1	2	3
1K pellet	17	12	22	24
10K pellet 23	29	31	37	
MICROSOMES	6	5	4	5
SOLUBLES	54	54	43	34

*Table 11 Comparison of the percent distribution of ^{35}S-methionine label in
sub-microsomal fractions*

Counts in each fraction are given as a percent of total counts in microsomes
obtained from sucrose step gradient. (PM: Plasma membrane, TPGER; fraction
enriched in tonoplast, Golgi and ER. SGR; pellet from sucrose gradient remaining
after collecting the bands. MITO; pellet at the bottom of the gradient presumbly
containing mitochondria)

	# of days in low-K^+			
Fraction	0	1	2	3
PM	5	8	8	5
TPGER	8	6	12	4
SGR	7	9	6	6
MITO	48	20	22	8
Not recovered	32	57	52	67

IV THE HIGH AFFINITY K$^+$ TRANSPORT SYSTEM IN BARLEY: LINKAGE WITH THE
PLASMAMEMBRANE H$^+$-ATPASE OR INVOLVEMENT OF A K$^+$-ATPase
SIMILAR TO THE Kdp SYSTEM OF E.coli ?

INTRODUCTION

During their life cycles, plants are frequently subjected to harsh environmental stresses such as extremes of temperature, shortage of water, and inadequate nutrient supply. Such stress typically evokes physiological and biochemical responses which are adaptive in nature; they enable the plant to compensate for the perturbations caused by the imposed stress. These responses may involve both qualitative and quantitative changes.

In some cases plant cells may sense and respond to imposed stresses very rapidly, by means of changes to existing structures. For example, nyctinastic leaf movements and touch-mediated responses of leaflets of *Mimosa pudica* are mediated by rapid turgor changes (see section 1.2.3.), driven by K$^+$ fluxes between specialized motor cells and surrounding tissues. Plant cells may also show rapid changes, of a complex nature in response to environmental perturbation. For instance, K$^+$ uptake (Enoch and Glinka, 1981; Perry et al., 1987) and phosphorylation of a 39 kDa polypeptide in plasma membrane enriched fractions of storage tissue (*Daucus carota* and *Beta vulgaris*) have been shown to increase in parallel as a response to hypertonic mannitol exposure (Srivastava et al.,1989). Phosphorylation of this polypeptide was responsible for regulating the turnover of phosphatidyl inositol-phosphate (PIP$_2$). The latter compound is well known as a signal transducer in animal (Rasmussen and Barrett, 1984) and in plant cells (Kauss, 1987).

Other plant responses to environmental perturbations are expressed on a time scale of hours to days rather than mere seconds or minutes. For example, changes such as increased capacity to reduce protein disulphide bonds, increased accumulation of membrane proteins and the incorporation of unsaturated fatty

acids into membranes as well as the accumulation of sugars or other solutes are all associated with the development of frost hardiness (see Levitt 1980). Such changes, it is generally accepted, arise from altered gene expression which may bring about changes in the rates of synthesis or turnover of particular proteins. There are many reports in the literature documenting dramatic responses to heat shock or to anaerobiosis; these stresses may lead to termination of synthesis of specific proteins and induction of novel protein synthesis (Hochachka and Somero, 1984; Nagata et al., 1988).

Some other environmental stresses which results in altered expression of particular proteins include salt stress (Weimberg, 1970; Hurkman and Tanaka, 1987; Ramgopal, 1987), osmotic shock (Srivastava et.al.,1989), water stress (Dasgupta and Bewley, 1984) and cold stress (see Levitt, 1980). By far the greatest number of reported studies have dealt with salt stress, osmotic shock and water stress. Categories of salt stress and osmotic shock are usually interpreted in terms of exposure to excess amounts of solutes, particularly those, such as NaCl or $MgSO_4$, which are significant environmental hazards for plant growth. However, salt stress may also be considered in terms of undersupply of inorganic nutrients, a condition which has long been associated with characteristic deficiency symptoms for specific elements.

Because the plasma membrane is of such critical importance as a region of interface between the cell and its environment, it is a prime locus for the placement of sensory systems and adaptive mechanisms designed to counteract environmental perturbation, particularly those associated with ion transport. Unfortunately, other than those of the chloroplast and mitochondria, only a limited number of membrane proteins have been characterized in higher plants. Of plasma membrane polypeptides, the best defined is the H^+-translocating ATPase (E.C.3.6.1.35). This enzyme is responsible for maintaining cytoplasmic pH (Raven

and Smith, 1973), membrane electrical potentials (Poole, 1978) for participating in cell elongation (Cleland and Lomax, 1977) and for the provision of the proton motive force which drives solute transport across the plasma membrane (see Briskin and Poole, 1984). This protein which is considered to consist of two closely associated polypeptides in the 100 kDa region on SDS-PAGE, is known to have an isoelectric pH = 6.5 (Gallagher and Leonard, 1987a; Leonard, 1988; Grouzis et al., 1990). This polypeptide has been purified from a number of plant species, sequenced and the gene cloned from various species including *Arabidopsis thaliana* (see Serrano, 1989). Sequence comparisons reveal that the gene has much in common with genes coding for other ATPases such as the Na^+,K^+ ATPase of animal cells. In recent years two other H^+ translocating enzymes have been characterized in some detail, namely the tonoplast H^+-ATPase and the tonoplast pyrophosphatase (see Sze, 1985; see Baker and Hall, 1988).

To investigate the possibility that K^+ transport was mediated by a plasma membrane ATPase, the effects of K^+ upon ATPase activity of isolated membranes or semi-purified ATPase preparations was extensively examined by several research groups (Fisher and Hodges, 1969; Hodges et al., 1972; Leonard, 1983, 1984, 1988). A characteristic property of plasma membrane ATPases isolated from several plant species was their stimulation by K^+ (Leonard, 1984 and references therein). In ATPases such as the animal cell Na,K-ATPase (see Glynn and Ellory, 1984) and gastric mucosal H^+, K^+-ATPase (De Pont et al., 1985) stimulation of activity by K^+ reflects the direct involvement of this ATPase in the transport of K^+. Initially, the observed stimulation of plant ATPases by K^+ was interpreted to indicate that these ATPases, like their animal counterparts, were directly involved in K^+ transport (Hodges, 1976; Fisher et al., 1973). This hypothesis was further supported by similarities between the kinetics of K^+ stimulation of ATPase activity and the kinetics of K^+ uptake (Fisher et al., 1973;

Leonard, 1984;). However, the degree of K^+-stimulation is quite often low when compared to the total level of ATPase activity (Leonard, 1984) and in some species it is absent (Briskin et al., 1985). Subsequently it became apparent that the isolated ATPases were, in fact, H^+-ATPases. Their involvement in K^+ transport is still a matter of controversy (see section 1.3) and at least 4 different coupling modes are theoretically possible (Lüttge and Clarkson, 1989).

The goal of this study was to investigate the nature of the high affinity K^+ transport system through biochemical methods. The well documented increase of K^+ influx associated with K^+ deprivation was exploited in an attempt to isolate the polypeptide(s) involved in K^+ influx. In addition, two hypothesis regarding the mechanisms responsible for increased influx were tested.

1. *In vitro* H^+-ATPase activity of plasma membrane preparations from roots of K^+-replete and K^+-deprived plants were compared. In addition, the expression of the polypeptides responsible for the H^+-ATPase activity of plasma membrane and tonoplast from roots differentially pretreated with respect to K^+ were compared by means of SDS-PAGE, followed by probing with antibodies raised against the plasma membrane and tonoplast H^+-ATPase of corn, respectively. The purpose of this experiment was to evaluate the hypothesis that increased K^+ influx was the result of increased H^+-ATPase enzyme.

2. The cross-reactivities of membrane preparations from K^+-replete and K^+-deprived roots to antibodies raised against the high affinity K^+ transport system of *E. coli* (a K^+-ATPase refered to as the Kdp system), were evaluated with a view to testing the hypothesis that higher plants possess a specific K^+-ATPase transport system homologous with the Kdp system of *E.coli*.

2 MATERIALS AND METHODS

2.1 Growth of plants and isolation of membrane vesicles from barley roots

Plant growth conditions and procedures used in the preparation of membrane vesicles (sucrose step gradient fractionation described section 2.3.2) were discussed in detail in Chapter 3.

2.2. Growth conditions and isolation of membrane fractions from *E. coli*

Strains of *E. coli* were gifts from Dr. P. Bragg in the Department of Biochemistry, UBC. The genes for the high affinity K^+ uptake system (Kdp ABC) in strain *AN 180* are repressed when *E. coli* is grown at high external [K^+] = 102 mM). If grown at low external K^+ (600 μM or less) just enough to sustain growth, the Kdp system is derepressed. This treatment (i.e. low [K^+]) was used as a positive control for the Kdp antisera. Cells grown at high [K^+] (102 mM) and another strain (*TK 3D11*) (a mutant line which fails to express the Kdp transport system) were used as negative controls in testing the Kdp antisera. Culture of these bacteria and preparation of membrane samples were according to methods used by Bragg et al. (1972) with slight modifications. Both strains had been grown originally on Difco's " antibiotic medium 3 " which contains $\sim$ 52 mM K^+. Minimal media used for *AN 180* cultures consisted of K_2HPO_4, 7.0g/l; KH_2PO_4, 3.0 g/l; Na-citrate.$2H_2O$, 0.5g/l; $MgSO_4.7H_2O$, 0.2g/l; $(NH_4)_2SO^4$, 1.0g/l; Fe.citrate, 3.5mg/l; glucose 0.4% (autoclaved separately and added), thiamine 1mg/l and arginine 50mg/l. This medium therefore contained 102 mM K^+. K^+ deficient medium for *AN 180* had Na_2HPO_4 5.68g/l and NaH_2PO_4 3.03g/l to replace the K^+ salts and in order to maintain the osmotic balance and PO_4^{3-} supply. Sterilized cultures (2 l each) were inocculated under sterile conditions and incubated at 37°C on orbital shakers for 2-3 days.

When the cultures were fully turbid (2-3 days), the cells were suspended in buffer at a ratio of 1 g of cells : 3-10 ml buffer (50 mM Tris-SO$_4$ pH 7.8, 10 mM MgCl$_2$) at 4°C and a few crystals of DNAase were added. Cells were ruptured in a chilled French press at 20,000 psi, and the homogenate was centrifuged at 10,000 x g$_{avg}$ for 10 min. The supernatant was then centrifuged at 180,000 x g$_{avg}$ for 2 h. The sedimented membranes were resuspended in the same buffer and resedimented to give "washed membranes". A low ionic strength wash was given by dialyzing the membranes suspended in "dialysis buffer " (1 mM Tris HCl pH 7.5, 10% glycerol, 0.5 mM EDTA and 0.1 mM DTT) at a rate of 1g original cells: 1 ml of buffer. Dialysis was performed in a large volume (e.g. 40g cells/40 ml of buffer dialysed in 4 l of buffer) of the same buffer at 4°C overnight. The membranes were then diluted ~ 2 fold in the same buffer and centrifuged at 180,000 x g$_{avg}$ for 2 h. Membranes were then resuspended in the same buffer and resedimented to give " dialysed membranes". These were run in SDS-PAGE and used for western blotting.

2.3 Vanadate sensitive H$^+$-ATPase assay

The assay used has been described in section 2.5.1.

2.4 Immunological studies using antibodies raised against the plasma membrane and tonoplast H$^+$-ATPases from corn and the Kdp system from E.coli

Equal amounts of protein (20-25 μg) from plasma membrane and tonoplast enriched fractions were heat-denatured in the sample buffer (which consisted of 0.125 M Tris-HCl, 25 % glycerol, 2.5 % SDS, either 2.5 % 2-mercaptoethanol or 50 mM dithiothreitol and 0.05 % bromophenol blue) and subjected to electrophoresis in 10% acrylamide gels under conditions described in chapter 5 (section 2.3). Western blotting was carried out according to White

(1987) with slight modifications. Antisera for plasma membrane H^+-ATPase and tonoplast H^+-ATPase from corn and for Kdp system from *E. coli* were gifts from Drs. R.T. Leonard, L.Taiz and A.Siebers respectively, who are gratefully acknowledged.

Transfer of proteins from the SDS-PAGE onto nitrocellulose was carried out overnight at 8 V and 220 mA in 50 mM Na acetate (pH 7.0) at 4°C using an apparatus of the Bio-Rad "Trans-Blot" type. The Western blots were incubated in blocker (3% (v/v) Norland Hipure fish skin gelatin [Saravis, 1984]) in phosphate buffered saline (PBS) (1.37 M NaCL, 27 mM KCL, 81 mM NaH_2PO_4, 15 mM KH_2PO_4 pH 7.4) for 1 h. Blots were then incubated in antiserum diluted 1:3000 (for plasma membrane H^+-ATPase antiserum), 1:5000 (for tonoplast H^+-ATPase antiserum), 1:500 (for anti-KdpA), 1:10,000 (for anti KdpABC and anti KdpB) and 1:100,000 for anti-KdpC) with PBS for 1 h, then washed 3 x in PBS (10 min. for each wash). This was followed by 1 h of incubation with alkaline phosphatase conjugated to goat anti-rabbit antibody (BRL) in blocker and another 3 washes in PBS. Blots were then washed in 50 mM Tris-HCl pH 8.0 followed by addition of substrate (0.1% w/v Naphthol AS MX phosphoric acid, disodium salt (Sigma) dissolved in the same buffer). Blots were developed until the optimum colour intensity was obtained (15-30 min or more) and dried between paper towels.

3 RESULTS AND DISCUSSION

ATPase activities, measured by the release of orthophosphate from ATP, were compared for microsomal and plasma membrane-enriched fractions obtained from roots of plants grown under low-K^+, low-NO_3^- and control (complete nutrient solution) conditions. Seedlings grown under low-NO_3^- conditions were used in order to examine the specificity of any responses to K^+ deprivation. ATPase activities of the microsomal fractions from high-K^+ roots were 2.5X and 2X greater than those obtained from low-K^+ and low-NO_3^- roots, respectively (Fig.7). However, when the inhibition by orthovanadate was compared for the three treatments, the microsomes from roots grown in high-K^+ showed the lowest sensitivity to the inhibitor indicating that some part of the observed activity was the result of non-specific phosphohydrolase activity (not withstanding the presence of phosphatase inhibitor, 1.0 mM molybdate) rather than of H^+-ATPase activity. Microsomal fractions obtained both from roots grown in low-K^+ and low-NO_3^- solutions showed 50-60 % inhibition by vanadate. These results agree well with those reported by Gallagher and Leonard (1982), and by Poole et al.(1984). K^+ stimulation of ATPase activities were low for all three treatments; 7% for low-NO_3^-, 10% for high-K^+ and 12% for low-K^+. Stimulation of activity by K^+, however, was low compared to values reported by Travis and Booz (1979) (50-100 %) for soybean root tissue. The amount of K^+-stimulation of H^+-ATPase activity varies according to cell type; meristematic regions show greater K^+ stimulation than the non-growing regions (Travis and Booz, 1979) and K^+ stimulation of H^+ ATPase activity also depends on the growth regime (Wignarajah et al., 1983; Lundborg et al., 1983). Although other inhibitors such as azide (for mitochondrial ATPases), nitrate (for tonoplast ATPases) and molybdate (for non-specific phosphatases) were included in the assay medium, the extent of their inhibition may not have been

100%. Hence, the real effects of vanadate and K^+ on plasma membrane H^+-ATPases may be partially masked. Therefore, plasma membrane enriched fractions, obtained from sucrose step gradients, were used in order to obtain a better assay of ATPase activities.

ATPase activities in plasma membrane-enriched fractions obtained from high- K^+ (16 μmol mg^{-1} protein h^{-1}) and low- K^+ (13 μmol mg^{-1} protein h$^-$^{1}) roots showed no significant differences. By contrast, the activity of plasma membrane fractions obtained from low-NO_3^- grown roots was very low; 7 μmol mg^{-1} protein h^{-1} (Fig. 8). These observations demonstrate that K^+ deprivation had not changed the amount of H^+-ATPase in the plasma membrane while lowering the [NO_3^-] of the growth media significantly reduced the amount and/or activity of this protein. The latter observation was not entirely unexpected, since NO_3^- was the only N source for protein synthesis in these experiments. K^+-stimulation of plasma membrane H^+-ATPase activity was relatively low in all treatments. In fact, in certain plant species stimulation of ATPase activity by K^+ was difficult to demonstrate (Leonard, 1983). Recently, Grouzis et al. (1990) reported that in the absence of an electrochemical potential gradient for H^+ (in the presence of gramicidin), K^+ stimulation was attributable to a direct effect of cation on plasma membrane proteins. K^+ stimulation of H^+-ATPase activity was greater (30%) in the plasma membrane fraction of low-K^+ roots than in those of high- K^+ (18%) or low-NO_3^- (22%) roots. The comparatively higher levels of K^+ stimulation in vesicles obtained from low-K^+ roots may even be an artifact. During homogenization the release of K^+ from the control (high- K^+) roots may have increased the background level of [K^+] in the homogenizing buffer, which originally had 10 mM K^+. After homogenization of roots grown in high-K^+, the [K^+] of the homogenate had increased to 60 mM from the K^+ released by the tissue. This would have increased the basal Mg-ATPase activity (activity measured in the

absence of K^+ in the assay buffer) of vesicles derived from roots grown in high-K^+, thereby masking the effect of the addition of 50 mM K^+ on the measured ATPase activity.

Vanadate inhibition in all treatments was 55-60%, which is slightly lower than the values reported previously (O'Neil and Spanswick, 1984). These authors have reported that inhibition by vanadate is directly proportional to the concentration of free Mg^{2+} in the assay medium and that the maximum inhibition of 80% was achieved in the presence of 6 mM $MgSO_4$. However, in the present study equimolar concentrations (3 mM) of Mg^{2+} and ATP were used. Also, 1 mM EDTA, which partially reversed the vanadate inhibition (O'Neil and Spanswick, 1984), was present in the assay medium. Therefore, the slightly lower inhibition observed in the present study might have been caused by the presence of 1 mM EDTA.

No significant changes were observed in the intensity of Coomassie blue staining of the 100 kDa region of SDS-polyacrylamide gels (Fig. 12, 13 and 14), suggesting that K^+ deprivation had not resulted in significant changes in the expression of the plasma membrane H^+-translocating ATPase. Typically 2-3 bands were discerned in the 100 kDa region (Fig. 12, 13 and 14). Biochemical heterogeneity, in terms of number of bands present and different isoelectric points (at pH 6.5 and 6.0) of polypeptides from corn roots in the 100 kDa region was first reported by Gallagher and Leonard (1987) and for barley by Dupont et al (1988). Purified H^+/K^+ ATPase from gastric mucosa plasma membrane (Takaya et al.,1987) showed an isoelectric point of pH 6.5. Based on these observations, Grouzis (1990) tested the possibility that, these two bands represented two different ATPases, with only one (similar to that in gastric mucosa), being stimulated by K^+. They concluded that, the K^+-dependent ATPase activity could not be limited to but one of the two polypeptides.
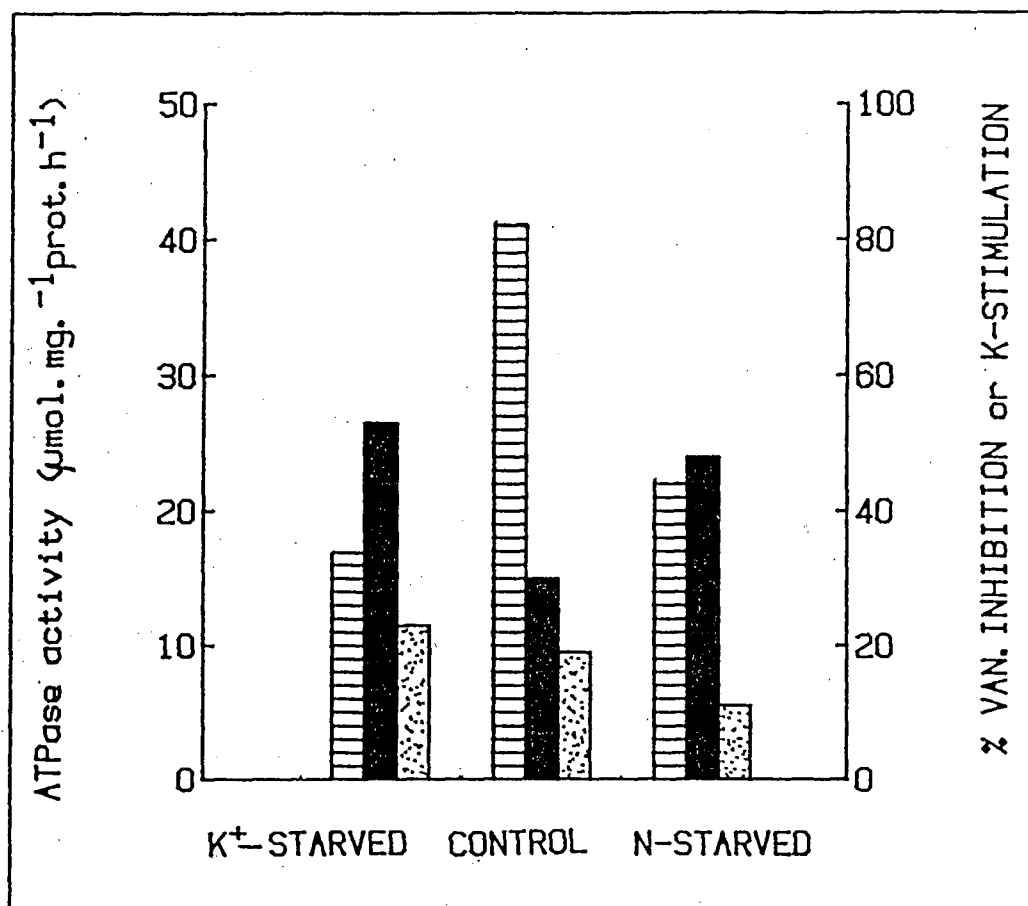
Fig 7 H^+-ATPase activities in microsomal membranes obtained from control
(high-K^+), low- K^+ and low-NO_3^- grown roots.

Effect of K^+ and NO_3^- deprivation on H^+-ATPase activity (▤), % inhibition of this activity by 500 μM vanadate (■) and % stimulation of this activity by 50 mM KCl (▨).
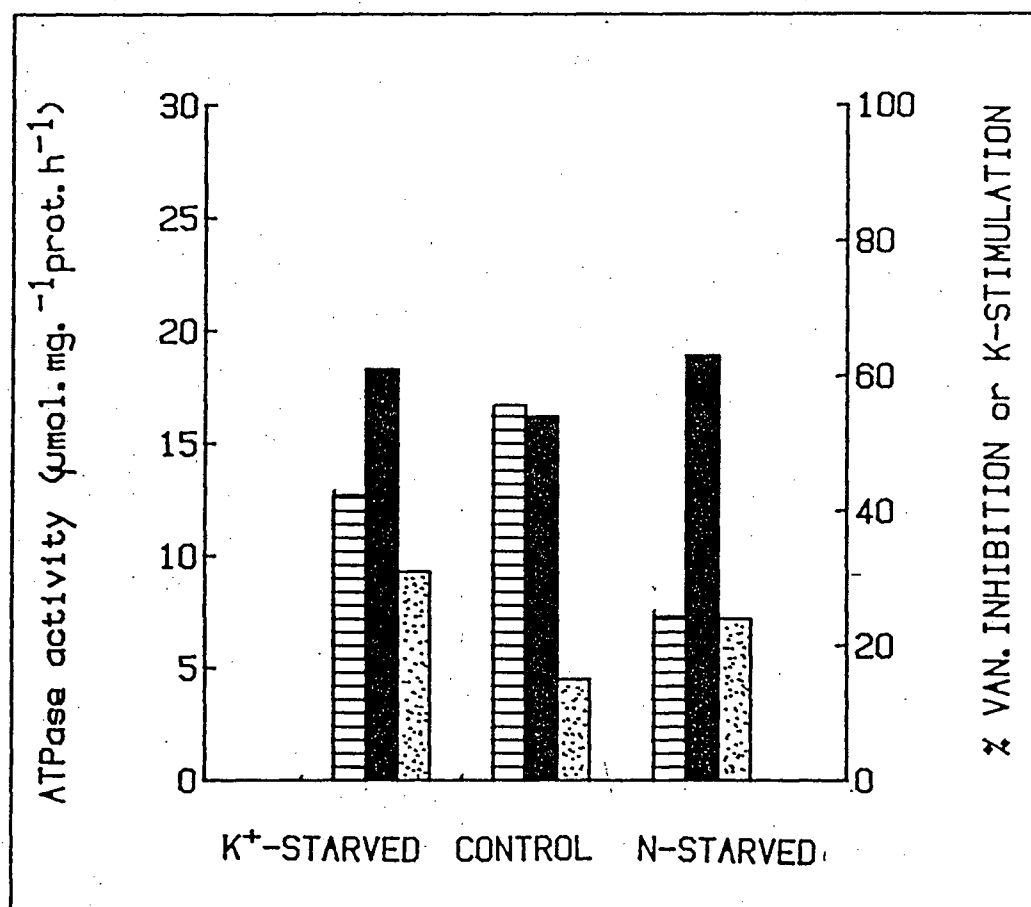
*Fig 8 H⁺-ATPase activities in plasma membranes obtained from control, low-K^+
and low-NO_3^- grown roots.*

Effect of K^+ and NO_3^- deprivation on H^+-ATPase activity (▤), % inhibition of this
activity by 500 μM vanadate (■) and % stimulation of this activity by 50 mM KCl
(▨).

The immunological cross reactivities with respect to H^+-ATPase antibodies failed to reveal significant increases in the activities of H^+-ATPase in plasma membranes (Fig. 9) or H^+ ATPase of the tonoplast (Fig. 10) of roots grown in low-K^+ solutions. This observation, if it can be accepted at face value, is consistent with the reported tight control of the amount of plasma membrane H^+-ATPase in yeast during changes of growth conditions and gene dosage (Eraso et al., 1987). According to Serrano (1989) regulation of the activity of the proton pump is most likely achieved through phosphorylation by protein kinases. Therefore, the apparent lack of increased activity observed in the present study does not exclude the possibility that *in vivo* activity has been increased. The conclusion that increased K^+ influx is not associated with increased activity of the plasma membrane H^+-ATPase is difficult to test unequivocally.

Villalobo (1982), suggested that K^+ transport into proteoliposomes derived from yeast cells is mediated by a plasma membrane Mg^{2+}-dependent H^+-ATPase which contains a voltage sensitive gate for K^+. This channel is opened in response to membrane potential hyperpolarization. Hedrich and Schroeder (1989) have suggested that in *Neurospora*, K^+ influx, even from low K^+ concentrations may be an energy-dependent (but passive) entry through a K^+-specific channel, if the electrical potential difference is sufficiently negative. Also, patch-clamp methodologies have demonstrated the presence of K^+ selective channels in yeast and in higher plant plasma membranes (*Vicia faba*) which are activated by hyperpolarization (see Serrano, 1989). The latter author reported that ATPases from *Saccharomyces pombe*, when purified to homogeneity and reconstituted into proteoliposomes, failed to pump K^+ in the course of ATP hydrolysis, contained a voltage sensitive K^+ channel. These studies suggest that the ATPase molecule is tightly associated with the K^+ channel and may even contain it
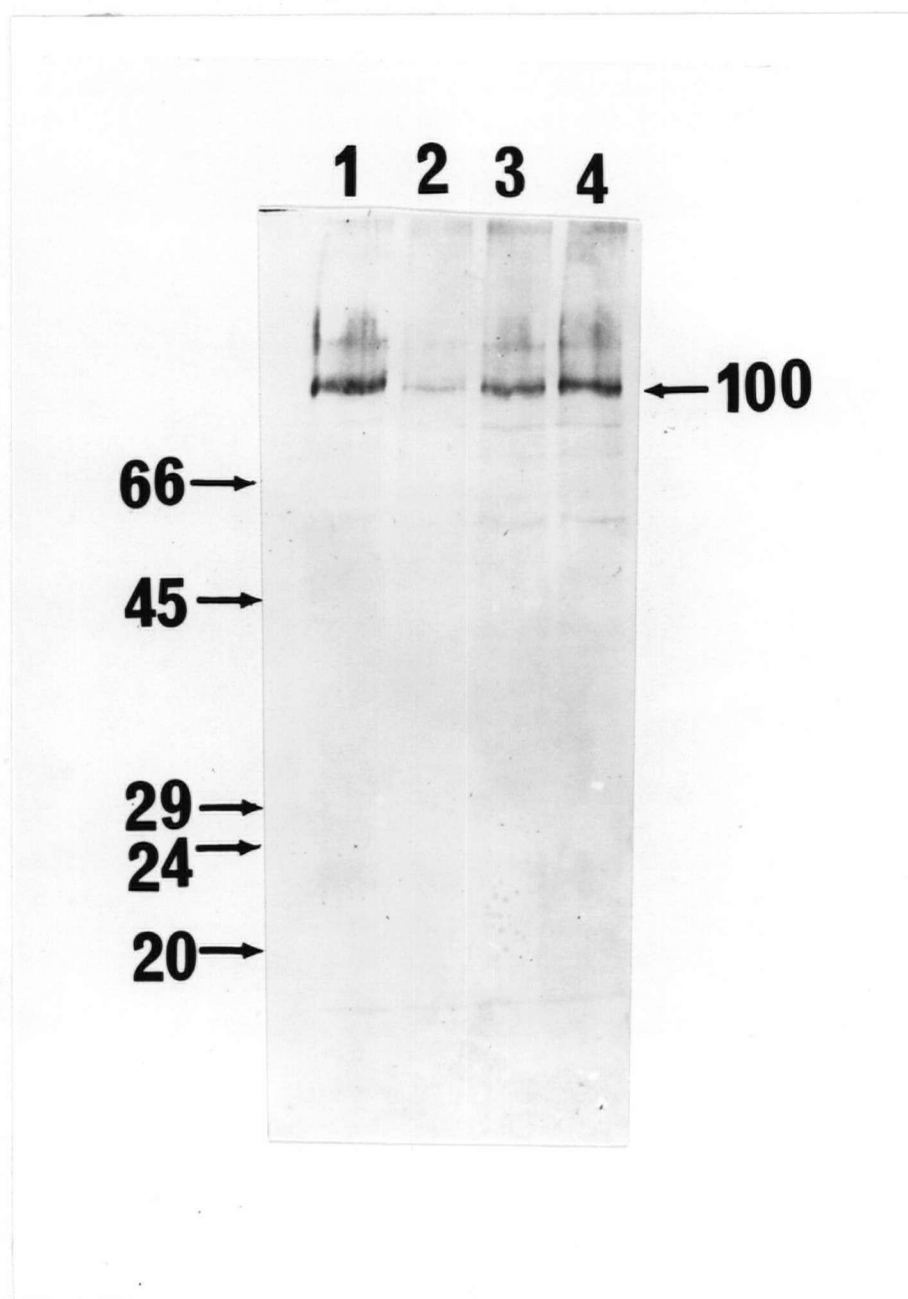
Fig 9 Western blot of plasma membrane proteins of barley

Plasma membrane proteins (25 μg/lane) cross-reacted with antibodies raised against the 100 kDa plasma membrane H^+-ATPase of corn root plasma membrane. Lane 1 (HK), lane 2 (1 day LK), lane 3 (2 days LK) and lane 4 (3 days LK) treatments.
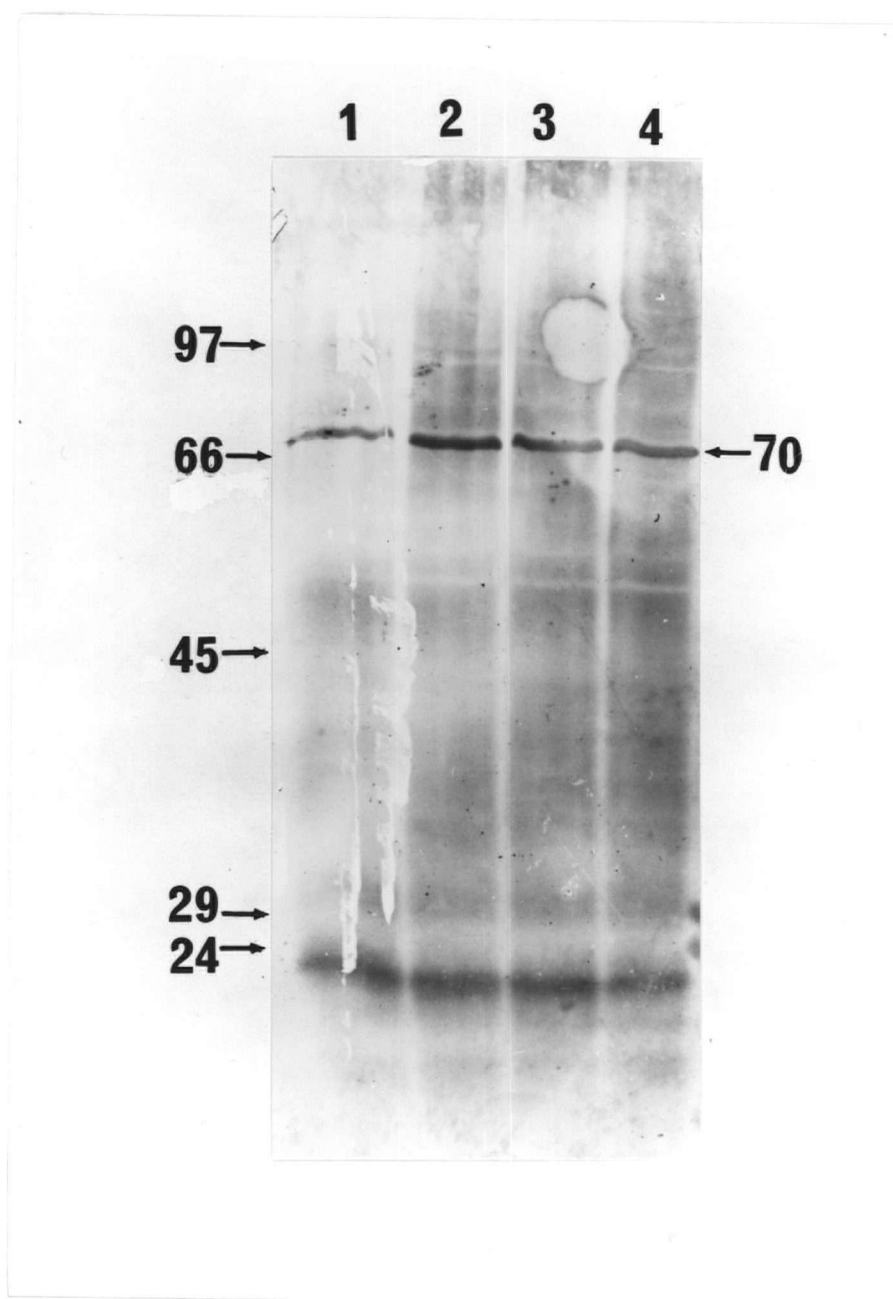
Fig 10 Western blots of tonoplast proteins of barley

Tonoplast proteins (25 μg/lane) were electrophoresed and transferred onto nitrocellulose paper. This was cross-reacted with antibodies raised against the 70 kDa polypeptide of corn root tonoplast H^+-ATPase. Lane 1 (HK), lane 2 (1 day LK), lane 3 (2 days LK) and lane 4 (3 days LK) treatments.
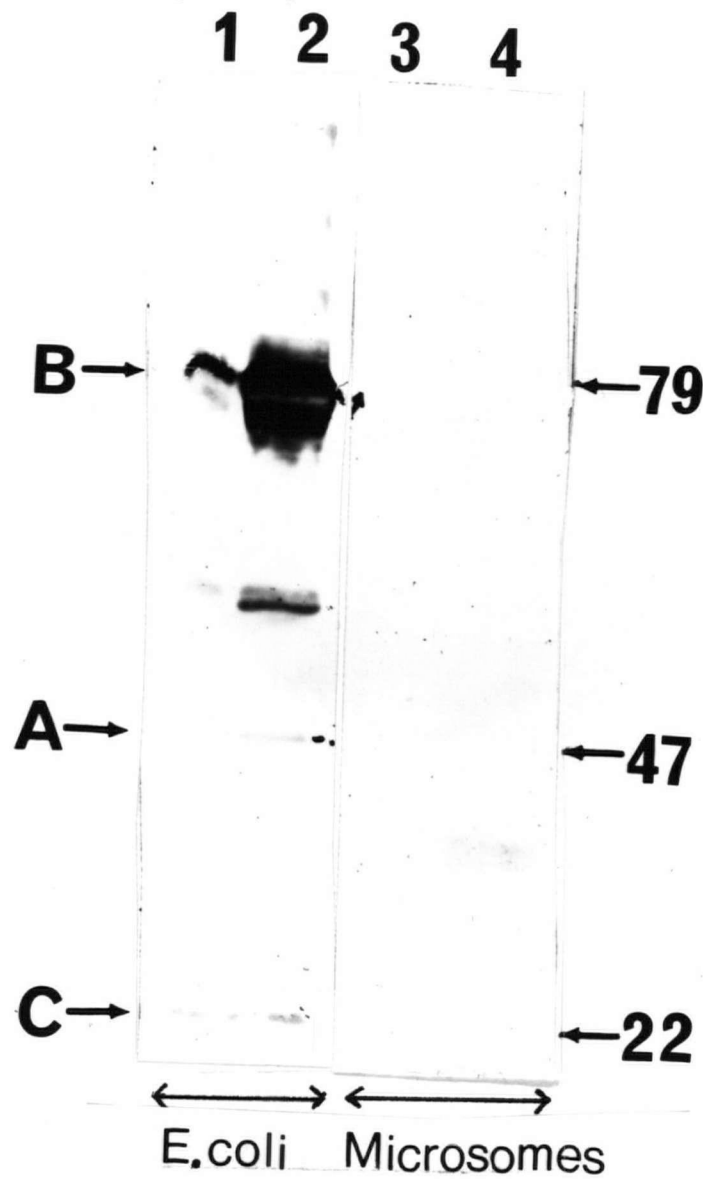
Fig 11 Western blots of barley microsomal and E. coli proteins

Barley root microsomal (~ 250 μg/lane) and E. coli membrane proteins (25 μg/lane) cross-reacted with a mixture of antibodies raised against the Kdp A, the Kdp B and the Kdp C polypeptides of E. coli K$^+$-ATPase. Molecular mass of these polypeptides are, 79, 47 and 22 kDa for B, A & C, respectively.

(Serrano, 1989). According to the latter author, an evolutionary linkage between an ancestral H^+, K^+-exchanging ATPase and H^+-ATPase may have persisted, and therefore some relationship between H^+-ATPase and K^+-transport can be expected.

In higher plant systems, Glass and Siddiqi (1982) and Kochian et al (1989) demonstrated a lack of sensitivity of the high affinity K^+ uptake system in barley and corn roots, respectively, to changes in pH (between 5 - 8) of the external solution. They argued that the high affinity K^+ -transport system in barley and corn roots is not mediated by a K^+/H^+ exchange mechanism. Rather, they suggest that influx of K^+ occurs either by means of a K^+-H^+ symport system (which may have a very high affinity to H^+), or a Kdp-type K^+-ATPase. Giannini et al.(1987b) found that in vesicles isolated from red beet, there is ATP dependent K^+ transport even when the H^+-free energy gradient has been eliminated by use of proton conductors (CCCP), suggesting that K^+ transport in these vesicles was directly driven by an ATPase. This may agree with the suggestion made by Villalobo (1982) as mentioned earlier. However, to resolve this question it would be necessary to demonstrate that, the ATP-driven K^+ transport occurs in the absence of a $\Delta\mu H+$ by using reconstituted polypeptides consisting only of the plasma membrane H^+-ATPase.

On the other hand it is difficult to rule out the possibility of the presence of an additional ATPase in the plasma membrane fraction which is directly involved in K^+ transport, analogous (or homologous) to the Kdp system in *E. coli*. The immunological cross-reactivity studies performed using antibodies raised against the high affinity K^+-ATPase system (Kdp system) from *E. coli* failed to indicate any cross reactivity with the membranes of low-K^+ grown barley (Fig.11). This may reflect only the substantial differences in function of these transport systems and the evolutionary distance between barley and the prokaryotes.

Although the evidence from the present studies is not unequivocal regarding the role of the plasma membrane H^+-ATPase in K^+ influx, there are several resons why a strict coupling would appear to be unlikely.

1. Lack of specificity: since the H^+ pump fuels the transport of several inorganic nutrients, an increase of its activity would be expected to increase fluxes of several ions. There is no indication that K^+ deprivation increases the influx of other ions. For an example, Smith (1973), reported that the influx of $H_2PO_4^-$, into barley, was unaffected by pretreatments with NO_3^-, SO_4^{2-}, $H_2PO_4^-$, HCO_3^-.

2. Thermodynamic control as a mean of regulating ion fluxes has been evaluated by Cram (1976), Glass (1977) and Raven (1977). These authors rejected thermodynamic control of the influx as a means for regulating the fluxes of Na^+, K^+ and Cl^-.

Despite uncertainty regarding the nature of the K^+ transport system as deduced from kinetic studies and putative coupling mechanisms with H^+ fluxes, an independent system for K^+ influx has always appeared more likely. This might be one or more of the following;

1. A uniport or channel (Fig. 1f),

2. A K^+ -ATPase of the Kdp type (Fig. 1e),

3. A K^+,H^+ symport (Fig. 1b). These provide for selective control of K^+ uptake; only cases 1 and 3 involve an indirect link to H^+-ATPase.

The next goal of the present work was to compare the polypeptide composition of the high-K^+ and low-K^+ barley roots in order to search for differences associated with the high K^+ influx, low-K^+ status of low-K^+ roots. First, the changes revealed by Coomassie blue-staining of membrane polypeptides will be discussed. Subsequently, changes resulting from very short-term K^+- deprivation (6- 12 h) by means of, ^{35}S-methionine labeling are described (Chapter 5).

V. CHANGES IN THE EXPRESSION OF MEMBRANE PROTEINS IN RELATION TO K^+ SUPPLY

INTRODUCTION

The membrane proteins responsible for the fluxes of important nutrient ions (e.g. K^+, NO_3^- and other macronutrients) have been inferred only on the basis of their physiological characteristics in higher plants. Preliminary observations have been reported for the inducible NO_3^- transporter of corn roots (McClure et al., 1987; Dhugga et al., 1988) and tobacco tissue culture (Zhang and McKeown, 1990). These workers all made use of the well documented "induction" of NO_3^- absorption which follows provision of NO_3^- to NO_3^--deprived cells, to search for parallel changes in the expression of specific polypeptides in various membrane fractions. McClure et al., (1987) reported increased ^{35}S-methionine incorporation into a 31 kDa polypeptide localized in tonoplast-enriched fractions. Dhugga et al. (1988), demonstrated that there were no significant differences in the Coomassie blue-staining pattern of plasma membrane polypeptides from uninduced and NO_3^-pretreated corn roots (induced for 2.5 and 5 h in 5 mM NO_3^-). Autoradiography of plasma membrane polypeptides labeled with ^{35}S-methionine in 1.25 mM $CaCl_2$ and 2.5 mM KCl solutions supplemented with 5 mM NO_3^- however, showed the synthesis of 4 polypeptides with M_r 165, 95, 70 and 40 kDa after 2.5 and 5 h pretreatment. Triton-X-114 fractionation of the plasma membrane polypeptides revealed that all four polypeptides were integral. Zhang and MacKown (1990) showed Coomassie stained polypeptides in nitrate-induced tobacco tissue culture. Three membrane polypeptides of M_r 60 kDa (tonoplast), 75 kDa (tonoplast) and 105 kDa (plasma membrane) were present only in nitrate-induced cells (5 h after addition of NO_3^-). A correlation was implied between the increased nitrate uptake

after transfer from ammonium to nitrate media and the expression of these polypeptides.

Work on the identity of the NO_3^- transporter in lower organisms (prokaryotes and lower eukaryotes) has been more fruitful. Recently, Sivak et al. (1989) reported enhanced expression of a 47 kDa polypeptide in the cell membrane of the cyanobacterium *Anacystis nidulans* following provision of nitrate. Synthesis of this polypeptide and nitrate transport were repressed by supplying NH_4^+ to this organism. Transfer of ammonium-grown cells to media containing nitrate as the sole source of nitrogen resulted in a parallel increase in the expression of the 47 kDa polypeptide and nitrate transport activity. It was suggested that this 47 kDa polypeptide might play a role in NO_3^- transport. In a series of very elegant experiments, Omata et al.(1989) have established strong evidence that in *Synechococcus* PCC 7942 (*Anacystis nidulans*) the NO_3^- transporter is a 45 kDa polypeptide. A mutant (M45), constructed by inactivating the gene encoding the 45 kDa protein, was unable to grow under conditions of low concentrations of nitrate; high NO_3^- concentrations supported growth of the mutant. When ammonium was provided as the nitrogen source, growth rate of M45 was as high as that of wild-type cells. It seems likely that the NO_3^- induced 47 kDa polypeptide reported by Sivak et al. (1989) and the 45 kDa polypeptide reported in the same species by Omata et al. (1989) are the same.

The transport of K^+ has been the subject of extensive studies. As a consequence, K^+ transport has been well characterized in animal cells (Matsui, 1982; Glynn, 1984), in bacteria (Epstein, 1985; Siebers and Altendorf, 1988), and in fungi such as *Saccharomyces cerevisiae* (Ramos and Rodriguez-Navarro, 1985). There is extensive physiological information on the kinetics of K^+ transport and the regulation of influx in higher plants, but, many details of the mechanism(s) of K^+ transport still remain unresolved and there is controversy concerning proposed

mechanisms. For example, in a recent review of K^+ nutrition, Lüttge and Clarkson (1989) listed 5 different mechanisms for K^+ uptake by plant roots (see Chapter 1)

To date, except for a single report in sunflower (Heimer et al., 1988), no information is available on the effect of K^+ stress on the synthesis of plant membrane proteins. The Heimer study failed to differentiate between changes in soluble and membrane proteins but described changes in total proteins.

This chapter describes the profiles of membrane polypeptides from K^+-replete and K^+-deprived roots, visualized by conventional staining techniques. This strategy was employed to explore the possibility that specific polypeptides would be expressed under conditions of K^+-deprivation. Further to these experiments, studies on ^{35}S-labeling of membrane polypeptides during the period of most rapid increase of K^+ influx, following removal of exogenous K^+, were undertaken. Also, in some experiments comparisons of polypeptide composition, especially those of plasma membrane and tonoplast, were made between two barley cultivars, Halcyon and Klondike. These experiments were performed in order to select a variety which could be used to replace cv. Halcyon, since the latter cultivar started showing growth anomalies possibly due to changes in the seed stock caused by prolonged storage (in April 1989).

The similarities between patterns of K^+ uptake (Chapter 2) and the time course of synthesis of membrane polypeptides in root tissue following K^+-deprivation indicate a possible involvement of these polypeptides as a part of the transport system. The results indicate changes in polypeptide composition both in plasma membrane and tonoplast at a very early stage of K^+-deprivation.

2 MATERIALS AND METHODS

2.1 Growth conditions and methods for in vivo ^{35}S-methionine labeling

Seedlings were grown under $+K^+$ or $-K^+$ conditions, as described in Chapter 2. The duration of K^+ deprivation and the periods of ^{35}S-methionine labeling varied according to the goals of the experiments and are given for individual experiments with the Figure and Table legends.

For labeling with ^{35}S-methionine, controls and K^+-deprived seedlings were transfered to 400 ml of well-aerated solutions containing ^{35}S-methionine (1 - 2 μCi ml^{-1}) in either 800 ml or 1 l plastic beakers, 6 - 12 h before harvesting. The temperature of these solutions and the inorganic composition were identical to that of the growth medium in which plants had been grown or pretreated. Solutions were aerated through needles inserted through the walls of the beakers. In labeling experiments lasting longer than 6 h, solutions were replaced after the first 6 h to prevent significant depletion of inorganic nutrients. At the end of labeling periods, roots were washed in cold distilled water to remove isotope from the Water Free Space. Roots were then blotted dry with paper towels, weighed and used for isolation of membranes immediately or frozen in liquid nitrogen and stored in a -80°C freezer.

2.2 Methods used for concentrating soluble proteins

Soluble proteins (designated hereafter as SN1), collected as the supernatant from the first 80,000 x g$_{avg}$ centrifugation and the supernatant of microsomal washes (hereafter called SN2) collected from the centrifugation of resuspended microsomes in buffer, were usually very dilute. In order to obtain sufficient protein to be visualized by Coomasie blue-staining it was necessary to concentrate these solutions. Several protein concentrating techniques, including TCA precipitation, freeze-drying, filtration through Amicon "Centricon" filter tubes,

acetone precipitation and ammonium sulphate precipitation were tried. Of these, TCA precipitation was the most successful. The method used was that described by Hurkman and Tanaka (1986) with slight modifications. To samples of the soluble fraction (usually 200 -250 μul), having equal amounts of proteins, 0.8- 1.0 ml of 12.5% ice-cold TCA was added. The mixture was shaken well and incubated on ice for 30 min, after which it was centrifuged (Eppendorf Microfuge model 5415) at 11,000 rpm for 10 min. After decanting the supernatant carefully, the pellet was washed with ethanol:ether (1:1) several times. The pellet was then carefully dissolved in the sample buffer. If the solution became yellow due to the presence of TCA, 1-2 μl of 1 M Tris was added. This solubilized protein was analysed by SDS-PAGE.

2.3 Electrophoresis and autoradiography

To fractionate soluble proteins and those of membrane origin, SDS-PAGE was performed in 10% or 12 - 17% gels, according to the method of Laemmli (1970) with slight modifications. All stock solutions including distilled water were millipore filtered and held at 4°C. For three 10% homogeneous gels, having 15 cm x 13 cm x 1.5 mm dimensions, 80 ml of resolving gel media were made. This gel consisted of 20 ml of 1.5 M Tris-HCl (pH 8.8) , 26.8 ml of acrylamide : bis acrylamide (30 : 0.8 % w/v, BDH electrophoresis grade), 0.8 ml of 10 % SDS (w/v) and 0.4 ml of freshly prepared 10 % (w/v) ammonium persulphate ($(NH_4)_2S_2O_8$) and 32 ml of distilled water. After degassing this mixture for 10 min, 28 μl of N,N,N'N'-tetramethyl ethylenediamine (TEMED) was added to initialize polymerization, with gentle swirling and the gel was poured between glass plates. A thin layer of iso-butanol was carefully layered over the gel to avoid exposure to air. When polymerization was completed, iso-butanol was carefully removed and 5 ml of stacking gel solution was poured. The composition of the stacking gel was, 3.75 ml of

0.5 M Tris-HCl (pH 6.8) 1.5 ml of acrylamide : bisacrylamide, 0.15 ml of 10 %

SDS, 0.2 ml of 10 % $(NH_4)_2S_2O_8$ and 15 μl of TEMED in 9.38 ml of distilled water.

Equal amounts of proteins, estimated according to Markwell (1978) or equal [35]S

counts (estimated by scintillation counting), were solubilized at 70°C for 20 min in

50 μl of sample buffer, which consisted of 0.125 M Tris-HCl (pH 6.8), 25 %

glycerol, 2.5 % SDS, either 2.5 % 2-mercaptoethanol or 50 mM dithiothreitol

(DTT) (reducing agents were added fresh) and 0.05 % bromophenol blue. After

solubilization, the solutions were centrifuged at 11,000 rpm for 3 min in an

Eppendorf microfuge and the supernatants were loaded onto the gels. Routinely,

stacking and separation of protein were carried out at 12 mA and at 25 mA

respectively, in running buffer consisting of 0.025 M Tris, 0.192 M glycine, 0.1 %

SDS at pH 8.3 for 6-10 h. Sigma standard molecular weight markers (cat # SDS-

6H) were used to determine the apparent molecular weights. Gels were either

stained and destained using conventional techniques (Coomassie blue or Silver

staining), or for radiolabeled proteins, soaked in " AMPLIFY" (Amersham) for 15

min, dried on Whatman # 3 filter paper in a Biorad gel drier and exposed to Kodak

X-OMAT 2R film. X-ray films were developed and fixed in KODAK developer and

rapid fixer, prepared according to the manufacturers guidelines. Quantification of

gels and fluorographs was performed by densitometric scanning using a Beckman

spectrophotometer (Model DU 64).

2.4 Triton-X-114 fractionation of membrane proteins to distinguish intrinsic from extrinsic proteins

The method used was similar to that described by Bordier (1981)

and Kjelbom et al.(1989). A solution of the nonionic detergent Triton-X-114 is

homogeneous at 0°C but separates into an aqueous phase and a detergent phase

above 20°C. The extent of this detergent phase separation increases with the

temperature and is sensitive to the presence of other surfactants. The

hydrophobicities or hydrophilicities of the membrane proteins determine their partitioning in a two phase system. Hydrophilic proteins partition exclusively in the aqueous phase, while integral proteins (being amphiphilic) are recovered in the detergent phase. This mixture (buffer A), containing 1% purified Triton-X-114, 150 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.6), separates on standing into 2 distinct phases. Fractionation of proteins into these two phases was achieved as follows:

1. Membrane vesicles were resuspended in distilled water to obtain a protein concentration of 4 mg/ml.

2. This suspension was mixed with an equal volume of buffer A and incubated at $0^{\circ}C$ for 10 min. The suspension was next mixed thoroughly by vortexing and allowed to stand on ice for a further 5 min.

3. The mixture was again vortexed and incubated at $37^{\circ}C$ for 15 min for phase separation .

4. The preparation was then centrifuged at 10,000 x g in a microfuge for 1 min.

5. To the resulting upper aqueous phase, Triton-X-114 was added to give a final concentration of 1%. Steps 2 to 5 were repeated, so as to remove any remaining intrinsic proteins not partitioned into the first Triton-X-114 phase.

6. The lower phase of the first separation was mixed with an equal volume of buffer A (containing no Triton-X-114) and steps 2-5 were repeated to remove any extrinsic proteins. The upper aqueous phases contain the peripheral or extrinsic membrane proteins while the lower detergent phases contain the integral or membrane spanning proteins.

3 RESULTS AND DISCUSSION

3.1 Changes in Coomassie blue-stained polypeptides of crude microsomal membrane

For most of the early work the barley cultivar Halcyon was employed. However, after April 1989, when this cultivar began to show growth anomalies, the cv. Klondike was used.

Lowering the $[K^+]$ from 6 mM to ~ 5 μM, caused several changes in the expression of polypeptides of crude microsomal (80,000 x g_{avg} pellet) fractions, separated by SDS-PAGE and detected by Coomassie blue-staining. The most obvious change observed was the increased expression of a polypeptide having a M_r of 45 kDa (lane 1, Fig. 12). This was first discernible at 1 day after withdrawal of K^+ and continued to increase up to 6 days (Fig. 13 and 14). This response is specific for K^+ deprivation and not a general response to nutrient stress because withholding NO_3^- or PO_4^{3-} for an equivalent duration caused no change in expression of this polypeptide (Fig. 12). Other polypeptides of M_r 34, and 30 kDa (lane 1, Fig. 12) and 37 kDa (lane 3, Fig. 13) also showed increased expression, although, to a lesser extent. Since in some instances there were two separate bands at the 45 kDa marker (possibly due to partial breakdown of the markers), the estimated molecular weights of 34 and 37 kDa polypeptides might have been under or overestimated. Therefore, there are possibilities that 34 and 37 kDa bands represent the same polypeptides. A 16 kDa polypeptide was also evident in freshly stained gels but was not well recorded by photographs. At least 3 days of K^+-deprivation was required to discern the latter polypeptides. These experiments, designed to evaluate the specificity of the 45 kDa response, also revealed a unique response to N deprivation. Lane 2 (Fig. 12) shows the pattern of microsomal polypeptides in roots of nitrate-starved plants. Enhanced expression of a polypeptide with(lane 1, Fig. 12) molecular mass 31 kDa is apparent. McClure et

al. (1987) reported increased incorporation of ^{35}S-methionine into a 31 kDa polypeptide in membrane fractions from corn roots during "induction" of NO_3^- transport following provision of NO_3^- to nitrate-starved roots. It is intriguing that NO_3^- deprivation in this study caused increased expression of a polypeptide with the same molecular weight. The effect of removing NO_3^- on subsequent NO_3^- influx is a matter of some controversy. Lee and Drew (1986) reported stimulated $^{13}NO_3^-$ influx when barley plants were transferred to N free solution for 3 d. More recent experiments (Siddiqi et al., 1989) have shown that removal of NO_3^- for 1 to 3 d caused $^{13}NO_3^-$ influx to decline rather than increase. However, if plants are briefly re-exposed to NO_3^- after periods of nitrate-deprivation prior to measuring $^{13}NO_3^-$ influx, enhanced influx was evident. However, this is only evident following the "re-induction" by NO_3^- treatment. The basis of these differences in results among research groups is not clear. It is possible that there are genotypic differences even within a single genus such as *Hordeum*.

There is also evidence for enhancement of a polypeptide with molecular mass ~ 50 kDa associated with PO_4^{3-} deprivation (lane 3 Fig. 12). The roles of these polypeptides clearly warrant further study.

Studies of K^+ (^{86}Rb) influx under similar conditions have revealed significant increases of influx within 6 to 12 h after removal of the K^+ supply (Fernando et al., 1987; Glass, 1975; Siddiqi and Glass, 1987; and Chapter 2 of this thesis) and influx typically reaches its peak value by 3 days. Further deprivation caused only a slight increase of K^+ (^{86}Rb) influx and may result in a decline of influx as metabolic effects of K^+ deficiency become apparent (Pettersson, 1975). When K^+ was resupplied after 3-6 days of deprivation, the intensity of the 45 kDa band decreased to less than 50% of control levels within 24 h (lane 1, Fig.14). By three days, intensity of this band was further reduced (lane 3, Fig.14) but its level of expression was still higher than in plants which had received K^+ continuously.
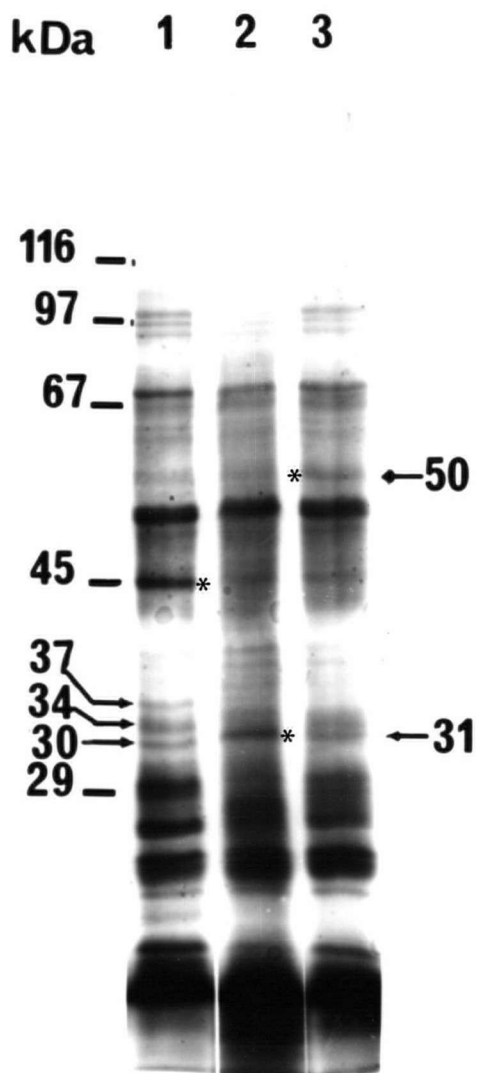
Fig.12 Effect of nutrient deprivation on SDS-PAGE patterns of microsomal proteins from barley roots

Coomassie blue-stained polypeptides of microsomes (70 μg/lane) of barley roots obtained from seedlings deprived of K^+ (lane 1); NO_3^- (lane 2) and P_i (lane 3) for 6 d. Masses of molecular weight markers (in kDa) are given at left.
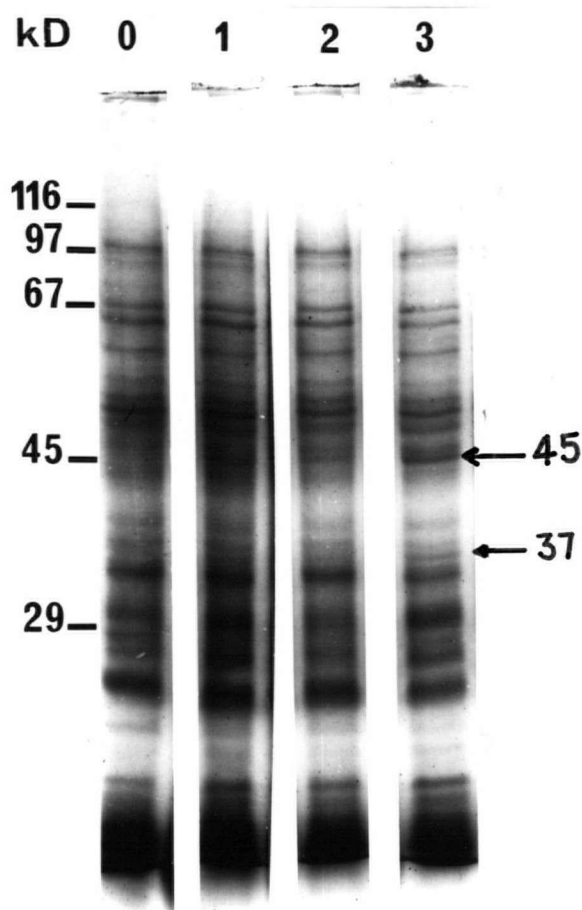
Fig.13 Duration of K^+ *deprivation on polypeptide profiles of microsomes from barley roots.*
Coomassie blue-stained microsomal proteins (50 μg/lane) obtained from roots of barley seedlings grown under control conditions (continuous K^+ supply) (lane 0), 1 day low-K^+ (lane 1), 2-days low-K^+ (lane 2) and 3-days low-K^+ (lane 3). At harvest seedlings were 11 days old. Masses of molecular weight markers (in kDa) are given at left.
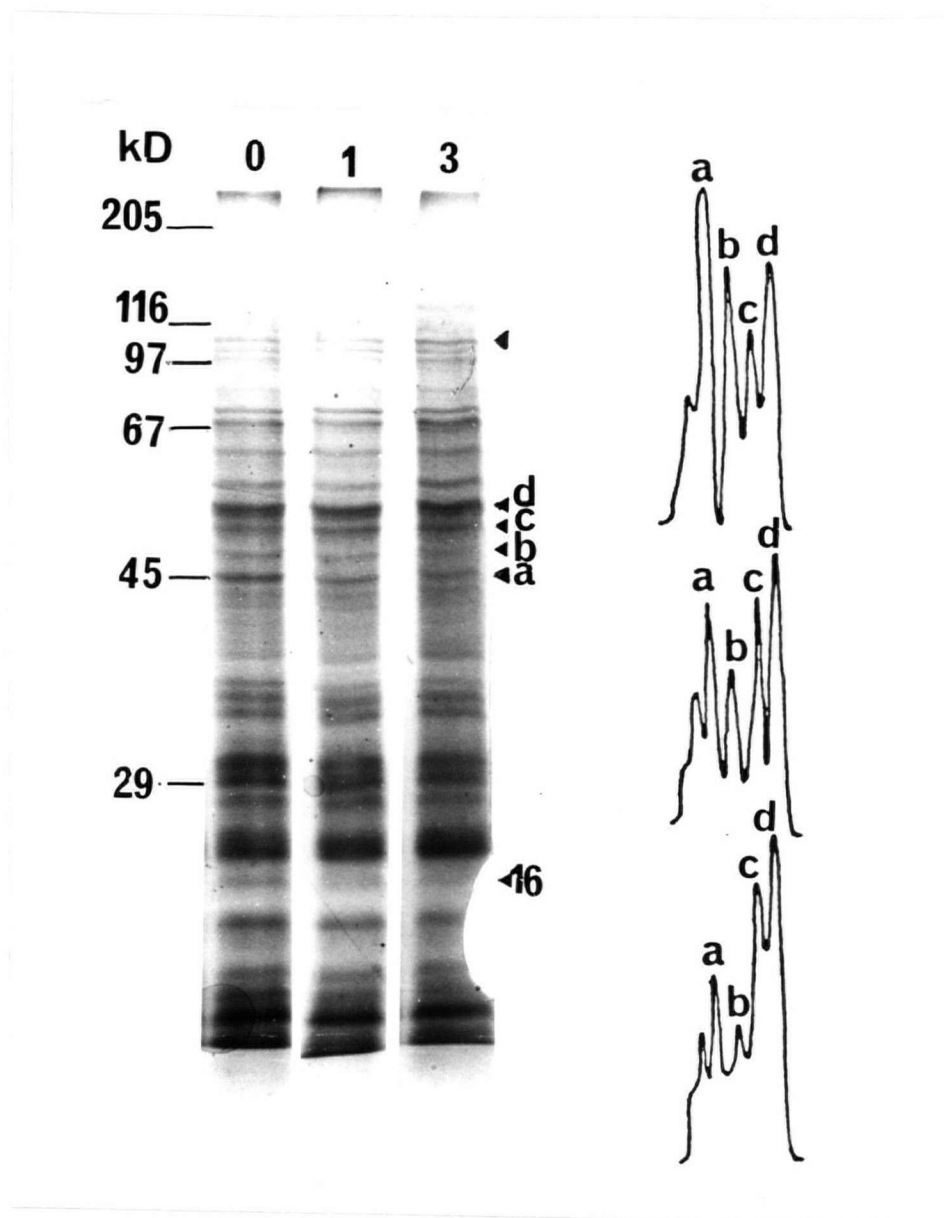
Fig.14. Effects of resupply of K^+ *to* K^+ *-deprived roots on polypeptide profiles of microsomes from barley roots.*

Coomassie blue staining pattern of microsomal proteins (50 μg each lane) from barley roots grown under low-K^+ conditions after resupply of K^+. Lane 0, plants deprived of K^+ for 6 d; lane 1, plants resupplied with K^+ for 1 d; lane 3, plants resupplied with K^+ for 3 days. Densitometer scans corresponding to lanes 0, 1 and 3 respectively (from top to bottom), of the 45 to 55 kDa region of the gel are shown at right. Masses of molecular weight markers (in kDa) are given at left.

This was an unexpected result because in previous studies (Glass , 1977; Siddiqi and Glass, 1983) it was reported that when K^+ is resupplied to K^+-deprived plants, K^+ (^{86}Rb) influx declines, within 1-2 d, to values equivalent to those observed in plants provided with a continuous supply of K^+. Nevertheless there is clearly a semiquantitative correlation between influx (and K^+ status of the root) and the level of expression of these 45 kDa and 34 kDa polypeptides. In most preparations the 45 kDa polypeptide was absent in microsomes derived from K^+-sufficient roots. However, in experiments where root $[K^+]$ in high K^+ roots was lower (60 -70 μmol g^{-1} fwt) than usual (90 -100 μmol g^{-1} fwt), the 45 kDa was expressed at low levels in unwashed microsomes. In experiments carried out using antibodies to the Kdp carrier (a K^+-ATPase) from *E.coli* (Chapter 4), it became evident that in bacteria grown at $[K^+]$ as high as 600 μM, the Kdp system was still expressed, albeit at a much lower level than in K^+-starved bacteria (see section 4.2.4). The occasional expression of the 45 kDa polypeptide in K^+-sufficient grown roots may therefore be compared to the situation observed for the Kdp system. It is apparent that there is a threshhold value of internal $[K^+]$, below which the 45 kDa polypeptide is expressed. So long as the internal $[K^+]$ was high (90 - 100 μmol. g^{-1}fwt.) this polypeptide was absent from unwashed microsomes of roots grown with K^+ even in the presence of 2 mM PMSF (Fig.16, lane 3).

3.2 Effects of changes in composition of the resuspension buffer on the stability of the 45 kDa polypeptide

A notable feature, and source of considerable initial difficulty in this study, was the apparent loss of the 45 kDa polypeptide on washing microsomes (see below). To determine what factors were responsible for the loss of this polypeptide, several changes were made in the composition of the resuspension buffer (listed below) in subsequent experiments:

1. nature and concentration of protease inhibitors,

2. presence and absence of chelating reagents and

3. changes in ionic strength.

Figure 15 shows Coomassie blue-stained gels of the washed and unwashed microsomes from roots grown with or without K^+ for 6 days. Buffers used in the preparation of these membrane vesicles had either 0.2 or 4 mM PMSF. Lanes 2 - 5 (unwashed microsomes) reveal that the 45 and 31 kDa polypeptides are present in both high-K^+ and low-K^+ treatments although these were more strongly expressed in the low-K^+ treatments (lanes 4 and 5). The highest intensity staining of the 45 kDa band was in the -K^+ microsomes in the presence of 4 mM PMSF (lane 5).

Later it became apparent that even 2 mM PMSF was adequate to protect these bands when added freshly prepared to the buffers (lane 4, Fig. 16A; lanes 4 and 5 Fig 17). In the absence of protease inhibitors there was a total loss of these bands even from the low-K^+ microsomes (lane 2, Fig 16A), while 25 ug/ml α-macroglobulin, (universal protease inhibitor) protected the bands of interest to the same extent as 2 mM PMSF (lanes 4 and 6, Fig. 16A). These results demonstrate that, proteolytic degradation of these polypeptides was virtually assured in the absence of appropriate levels of protease inhibitors. Therefore, we considered that the lack of expression of the 45 kDa polypeptide in unwashed microsomes from roots grown in +K^+ solutions might have been due to insufficient protection from proteolytic activity.

Figure 16A also shows that the 45 kDa polypeptide was absent in the washed microsomal preparations from K^+-sufficient roots, even in the presence of freshly added 2 mM PMSF. It was hypothesized that some changes in the composition of the buffers used to wash the microsomes may have been responsible for the loss of the 45 kDa polypeptide.
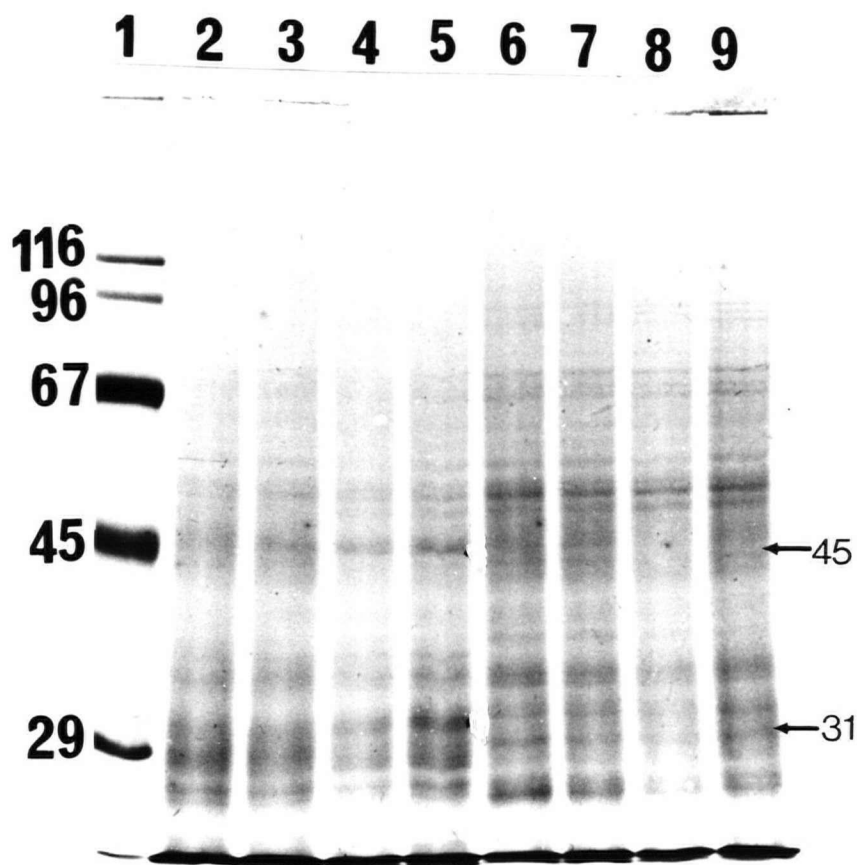
Fig.15 Effect of changes in protease inhibitor concentrations in the buffers on SDS-PAGE patterns of microsomal proteins from barley roots

Coomassie blue-stained polypeptide (30 μg/lane) profiles of unwashed (lanes 2-5) microsomes and those washed in the presence of 1 mM EDTA (lanes 6-9). Lanes 2, 3, 6 and 7 represent those obtained form $+K^+$ grown roots, while lanes 3,4,8 and 9 represent those obtained from $-K^+$ grown roots. Lanes 2, 4, 6 and 8 represent microsomes exposed to 0.2 mM PMSF while those in lanes 3, 5, 7 and 9 received 4.0 mM PMSF in the buffers.

Therefore, the composition of the buffers was scrutinized with a view to identifying the agent responsible for this loss. It is well known that high ionic strength of buffers can cause loosely-associated or peripheral membrane proteins to be displaced. When roots grown under high K^+ conditions are ground in buffer, K^+ released from the vacuoles increases the $[K^+]$ of the homogenization buffer from 10 to 60 mM. Therefore, $+K^+$ and $-K^+$ roots had 60 and 10 mM K^+, respectively in their homogenizing buffers. The possibility was considered, therefore, that the low level of expression of the 45 kDa polypeptide in $+K^+$ roots might have arisen as an artifact associated with the high level of $[K^+]$ in the buffer after homogenizing. Loss of the 45 kDa polypeptide may have resulted from an ionic effect or even from a K^+ activated membrane-bound protease. To test this hypothesis, the $[K^+]$ of the homogenizing buffer was increased to 60 mM for roots grown under $-K^+$ conditions. This removed any difference in the $[K^+]$ of the homogenates of $+K^+$ and $-K^+$ roots. However, SDS-PAGE revealed no differences in the level of expression of the 45 kDa polypeptide in microsomes from $-K^+$ roots amended or not amended with additional K^+ during homogenization (Fig 17, lanes 4 and 5). Furthermore, washing the microsomes in 0, 10 or 150 mM KCl failed to influence the level of the 45 kDa polypeptide (Fig. 18) detected by Coomassie blue-staining of gels.

Major differences in the composition of homogenizing and resuspension buffers which might have caused displacement of the 45 kDa polypeptide, were the presence of salicylhydroxamic acid (SHAM), polyvinylpyrrolidine (PVP), which were absent from the resuspension buffer and the presence of EDTA in resuspension buffer (the compositions of these buffers are given in detail in Chapter 3). To check whether these differences might have caused the loss of the 45 kDa polypeptide, membrane fractions were prepared in the presence or absence of each of these substances in the resuspension buffer. Neither

SHAM nor PVP exerted any significant influence on the loss of the 45 kDa polypeptide.

Among other components of the resuspension buffer which might have been responsible for the loss of the 45 kDa polypeptide, EDTA was the next focus of attention. Resuspension buffer used in the present work routinely contained 1 mM EDTA in order to avoid membrane aggregation. Mg^{2+} is known to be critical for the binding of ribosomes to the endoplasmic reticulum (ER) (Jones, 1985); the binding of ribosomes to ER (generating RER) causes RER vesicles to become more dense, and they tend to equilibrate isopycnically with plasma membrane vesicles. The chelation of Mg^{2+} by EDTA leads to the so-called " Mg^{2+} shift" of ER vesicles in the sucrose gradient, due to the formation of smooth vesicles (SER) which are lighter than RER. As a result, plasma membrane is obtained free from contamination by RER. Washing of the microsomal pellet with resuspension buffer in the presence or absence of 1 mM EDTA followed by centrifugation at 80,000 x g_{avg} typically caused loss of the 45 kDa polypeptide only in the presence of EDTA (lanes 8 & 9, Fig.15 ; lanes 8 & 10, Fig.16A; lane 2, Fig. 16B). Similar effects of EDTA have been reported for chloroplast thylakoid proteins (Park and Pfeifhofer and references therein, 1969; Lineberger and Steponkus, 1980). They reported that, when washed in 1 mM EDTA, the particles on the A' surface of the thylakoid were largely removed. These particles, initially suggested to be the so-called "quantosomes" were subsequently identified as the Ca^{2+}-dependent ATPase (Park and Pfeifhofer and references therein, 1969).

There are several possible explanations for the observed EDTA effect. It is possible that the 45 kDa protein is a soluble protein (SN1) trapped in the microsomal vesicles during homogenization. Changes in the permeability properties or integrity of microsomal vesicles due to changes in the $[Ca^{2+}]$ or $[Mg^{2+}]$ of the buffers (due to the presence of EDTA) might result in release of

trapped proteins. If this were the case, such proteins should be found in the soluble protein fraction (SN1) and in the second 80,000 x g_{avg} supernatant (SN2) when microsomes are treated with EDTA. If not released during washing, trapped proteins would be released during solubilization of membrane vesicles for SDS-PAGE and will be present in the gels.

However, neither SN1 (Fig.19, lanes 2-5) nor SN2 revealed the presence of a Coomassie blue-stained band at 45 kDa. The concentrated SN2 when stained with silver-stain, indicated the presence of two very lightly stained bands with molecular weights ~ 18 & 20 kDa, respectively (data not shown). These may be degradation products of the 45 kDa polypeptide by a specific protease, released from the vesicles or present as a membrane-bound protease. Increased susceptibility of this polypeptide to the protease, in the absence of bulk soluble protein (following separation of microsomes from the soluble proteins) may have caused the loss of this polypeptide. However, this is unlikely since the 45 kDa was lost even when unwashed microsomes were washed in the presence of various protease inhibitors including a-macro globulin and PMSF.

The ease with which the 45 kDa polypeptide was washed off by 1 mM EDTA indicates the importance of divalent cations for the proper association of this polypeptide with microsomal membranes (Fernando et al., 1990). It can be assumed that the 45 kDa polypeptide is a loosely-associated, peripheral membrane protein.

Further fractionation of microsomes by sucrose gradient fractionation in the presence of 1 mM EDTA, revealed the presence of a 47 kDa polypeptide in the "tonoplast enriched" fraction. The intensity of this band appeared to increase with the duration of K^+-deprivation (lanes 5, 6, 7 and 8, Fig 20) and lane 2 (Fig 21). Usually the molecular weights estimated by regression of R_f vs. log molecular weights were ± 2 kDa.
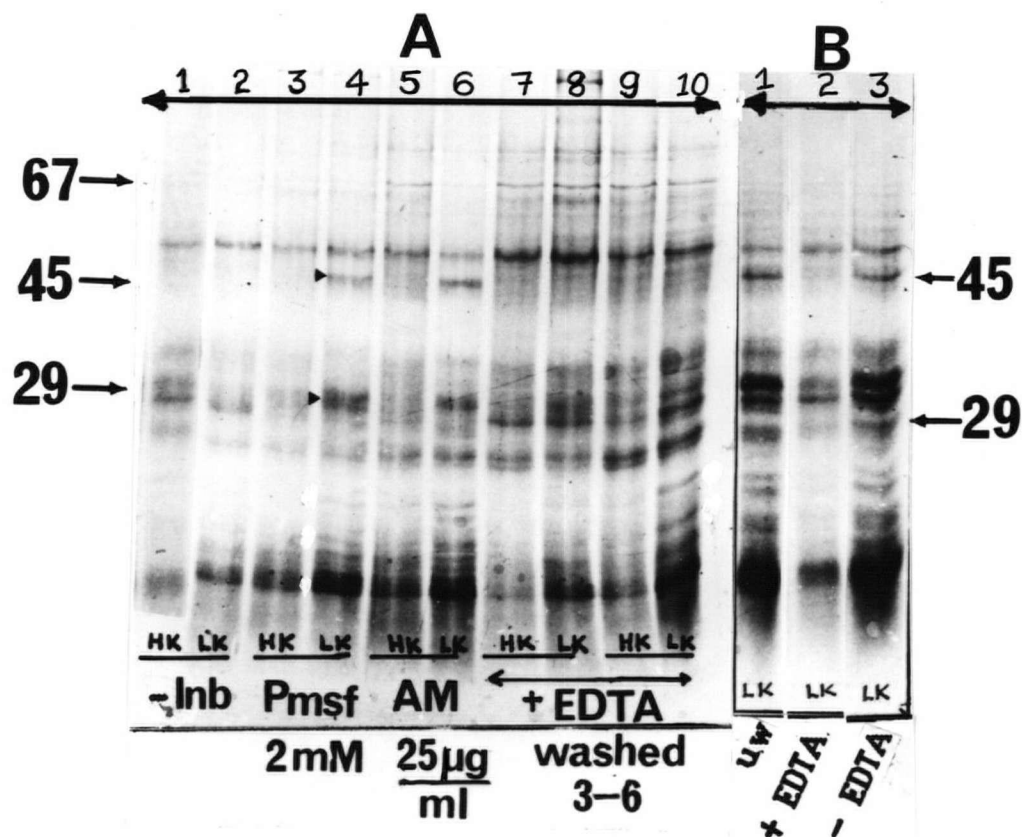
Fig. 16. Comparison of the effects of two protease inhibitors and presence and absence of 1 mM EDTA on the stability of the 45 kDa polypeptide

Coomassie blue-stained polypeptide (50 μg/lane) patterns of microsomes obtained from +K$^+$ and -K$^+$ roots.

16A: Lanes 1, 3, 5, 7 & 9 represent those obtained from +K$^+$ roots while lanes 2, 4, 6, 8 & 10 represent those obtained from -K$^+$ roots. Microsomes in Lanes 1 and 2 had no protease inhibitors in the buffers while those in lanes 3 and 4 received 2 mM PMSF, while those in lanes 5 and 6 were exposed to 25 μg/ml α-macroglobulin. Lanes 7, 8, 9 and 10 represent microsomal polypeptides corresponding to those in lanes 3, 4, 5 and 6, after washing in the presence of 1 mM EDTA.

16B: Lane 1 represents unwashed microsomes obtained from low-K$^+$ grown roots. Polypeptide patterns of these microsomes, when washed in the presence (lane 2) and in the absence (lane 3) of 1 mM EDTA.
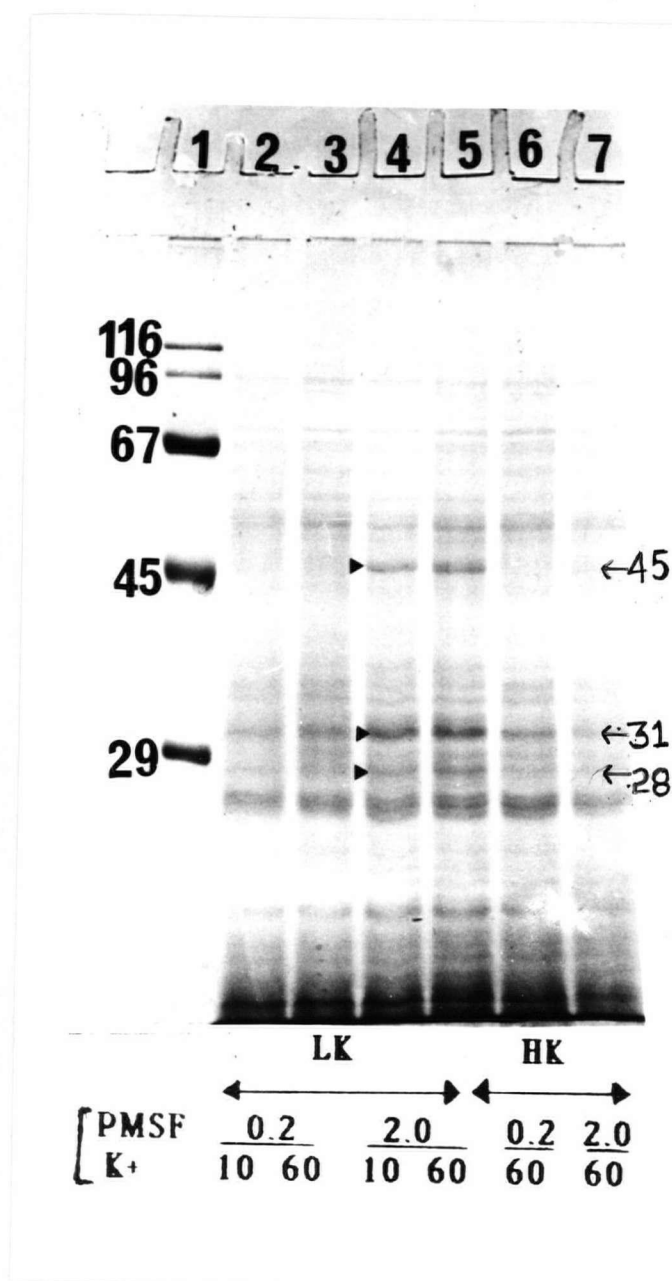
Fig 17. Effect of amending the [K$^+$] in the homogenizing buffer on the expression of 45 kDa polypeptide

Coomassie blue-stained polypeptides of microsomal proteins (50 μg/lane) obtained from low-K$^+$ (lanes 2, 3, 4 and 5) and high-K$^+$ (lanes 6 and 7) grown seedling roots. [PMSF] and [K$^+$] present in the buffers are shown under each lane.
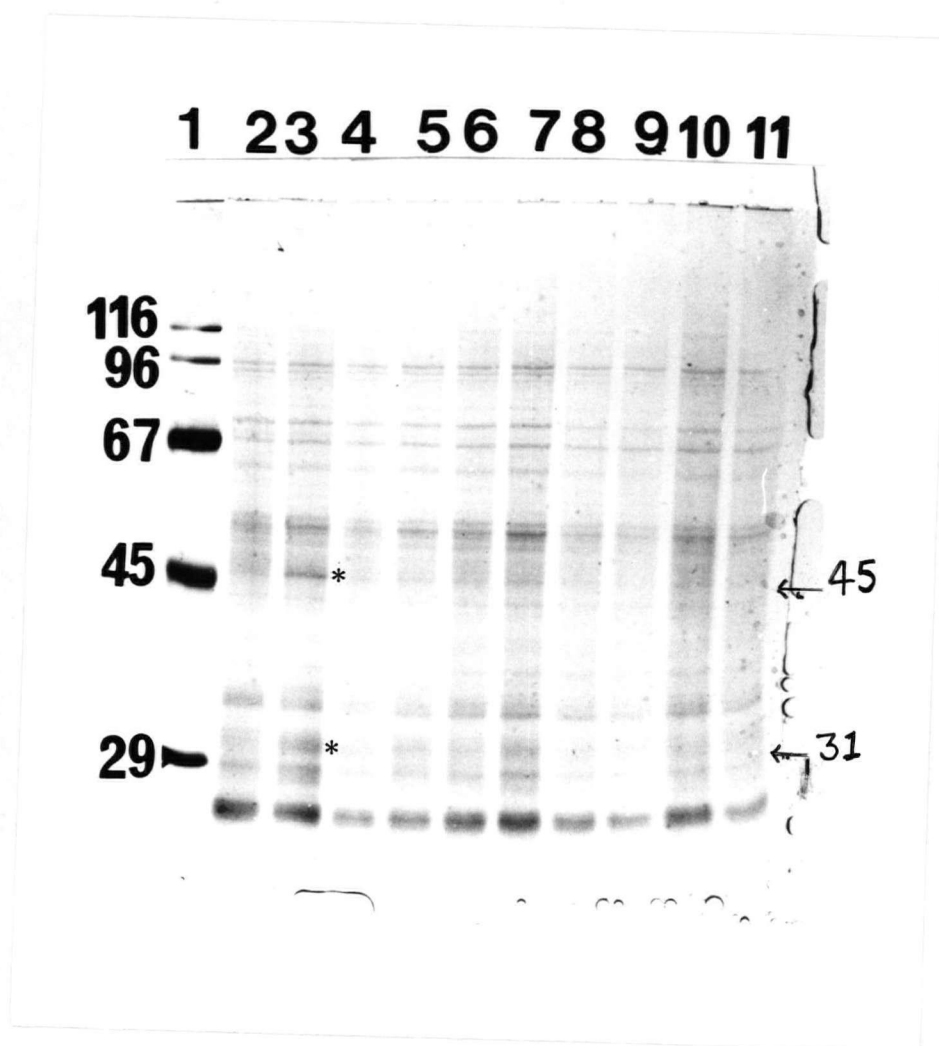
Fig 18. Effect of varying [K$^+$] in the washing buffer on the stability of the 45 kDa polypeptide

Coomassie blue-stained microsomal proteins (30 μg/lane) from +K$^+$-grown roots (lanes 2,4 and 6) and -K$^+$- roots (lanes 3,5 and 7). Lanes 2 and 3 represent the unwashed microsomes obtained using homogenization buffer amended for [K$^+$] (as described in Fig 17). These microsomes were washed in resuspension buffer in the absence of K$^+$ (lanes 4 and 5), or in the presence of 10 (lanes 6 and 7), 50 (lanes 8 and 9) and 150 (lanes 10 and 11) mM K$^+$.
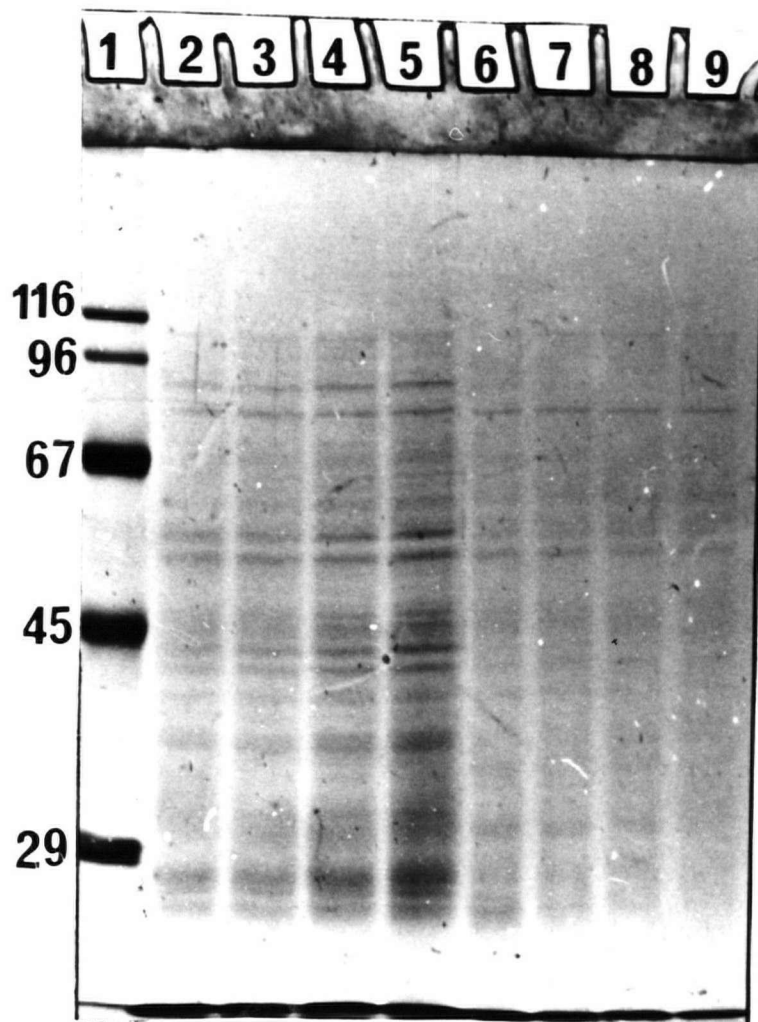
Fig. 19 K$^+$ *-related changes in Polypeptide profiles of soluble proteins*

Coomassie blue-stained polypeptides of soluble proteins (SN1) (lanes 2, 3, 4 and 5) (100,000 cpm/lane) and supernatant of microsomal wash (SN2) (lanes 6, 7, 8 and 9) (25,000 cpm/lane) obtained from roots of seedlings grown under K$^+$-deprived conditions for 0 (lanes 2 and 6), 6 (lanes 3 and 7), 12 (lanes 4 and 8) and 18 h (lanes 5 and 9) respectively. (Also see Fig 25 A & B for fluorographs of this gel).

Therefore, it is not unreasonable to assume that the 45 kDa (in microsomes) and 47 kDa (in tonoplast) polypeptides were the same, as may be the case for NO_3^- induced polypeptide in *Anacyctis nidulans* reported by Sivak et al. (1989) and Omura et al. (1989). If so, the expression of this polypeptide in the tonoplast fraction at a lower intensity than that in the microsomal fraction in the presence of EDTA, may have indicated a partial loss. Alternatively, the 45 kDa band may reside in other membranes than the tonoplast.

In experiments carried out to compare cultivar Klondike with Halcyon, no significant differences were observed in the polypeptide composition (Fig 20 and 21). However, most of the higher molecular weight polypeptides from cv. Klondike showed a greater degree of proteolytic degradation than those of cv. Halcyon. Both cultivars showed increased expression of the 47 kDa polypeptide in tonoplast enriched fractions (lanes 5-8, Fig 20; 0 & 96, Fig 21) isolated in the presence of 1 mM EDTA, with increased duration of K^+-deprivation. Several more differences, are apparent in tonoplast derived from cv. Klondike which are not associated with K^+ status, but may be genotypic in origin.

Better separation of polypeptides in the 45 kDa region was achieved in the material shown in Fig 21, which represents cv. Klondike. The well-stained band just below the 45 kDa marker (44 kDa) may be unique to cv. Klondike and may not represent the 45 kDa polypeptide in the unwashed microsomes of cv. Halcyon, since this 44 kDa band did not show K^+ related changes, and was not washed off in the presence of EDTA (see Fig. 24A). These results indicate that only the lightly stained 47 kDa band may be a K^+ related polypeptide and may be identical to the 45 kDa band seen in the microsomes. However, since the 45 kDa band was washed off by 1 mM EDTA, the tonoplast polypeptide may be distinct from the microsomal polypeptide. Since these are one dimensional gels, such slight differences in molecular weights should, perhaps, not be considered significant.

Alternatively the lower level of expression of the 47 kDa band in tonoplast preparations may represent a small fraction of the total microsomal protein and therefore may be at the limits of detection by Coomassie blue staining. Therefore, this band may not be obvious at the microsomal level. Unfortunately, attempts made to isolate tonoplasts without EDTA in the buffers were unsuccessful. In the absence of EDTA only 2 bands were present in the 4 step sucrose gradient instead of 3 bands, indicating the equilibration of the 30-34 % interphase band at another region. This might have been due to the "Mg^{2+}-shift" discussed in section 3.2.

In conclusion, it appears that the expression of the 45 kDa polypeptide is a specific response to K^+ deprivation; it can be considered to be a peripheral polypeptide and may be localized in the tonoplast.

The manner in which this polypeptide functions in barley root cells may be similar to the "apo-carriers" described in *E.coli*. In amino acid transport in *E.coli*, some of the porters have been shown to require specific transport-assisting proteins in the periplasm. These proteins, by reversibly binding specific amino acids, perform the function of "buffers" to ensure a constant supply of the amino acid in the immediate vicinity of the porter or carrier (Skulashev, 1988). Because of their involvement in transport these proteins have been termed "apo-carriers". They first bind the appropriate amino acids in the periplasm and then form a complex with an integral protein of the cytoplasmic membrane. Likewise, very recently Laudenbach et al. (1991) has demonstrated that a sulphate binding protein is expressed in large amounts in the periplasmic space when the cyanobacteria *Synechococcus* sp (strain PCC7942) was deprived of sulfur. Similarly, de Hostos et al. (1988) reported that *Chlamydomonas reinhardtii* developed a capacity to transport sulfate more rapidly during sulfate deprivation by producing an arylsulfatase which was released from the cytoplasm (according to the latter authors' terminology, "periplasm").
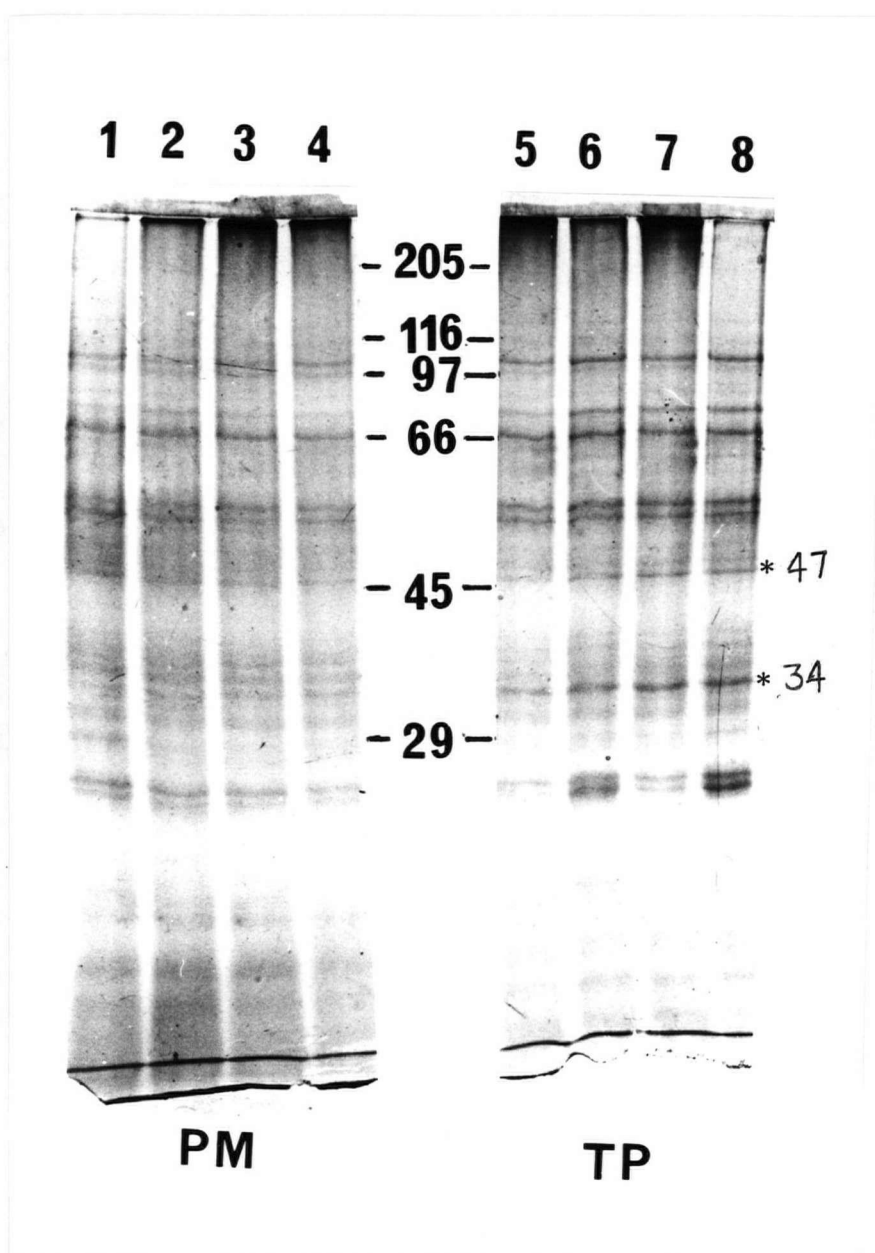
Fig. 20 Comparison of Coomassie blue-staining patterns of plasma membrane and tonoplast polypeptides of cv. Halcyon.

Coomassie blue-stained 10% gel of plasma membrane (lanes 1, 2, 3 & 4) (50 μg/lane) and tonoplast (lanes 5, 6 7 & 8) (50 ug/lane) obtained from roots grown under K^+ deprived conditions for 0 (lanes 1 & 5), 1 (lanes 2 & 6), 2 (lanes 3 & 7) and 4 (lanes 4 & 8) days. These membranes were isolated in the presence of 1 mM EDTA.
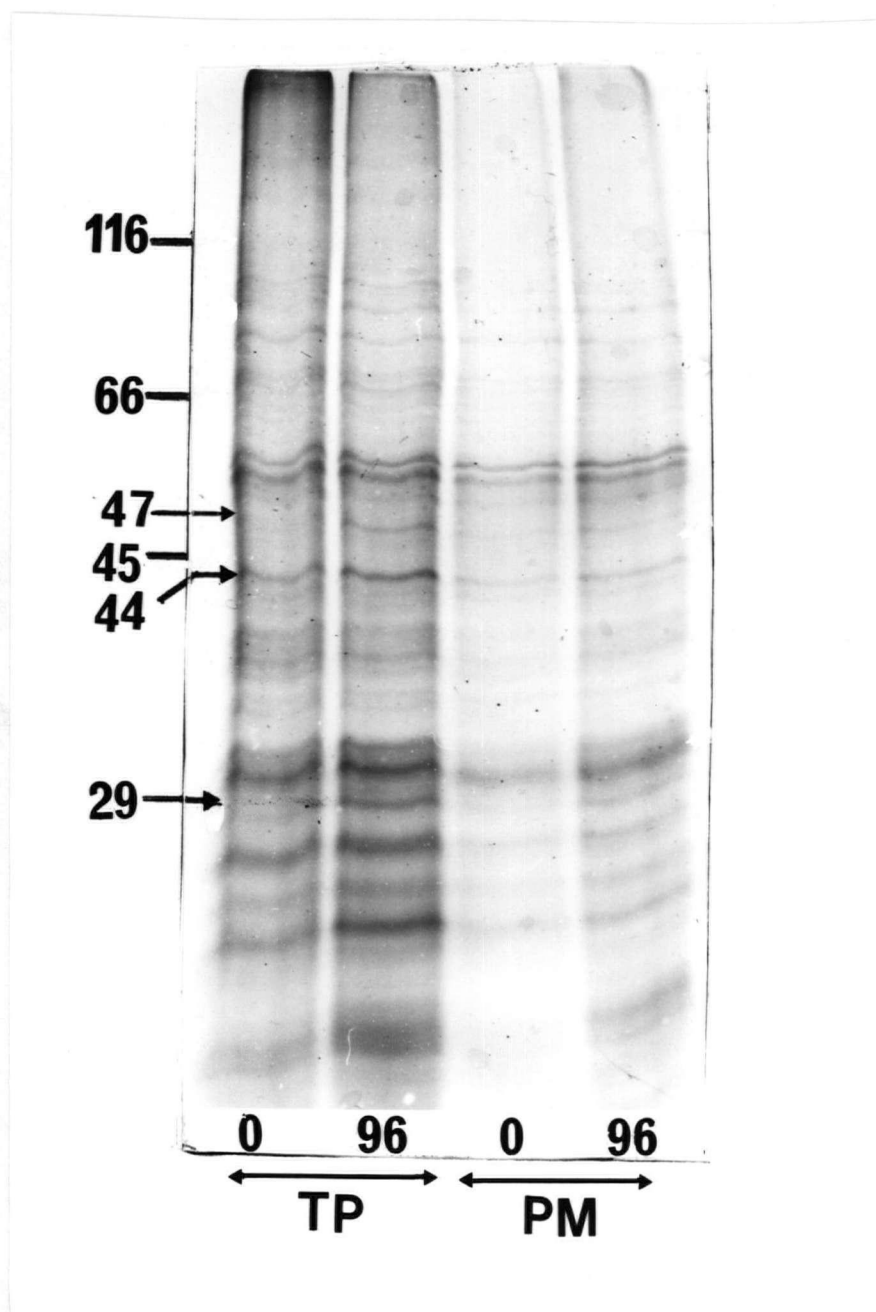
*Fig. 21 Comparison of plasma membrane and tonoplast polypeptides of
     cv. Klondike stained with Coomassie blue.*

Coomassie blue-stained 10% gel of tonoplast (TP) and plasma membrane (PM)
(50 μg/lane) obtained from roots grown without K^+ for 0 (0) and 4 days (96 h).
These membranes were isolated in the presence of 1 mM EDTA.

Burn (1988) has described similar types of cytosolic polypeptides which show reversible association with lipids under appropriate conditions. The latter author has classified such proteins as " Amphitropic ". These proteins are suggested to be important in cytoskeleton-membrane interactions, transmembrane signaling and in organization of the cytoskeleton.

The failure to recover the 45 kDa polypeptide in the soluble fraction (SN2) following its displacement by EDTA treatment may indicate that, when removed from its association with tonoplast or plasma membrane fractions, the polypeptide is much more susceptible to degradation. Efforts to concentrate the SN2 fraction by TCA precipitation, acetone precipitation or by use of Centricon filtration units (Amicon ltd.) were without success. By contrast, preliminary studies using ^{35}S-methionine labeling of membrane polypeptides provided more promising results, especially in terms of detecting short-term responses to K^+ deprivation. These studies which are discussed in section 5.3.3 were therefore given priority over further efforts to characterize the 45 kDa protein.

3.3 Time course of ^{35}S-labeling of membrane polypeptides in relation to K^+-deprivation

The time course of synthesis of membrane polypeptides in different sub-cellular fractions, in relation to the durations of K^+-deprivation, was studied using *in vivo* ^{35}S-methionine labeling. Two factors were considered in determining the time and the duration of seedling exposure to ^{35}S-methionine. First, the shortest time required to obtain significant labeling of membrane polypeptides and second, the feasibility of scheduling convenient times for membrane fractionation. Three labeling strategies were investigated in order to determine the patterns of polypeptide synthesis following removal of K^+.

Method 1: ^{35}S-methionine labeling during the last 6 h before harvesting

Labeling was performed during the last 6 h prior to harvest. Preliminary experiments indicated that seedlings took up 80-90% of the available radioactivity within 6 h of exposure to ^{35}S-methionine. When roots were harvested at the end of this 6 h period, the amount of radioactivity taken up per unit root weight between high-K$^+$ and low-K$^+$ roots revealed slight differences (Table 12). TCA precipitation of radioactive proteins indicated that the amount of absorbed radioactivity incorporated into proteins was highest in the soluble fractions (Table 12).

Method 2: Labeling for 6 h followed by a 12 h chase before harvesting

In order to obtain higher incorporation of label into membrane proteins, ^{35}S-methionine was administered 18 h prior to harvest. After 6 h, when 80 - 90 % of the available label had been absorbed, plants were transferred to non-labeled growth media for a further 12 h. Plants were exposed to the same conditions, with respect to [K$^+$], during this 12 h "chase" as had been provided during the labeling period. Although the duration of exposure (6 h) to radiolabel in this method was identical to that of the former method (Method 1), the pattern of labeling revealed novel differences.

Method 3: 12 h labeling prior to harvesting

In this method, labeling was administered for 12 h. prior to harvesting.

At the end of the labeling or "chase" periods all roots were washed with distilled water to remove label in the cell wall space, frozen in liquid nitrogen and held at -70°C until used for membrane preparation.

3.3.1 Comparison of microsomal polypeptide profiles

Coomassie blue-staining (A) and labeling patterns (B) of microsomal polypeptides before and after washing with or without 1 mM EDTA are shown in

Figures 22 (labeling method 1), 23 (labeling method 2) and 24 (labeling method 3). Coomassie blue-staining of the microsomes from roots deprived of K^+ for 4 days revealed stronger expression of the 45 kDa polypeptide (Fig 22 A) as well as the appearence of a second, lighter band at a M_r of 47 kDa. Both of these polypeptides disappeared after washing of microsomes in the presence of 1 mM EDTA in the resuspension buffer. However, a polypeptide having M_r 43 kDa (detected by significant incorporation of ^{35}S-methionine) was expressed as early as 12-18 h after K^+ was removed from the growth media. This polypeptide is very evident in some instances (Fig 24 B), while it was expressed to a lesser extent in other experiments (Fig 22 B, & 23). It was, at first, considered to be the radiolabeled polypeptide corresponding to the 45 kDa protein described in section 5.3.1. However, as shown in Fig 24 B the presence of this polypeptide was not influenced by EDTA washing and it must therefore be distinct from the former protein. A second heavily labeled polypeptide with M_r 49 kDa, (lane 3, Fig 22 B; lane 2, Fig 23), showing equal intensities in both unwashed and washed microsomes, was also synthesized in response to K^+ deprivation and revealed by labeling strategies 1 and 2. This labeling can be seen in Figures 22B, 23 and 24B. The expression of the 49 kDa polypeptide within 12 - 24 h indicates that this polypeptide may play a significant role in the increased K^+ uptake of K^+-deprived seedlings.

Other changes observed in the microsomal fractions were minor compared to those discussed above. Although K^+ deprivation caused no differences in Coomassie blue-stainable polypeptides in the 67 and 55 kDa regions of the gel (Fig 22A and also 27), microsomes from roots grown under high-K^+ conditions showed labeling of polypeptides corresponding to these regions of the gel (lane 1, Fig 22B); these polypeptides were not labeled in the -K^+ treatments.

Table 12. Amount of radio label taken up and incorporated into various fractions

Amount of radioactivity taken up (cpm g^{-1} fwt.) by roots of controls (0 days), and by roots deprived of K^+ for 3 and 6 days. TCA precipitable counts are given as % of total counts in root homogenate. (Counts in homogenate as a % of counts in the labeling solutions are given in parentheses)

Days in low-K^+	Total counts homogenate	TCA precipitable counts %	
		soluble	microsomal
0	5×10^8 (98)	79	10
3	4×10^8 (97)	70	10
6	4×10^8 (97)	83	11
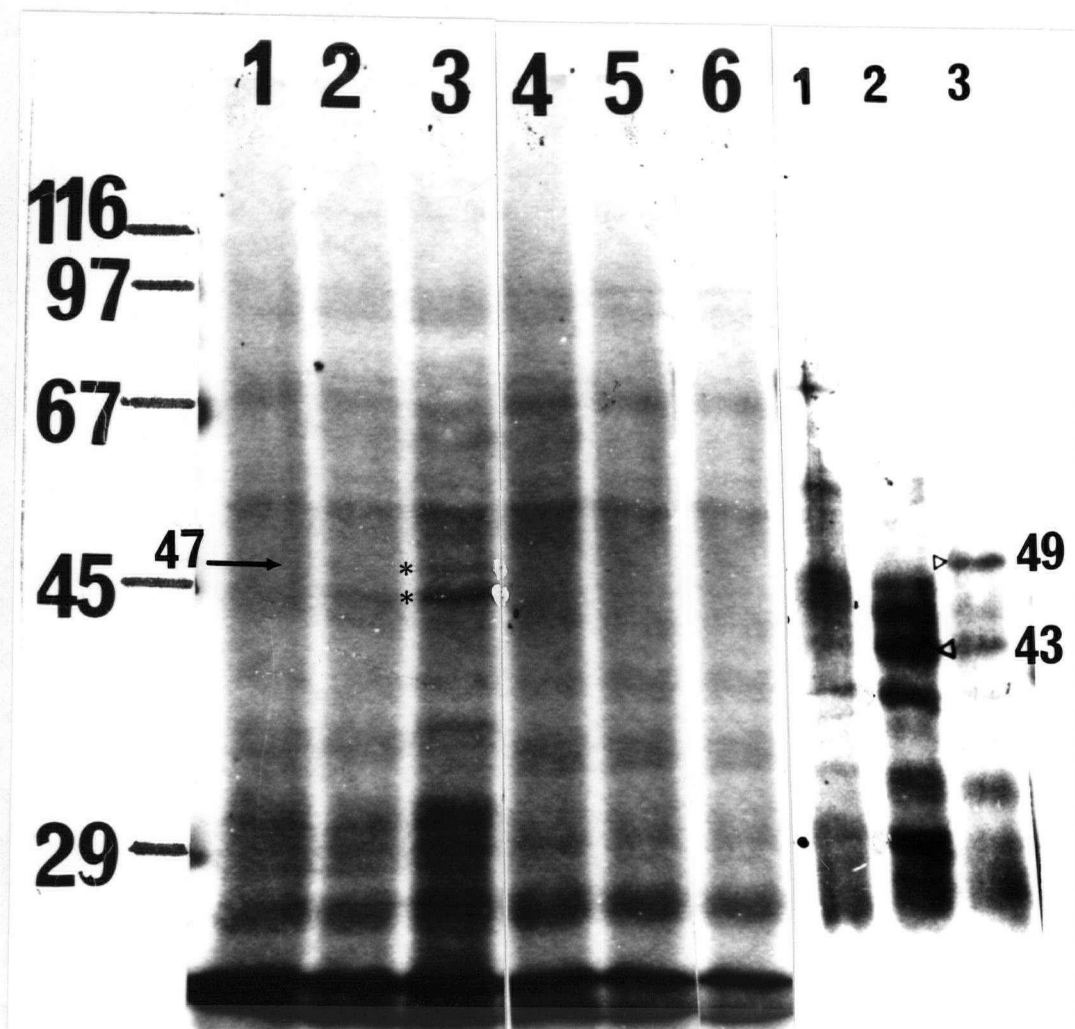
Fig 22 *Coomassie blue stained (A) and Fluorographs (B) of microsomal proteins of the same gel. Labeling method 1 was employed for in vivo labeling with ^{35}S-methionine*

Microsomal polypeptides (100,000 cpm/lane) of roots from seedlings deprived of K^+ for 0 h (lane 1A, 4A& 1B), 24 h (lanes 2A, 5A & 2B) and 96 h (lanes 3A, 6A & 3B). Lanes 1-3A represent the unwashed microsomes, lanes 4-6A and 1-3B represent the microsomes washed in the presence of EDTA. Masses of molecular weight markers are given at left.
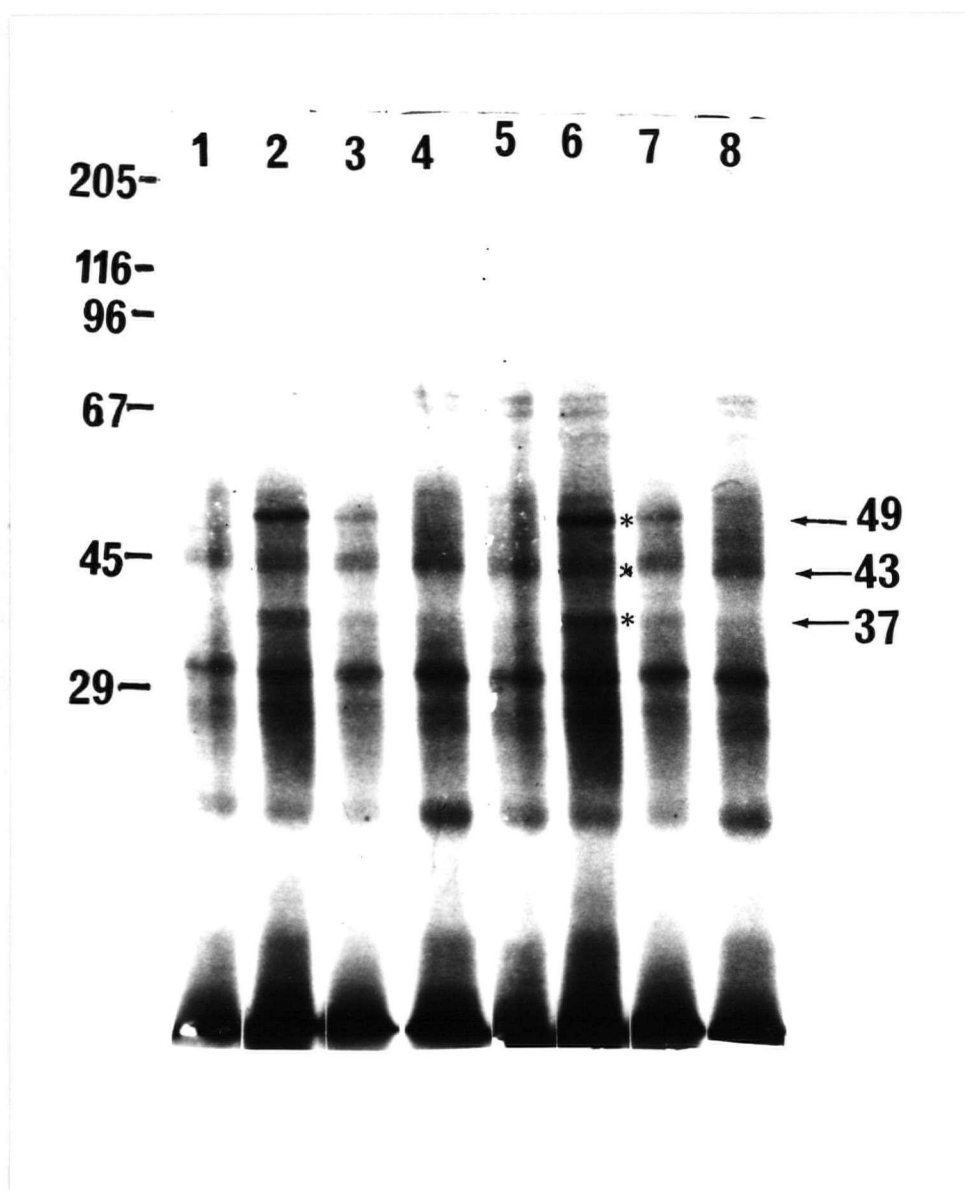
*Fig 23 Fluorographs of microsomal polypeptides. Labeling method 2 was
          employed for labeling with ^{35}S-methionine.*

Profiles of microsomal polypeptides (100,000 cpm/lane) of roots from seedlings
deprived of K$^+$ for 0 h (lane 1), 24 h (lane 2), 48 h (lane 3) and 96 h (lanes 4).
Lanes 1-4 represent the unwashed microsomes and 5-8 represent the microsomes
washed in the presence of EDTA. Masses of molecular weight markers are given at
left.

This may indicate that K^+-deprivation results in both the repression and derepression of specific membrane polypeptides.

Figure 24 (A and B) shows microsomal proteins separated on a 12 - 17% SDS gel. A Coomassie blue-stainable polypeptide of M_r 44 kDa was present in all the treatments including the high-K^+ "control". By contrast, the 43 kDa radiolabeled polypeptide was expressed only in -K^+ treatments, with highest intensity evident after 12 h of -K^+ treatment (Fig 24B). The possibility that the 44 and 43 kDa polypeptides are the same is quite unlikely, for two reasons:

1. The 43 radiolabeled band was detected only in -K^+ treatment (Fig 24B) and

2. careful alignment of the fluorograph (X-ray film) on the original dried gel showed that these polypeptides migrated quite differently.

Another polypeptide of M_r 81 kDa showed intense labeling after 96 h of K^+-deprivation (Fig 24B). Recently, Li et al. (1990) have purified an 82 kDa protein from rat liver mitochondria. They demonstrated that this protein, when reconstituted into proteoliposomes, had the ability to catalyze electroneutral K^+ uptake into liposomes. They have reported that this protein is similar to the native mitochondrial K^+/H^+ antiporter. It is intriguing to see the close proximity of the molecular weight of the 81 kDa polypeptide seen in the microsomes from barley roots deprived of K^+ for 96 h with that of the rat mitochondrial (K^+/H^+) antiporter. Figure 24C shows a densitometric scanning of the fluorograph represented in Figure 24B and the calculated areas of the peaks of interest.

Some differences were also observed in the soluble proteins when seedlings were exposed to -K^+ conditions for durations as short as 12 h. The fluorographs in Figures 25A & B were obtained by exposing X-ray film to the dried gel shown in Figure 19. The most prominent polypeptides had values of Mr 29 and

85 kDa, and these were intensely labeled during the 12 h period of K^+ deprivation (Fig 25A and 25B). A lack of correspondence in the polypeptide profiles of soluble (Fig 25A) and microsomal membrane fractions suggests that contamination of the latter by soluble proteins was not a significant problem. Fig 25B represents the SN2, the supernatant resulting from microsomal washing. Some of the polypeptides present in SN2, correspond in molecular weight to those in the soluble fraction (SN1). The latter polypeptides may represent those which are specifically associated with the microsomes.

3.3.2 Polypeptide profiles of plasma membrane and tonoplast

Coomassie blue-stained plasma membrane and tonoplast preparations from roots grown with or without K^+ for up to 4 days revealed only minor differences in one dimensional SDS-PAGE (Fig 26). By contrast fluorograms of the same gel revealed qualitative and quantitative differences in labeling pattern (Fig 26B). When labeling was carried out during the last 6 h of the 24 h of K^+-deprivation (method 1), both plasma membrane (PM) and tonoplast-enriched (TP) fractions showed similar patterns of labeling (Fig 26B). In plasma membranes derived from roots after 1 day of K^+-deprivation (Fig 26B), a 37 kDa polypeptide became strongly labeled (lane 2, Fig 26B). This polypeptide was also apparent in the plasma membrane fraction obtained after 3 days of K^+ deprivation in a separate experiment using labeling method 2 (Fig 27, lane 3). However, the difference between high-K^+ and low-K^+ treatments was not pronounced. The 43 kDa polypeptide which was strongly labeled in the microsomal preparations (Fig 24B) after 12 h of K^+ deprivation (labeling method 3) was clearly localized in the plasma membrane and tonoplast preparations (lanes 2 and 5, Fig 26B; lane 5, Fig 27). However, this band was not apparent in the corresponding plasma membrane
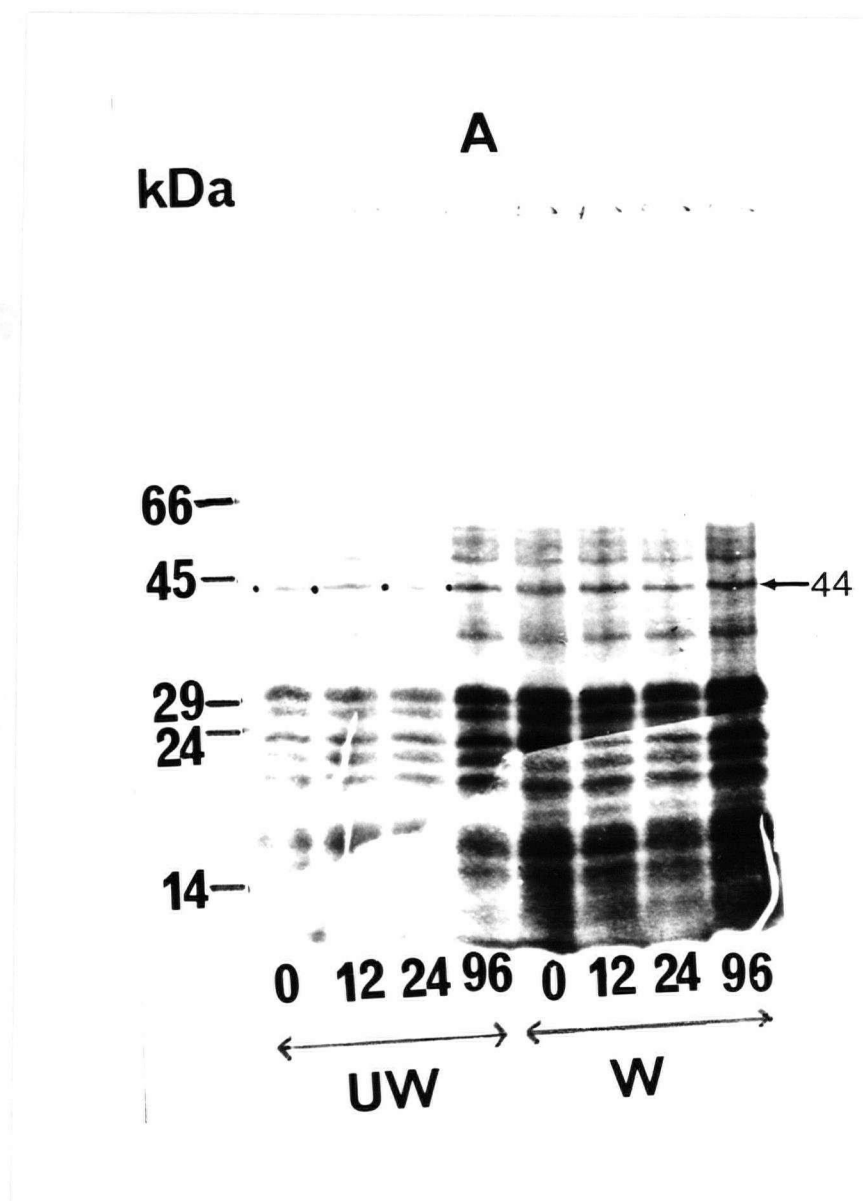
Fig 24A   Coomassie blue stained microsomal proteins.

Microsomal polypeptides (100,000 cpm/lane) of unwashed and washed microsomes obtained from roots of seedlings deprived of K^+ for 0, 12, 24 and 96 h. UW; unwashed microsomes, W: washed microsomes.
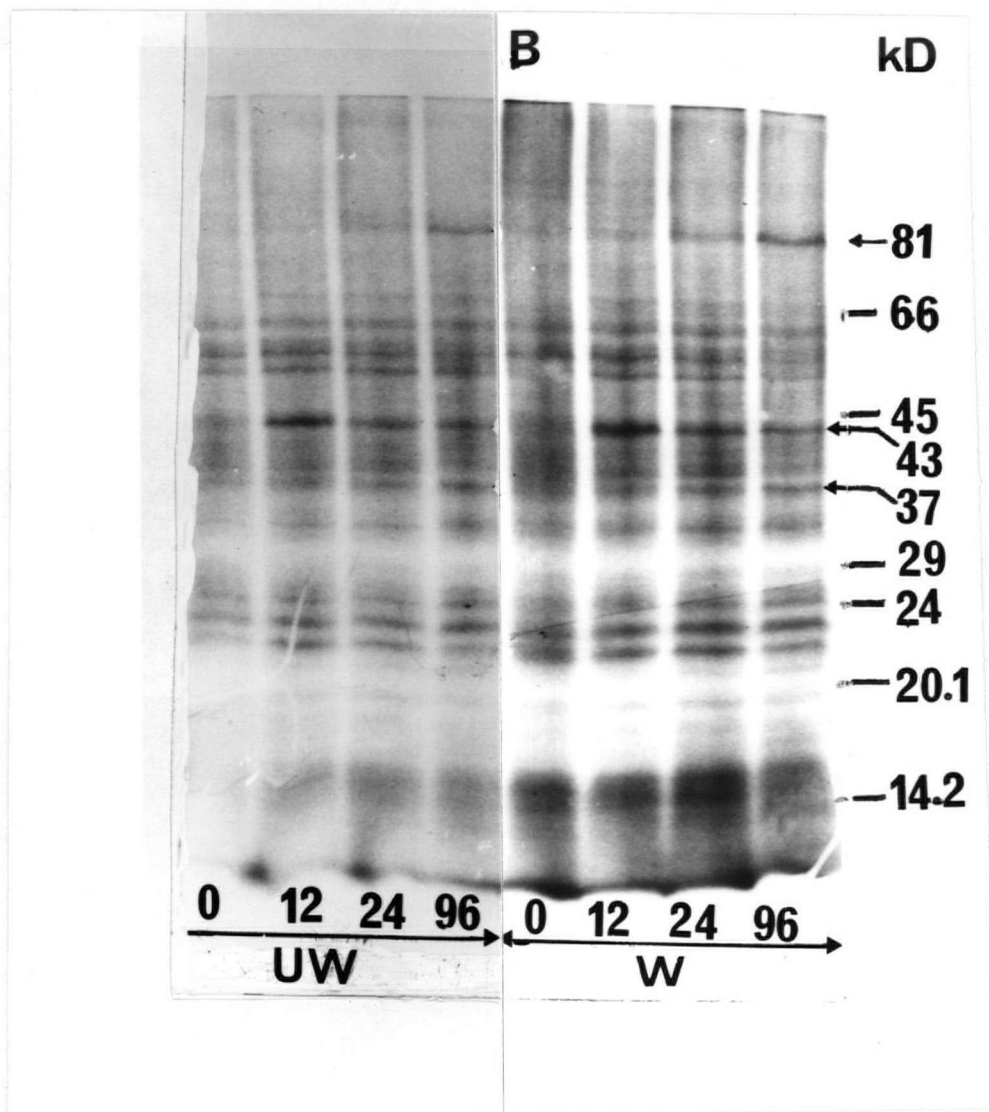
Fig 24B Fluorograph of microsomal proteins. Labeling method 3 was used for labeling
with ^{35}S-methionine

Microsomal protein (100,000 cpm/lane) of the unwashed and washed microsomes
obtained from roots of seedlings deprived of K$^+$ for 0, 12, 24 and 96 h. UW;
unwashed microsomes, W: washed microsomes.
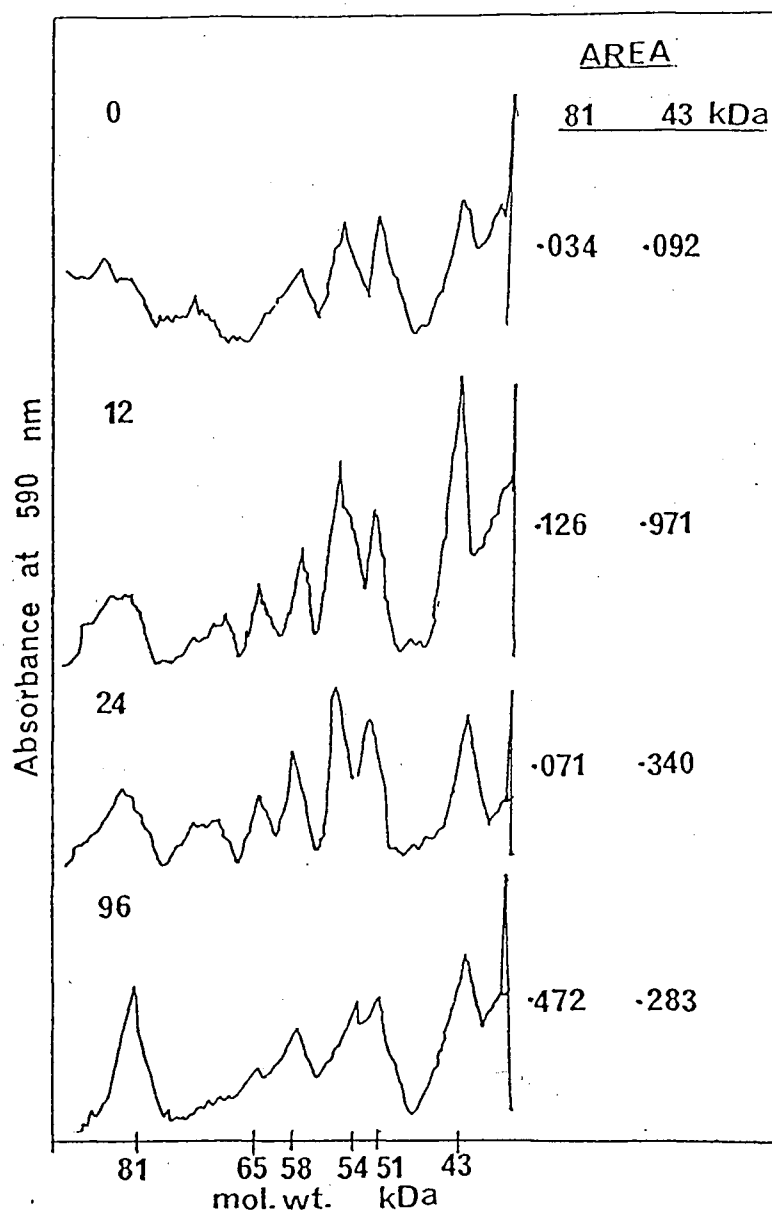
Fig 24C Densitometric scans of the fluorograph in fig 24B.

Densitometric scans of washed microsomes represented in Fig 24B. Scans were obtained using the Beckman spectrophotometer model DU-64. (from laboratory of Dr. Dane Roberts, B.C Research Corporation).
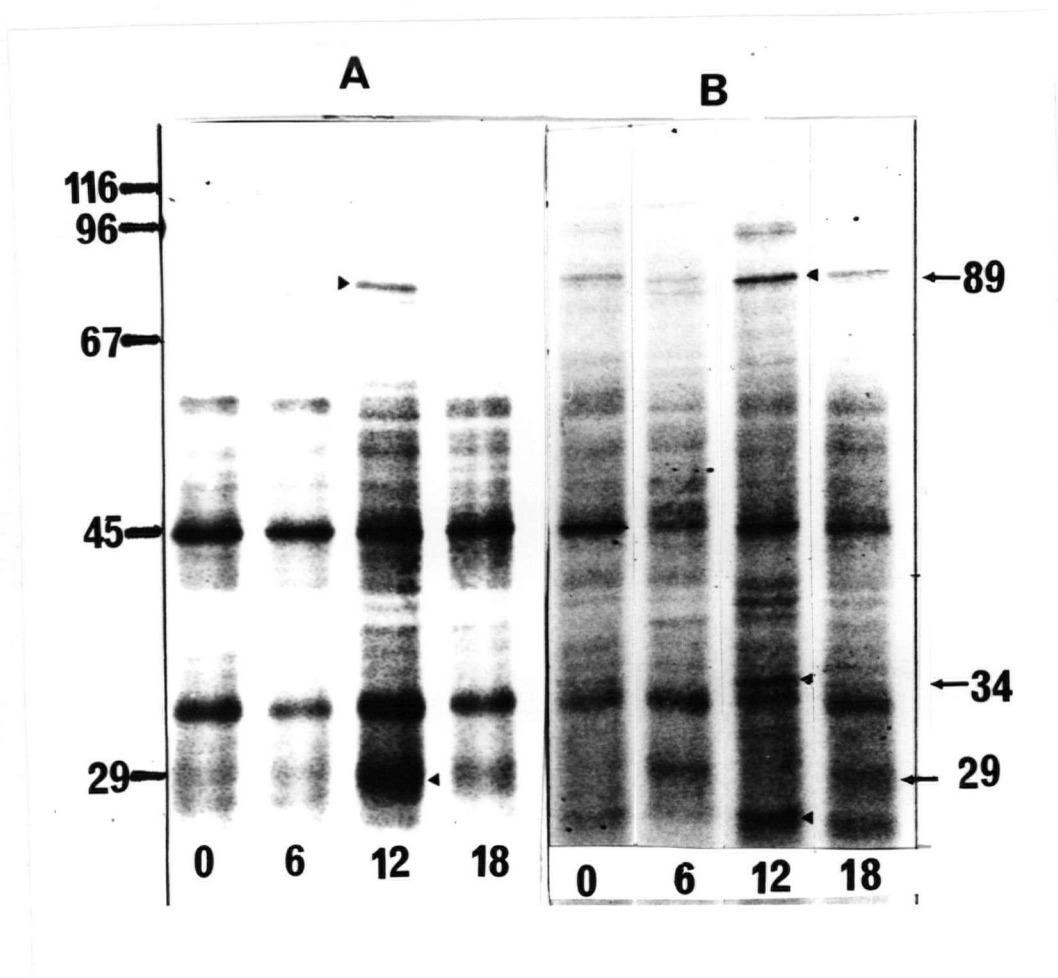
Fig 25. Fluorographs of soluble proteins (SN1) (A) and supernatant of microsomal wash (SN2) (B)..

TCA precipitated soluble (SN1) (50,000 cpm/lane) and supernatant of microsomal wash (SN2) (50,000 cpm/lane) of the roots grown in -K$^+$ conditions for 0 (0), 6 (6), 12 (12) and 18 (18) h. (also see Fig 19 for the Coomassie blue stained gel of these fluorographs).

by method 2. Subsequent experiments have revealed that this polypeptide was even more strongly labeled in a 6 h labeling

period following 6 h of K^+ deprivation.

Under these conditions the 43 kDa polypeptide was also evident in the plasma membrane and endoplasmic reticulum fractions (J. Mehroke, unpublished data). Interestingly, the 43 kDa band was also evident in microsomal preparations from oat roots deprived of K^+ for 6 h followed by ^{35}S methionine labeling for 6 h (Fig 28). Indeed, several polypeptides in the microsomal preparations were strongly labeled in both barley and oats under these conditions. These included the 43 kDa polypeptide and those with M_r = 24, 35, 71 and 84 kDa (J.Mehroke, unpublished data). The polypeptide estimated as M_r ~ 37 kDa in the earlier experiments (Fig 26 and 27) may represent the 35 kDa polypeptide shown in later experiments (Fig 28). An intensely labeled 49 kDa polypeptide apparently unique to the tonoplast was expressed after 24, 48 and 72 h (lanes 5, 6 and 7, Fig 27). This polypeptide was present in plasma membrane fraction only after 72 h of K^+ deprivation (lane 4, Fig 27).

Three polypeptides of M_r 52, 54 and 71 kDa were strongly labeled in plasma membrane and tonoplast preparations derived from roots grown in high-K^+, but these were not expressed in membranes derived from roots grown in low-K^+ conditions (lanes 1 and 4, 26). Two polypeptides of molecular weights similar to those labeled (54 and 71 kDa) were stained by Coomassie blue (lanes 4 and 5, Fig 26A). In this gel the 54 kDa polypeptide appeared to be expressed in plasma membrane and tonoplast independently of K^+ treatment (lanes 1-6, Fig 26). The 71 kDa polypeptide was most prominent in tonoplast preparations (lanes 4-6, Fig 26A). It was evident in the plasma membrane too but at very low levels (lanes 1-3 Fig 26A). By day 4 of the treatment (lane 6, Fig 26A), this polypeptide had diminished in quantity in the tonoplast preparations. Since these bands appeared

not to be synthesized in the low-K^+ treatment their continued presence in the Coomassie blue-stained gels may indicate that their turnover is quite slow. The concept of long-lived polypeptides in the membranes of barley roots was advanced some years ago by Schaefer et al. (1975) when these authors reported the relatively slow turnover of the carrier involved in K^+ uptake. The 52, 54 and 71 polypeptides reported here, also were rapidly synthesized under high K^+ conditions. It could be speculated that these proteins may represent a repressible system expressed rapidly when $[K^+]_i$ is increased. These may be considered to be compartmentalized in the vesicles formed during pinocytosis or membrane recycling, and therefore, their presence in the low K^+ after 1-4 days from the onset of K^+ deprivation (lane 2, 4, 5 and 6, Fig 26A) may not indicate they are still functional.

It is also interesting to note the appearence of two polypeptides in the 100 kDa region of the plasma membrane lanes. This region corresponds to the location of the plasma membrane H^+-ATPase. As mentioned earlier (Chapter 4), no significant difference in the extent of expression of these bands arose as a result of the different K^+ treatments.

3.3.3. Determination of intrinsic or extrinsic nature of the polypeptides of interest

The purification and characterization of the polypeptides identified by the present work, as well as their exact functions lies beyond the scope of this thesis. Such work is already underway in Dr. Glass' laboratory. However, an attempt was made to determine the hydrophobicities of these polypeptides using phase separation of integral membrane proteins in Triton-X-114 solutions (Bordier, 1981) as described in section 5.2.4.
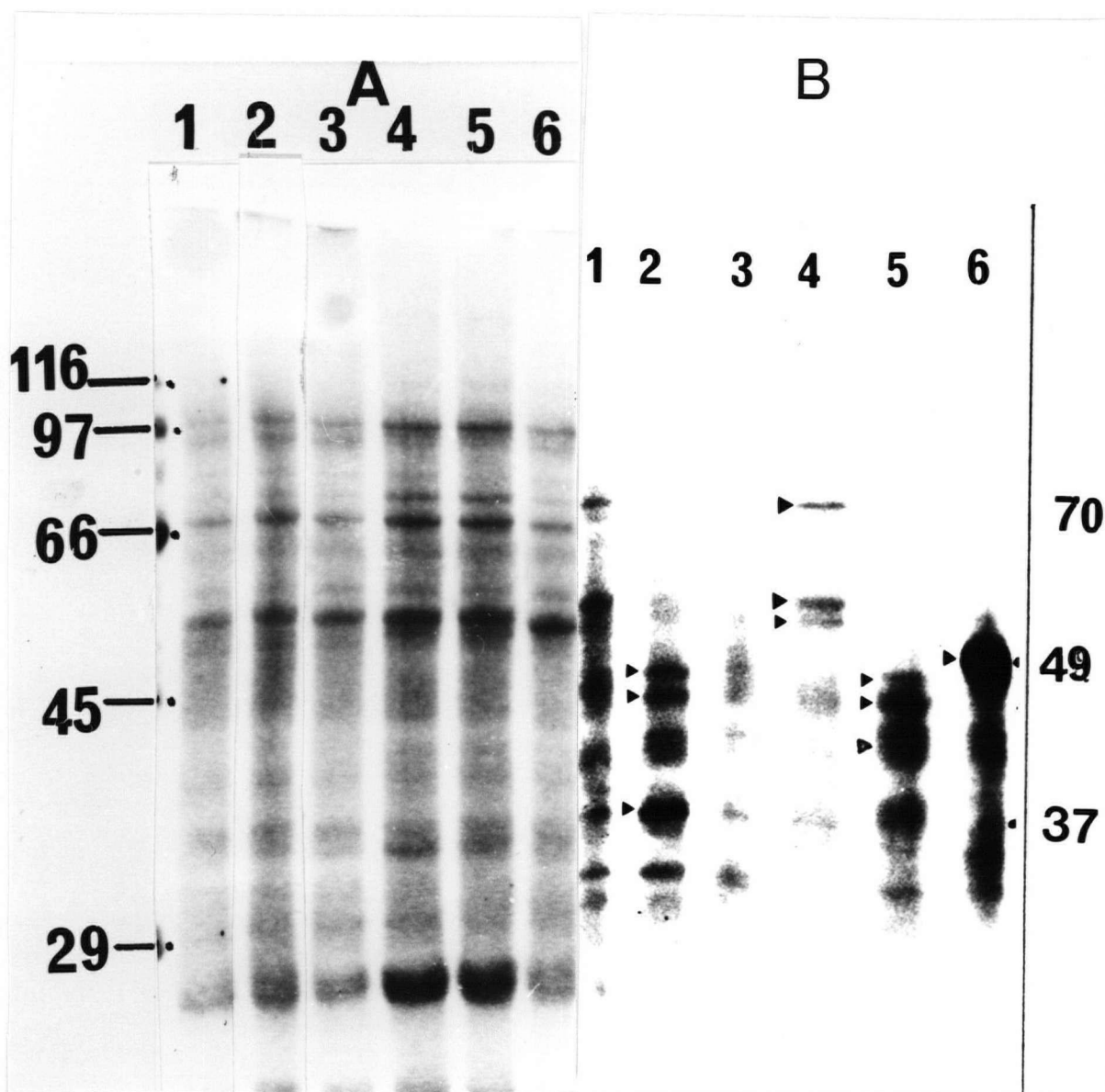
Fig. 26 *Coomassie blue-stained gels (A) and fluorograph (B) of plasma membrane and tonoplast polypeptides. Seedlings were subject to labeling method 1.*

Plasma membrane (lanes 1-3) and tonoplast (lanes 4-6) polypeptides obtained from roots grown in $+K^+$ and $-K^+$ conditions for 0 (lanes 1 & 4), 1 (lanes 2 & 5) and 4 (lanes 3 & 6) days. These membrane-enriched factions were obtained using sucrose step gradient.
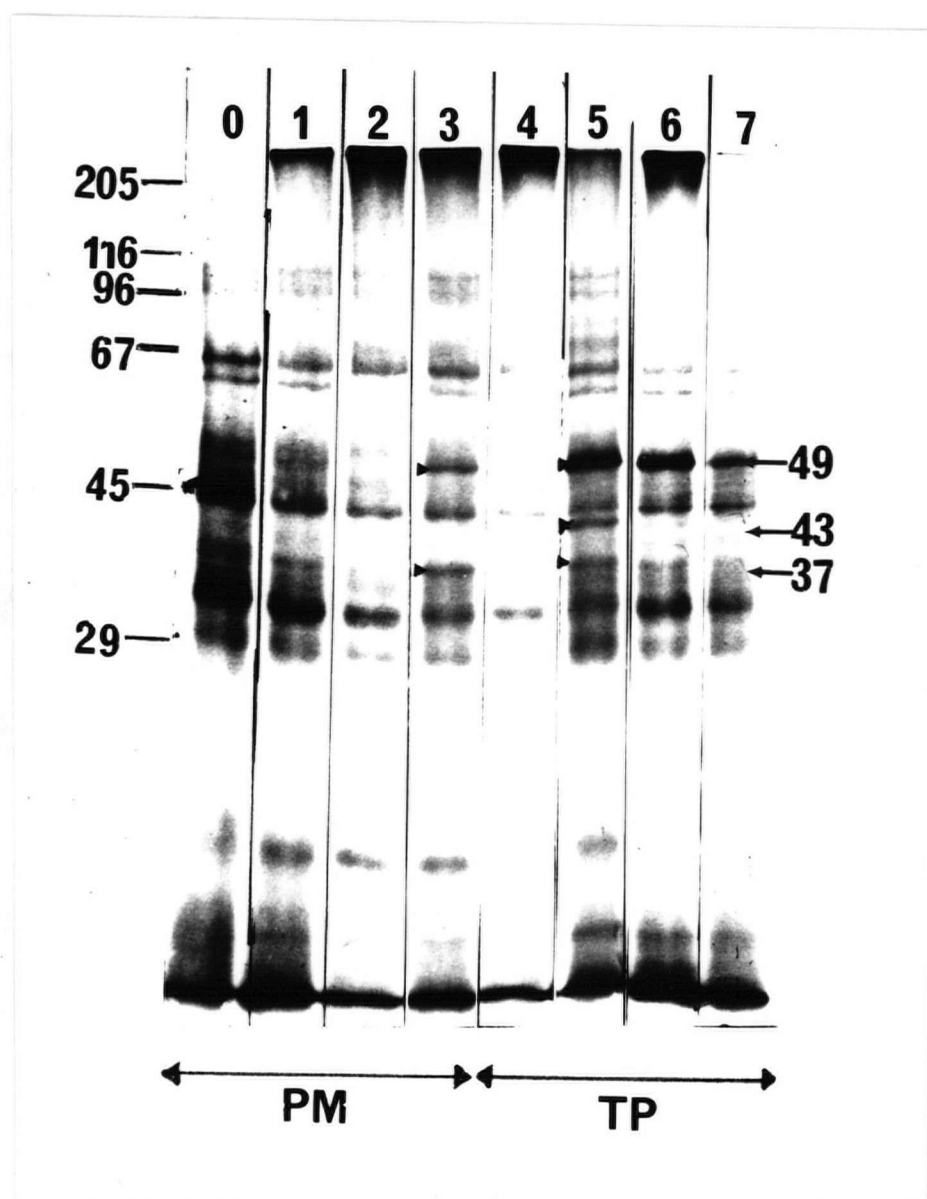
Fig.27   *Fluorograph of plasma membranes and tonoplast. Seedlings were subject to labeling method 2.*

Seedlings had been subjected to 0 (lanes 0 & 4), 1 (lanes 1 & 5), 2 (lanes 2 & 6) and 3 (lanes 3 & 7) days of K^+-deprivation. These membrane-enriched fractions were obtained from sucrose step gradient.
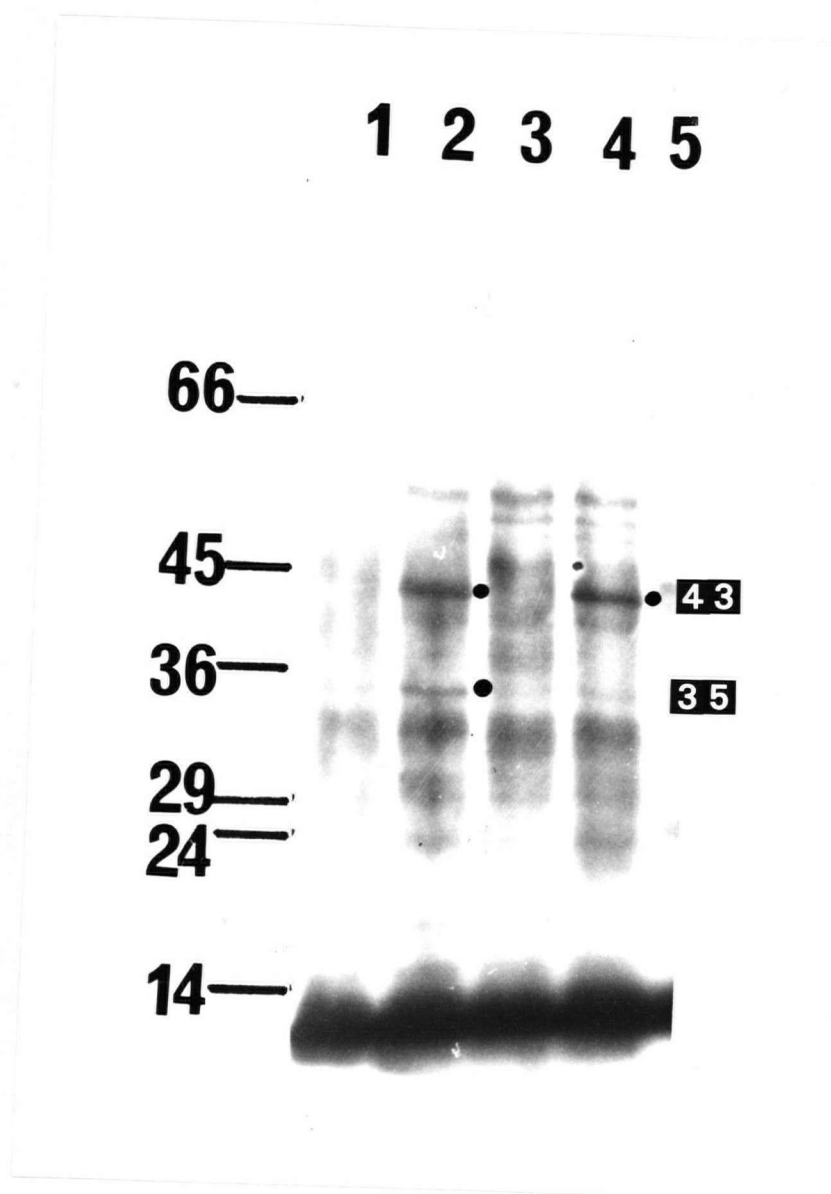
Fig 28   *Fluorograph of microsomal proteins from barley*
and oat roots.

Microsomes obtained from roots of barley (lanes 1 & 2) and oat (lanes 3 & 4)
seedlings grown in - K^+ solutions for 6 h followed by ^{35}S-methionine labeling for 6
h. Lanes 1 & 3 represent the controls on which the seedlings were grown
continuously (10 days) in the presence of K^+.

A silver-stained gel of the aqueous fraction after separating the intrinsic proteins into the Triton-X-114 phase is shown in Figure 29. This gel does not reveal any significant differences between the treatments. By contrast, the fluorograph of the same gel (Fig 30 A) revealed intriguing results. Several polypeptides of M_r = 85, 54, 37 and 31 kDa were distinctively labeled in the microsomes obtained from roots deprived of K^+ for 96 h. The labeling pattern in the Triton-X-114 phase (30B) was distinct from that of the aqueous phase (Fig 30A).

Major bands having M_r 81, 63, 43 and 41 kDa were observed in this phase. Of these, the 81 kDa polypeptide was more pronounced in the 96 h -K^+ treatment (lane 4, Fig 30B) while the 43 kDa polypeptide was strongly labeled in 12, 24 and 96 h of -K^+ treatments. (lanes 2,3 and 4, Fig 30B). There was no significant difference in the expression of the 63 kDa polypeptide among treatments, while the 41 kDa polypeptide was only present in the 24 h -K^+ treatment. The polypeptides partitioned into the detergent phase represent intrinsic proteins. Therefore, the 43 kDa polypeptide which was discussed previously (in section 3.3.2) appears to be intrinsic while the 49 kDa polypeptide appeared to be extrinsic or peripheral in nature.

Of the polypeptides detected, the 37 kDa in plasma membrane and tonoplast (Fig 26B), 43 kDa in microsomes, plasma membrane and tonoplast (Figs. 23, 24B, & 26B), 49 kDa in tonoplast (Figs. 26B & 27B), and the 81 kDa in microsomes (Fig 24B) polypeptides are of particular interest regarding the correlation of their expression with K^+-deprivation. Table 13 summarises the changes in expression of polypeptides associated with K^+ deprivation.

The rapidity of appearance of the 43 kDa polypeptide correlates well with the observed rapid increase of K^+ influx following K^+-deprivation. The integral nature of this polypeptide is consistent with involvement as part of a K^+ transporter. The hydrophobicity of this protein might account for its lack of

stainability with Coomassie blue. On the other hand, it may not have been expressed in sufficient amounts to be detected by Coomassie blue-stain. If this is the case, this protein appears to be synthesized in quantities much less than 1-5 μg g^{-1} fwt, which is typically below the limits of detection by Coomassie blue staining (unpublished data).

It is pertinent at this point to consider the nature of the proposed high affinity K$^+$ carriers of other organisms. Physiological, biochemical and genetic studies have provided evdence for the existence of two K$^+$ transport systems; the Kdp system in *E. coli* (representing the prokaryotes) and the TRK system in *Saccharomyces* (representing the eukaryotes). For higher plants, there is no evidence at the molecular level concerning the existence of a K$^+$ transport system.

The high affinity K$^+$ uptake system in *E.coli* has been shown to be a K$^+$-ATPase (Epstein et al., 1984) while in *Neurospora* it was suggested that the counterpart is a K$^+$-H$^+$ cotransport system (Blatt et al., 1987; Rodriguez-Navarro et al., 1986). If a K$^+$-H$^+$ cotransport system is operating in barley, the inward movement of K$^+$ would be coupled to the passive H$^+$ movement down the steep proton gradient generated by the proton ATPase. Such a system would be expected to be sensitive to changes in the pH gradient. However, Glass and Siddiqi (1982) and Kochian et al. (1989) demonstrated a lack of sensitivity of the high affinity K$^+$ transport system in barley and corn roots, respectively, to changes in the pH gradient.
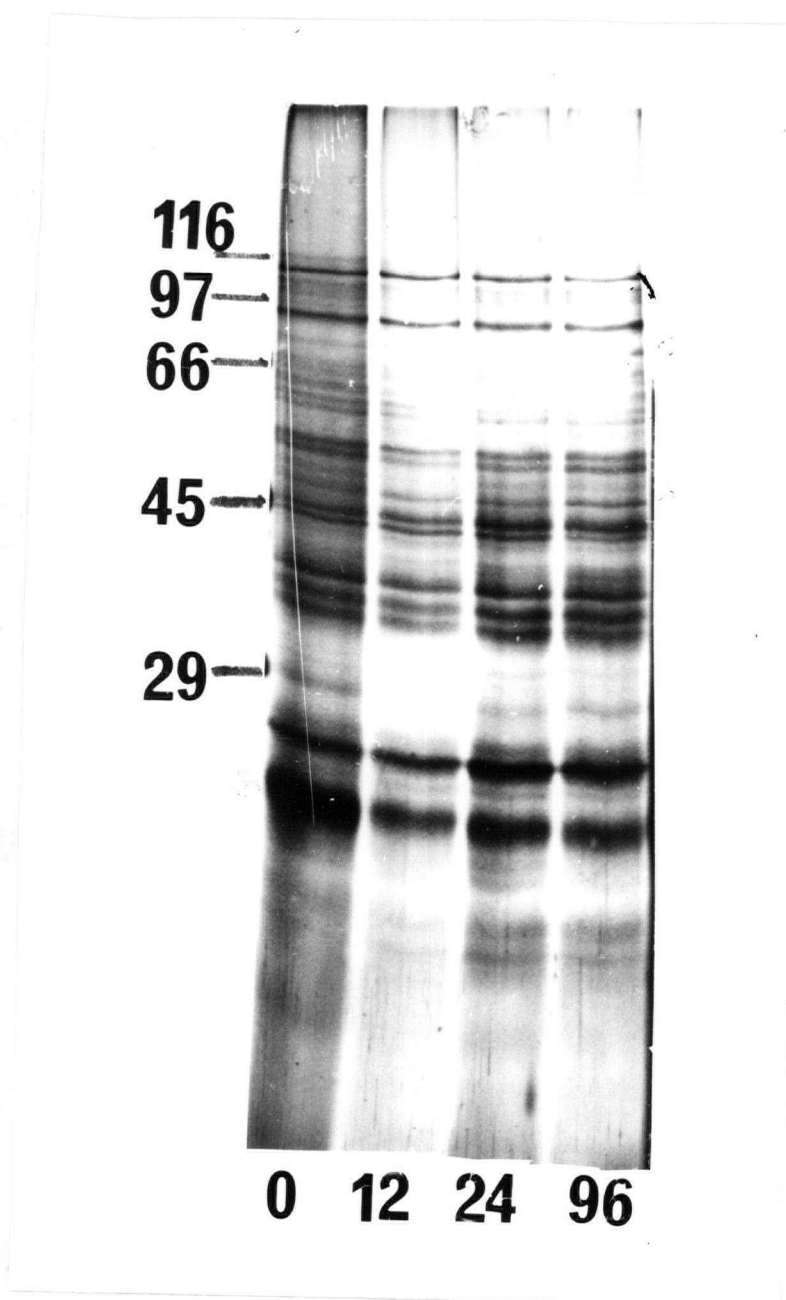
Fig.29 Silver stained gel of aqueous phase after detergent separation of intrinsic proteins.

Aqueous phase after Triton-X-114 phase partitioning of the microsomal proteins derived from seedling roots grown for 0, 12, 24 and 96 h without K^+.
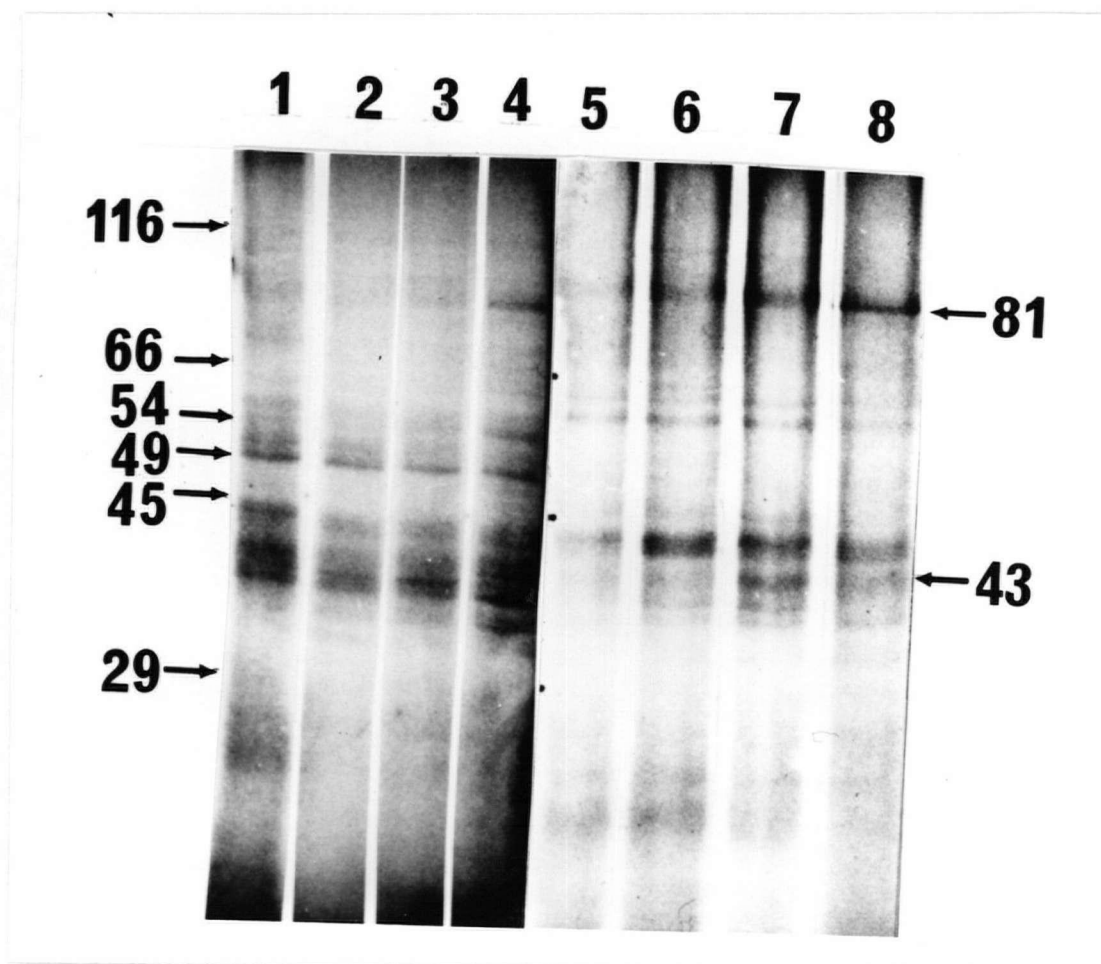
Fig 30 *Fluorograph of aqueous phase (lanes 1-4) (gel in fig 29) and detergent phase (lanes 5-8) after separation of microsomal proteins on Triton-X-114.*

Aqueous phase after Triton-X 114 phase partitioning of the microsomal proteins derived from seedling roots grown for 0 (lane 1 and 5), 12 (lane 2 and 6), 24 (lane 3 and 7) and 96 h (lane 4 and 8) without K^+.

Table 13   Summary of the observed changes in the expression of polypeptides

Polypeptides repressed (-ve) and derepressed (+ve) by the absence of K^+ in the external media. Ex: Extrinsic polypeptide, In: intrinsic polypeptides. Sol: solubles, Mic: microsomes, PM:plasma membrane and TP:tonoplast. Abbreviations given in parentheses next to molecular weights show the method of detection of the polypeptides. i.e. CB: Coomassie blue staining; Lab: ^{35}S-methionine labeling). nd: no difference or not detected. UK: unknown. Mol. wt. marked by asterics indicate polypeptides which showed the most prominant changes. ?: questionable.

Mol. Wt.	Fraction				
	Sol.	Mic.	PM	TP	Ex/In
28 (CB)	nd	+	nd	nd	uk
29 (lab)	+	nd	nd	nd	uk
34 (CB)	nd	+	nd	nd	uk
37 (lab)*	nd	nd	+	+	Ext
43 (lab)**	nd	nd	+	+	Int
45 (CB)**	nd	+	nd	nd	uk
49 (lab)*	nd	nd	+	+	Ext
52 (lab)	nd	nd	-	-	Ext
54 (lab)	nd	nd	-	-	Ext
70 (CB)	nd	nd	nd	-	uk
71 (lab)	nd	nd	-	-	uk
81 (lab)*	nd	+	nd	nd	uk
85 (lab)	+	nd	nd	nd	?

Earlier, Glass and Dunlop (1979) suggested that K^+ uptake is regulated kinetically rather than thermodynamically. Thus changes in pmf associated with pH changes might reduce influx only if the pmf were insufficient to drive K^+ influx. Nevertheless, if a K^+/H^+ cotransport system is in operation, it would be anticipated that under some conditions (e.g. at external pH=8) the pmf may be insufficient to drive K^+. Based on these observations and hypotheses, Kochian et al. (1989) speculated that the high affinity K^+ transport system in corn roots might be a K^+-ATPase, similar to that of *E.coli*. The higher plant plasma membrane H^+-ATPase shows 56-60 % similarity to that of protozoans and the fungal H^+-ATPases (see Serrano, 1989). Based on the significant similarities found between (E-P) ATPases, the latter author has depicted a tentative evolutionary tree for the whole family of (E-P) ATPases. If there are such similarities, it might be expected that at least some homology would occur between bacterial and higher plant systems. However, the lack of cross reaction of antibodies of the Kdp system to the barley root membrane proteins observed in the present study indicates these two systems are not closely related.

Another possibility for the K^+ uptake system in barley roots is a channel. Among the various types of ion transporters, channels represent the lowest number per cell. Typically, channels function at high external $[K^+]$ (see section 1.3.5). Using corn protoplasts, Ketchum et al. (1989) have demonstrated that the K^+ channel blocker TEA (tetraethylammonium) is effective in inhibiting outward K^+ rectified current and the inward time-dependent K^+ current. Kochian et al. (1990), using corn roots, also demonstrated that only the linear low affinity K^+ transport system (system II) in corn roots could be inhibited by TEA. However, the existence of channels capable of mediating K^+ uptake from low external concentrations has been suggested by Heidrich and Schroeder (1989). For instance, membrane potentials in *Neurospora*, incubated in K^+-free solutions, have been

reported to be as low as -305 mV (Rodriguez-Navarro et al.,1986). The latter authors showed that addition of only 0.6 μM K$^+$ in the extracellular space would allow steady state uptake of K$^+$, mediated via passive transport through K$^+$ channels, assuming 100 mM K$^+$ in the cytoplasm. The operation of the proton pump is required to maintain the electrical potential difference. Therefore, passive K$^+$ uptake could occur from K$^+$ solutions in the micromolar concentration range so long as the electrical potential difference is sufficiently negative. However, in higher plants the operation of high affinity and low affinity K$^+$ uptake systems, in response to low and high external [K$^+$], respectively, can only work efficiently if there are K$^+$ channels which are closed in low K$^+$ and open in high K$^+$ solutions (Ketchum, 1989). There are indications from measurements of resistance (Bates et al.,1982; Higinbotham et al., 1964) and of potentials (Bates et al., 1982; Cheeseman and Hanson, 1979) that this is the case.

The expression of the 37 and 49 kDa polypeptides only at a later stage (at least 4 days of K$^+$-deprivation) may indicate that, they are expressed as a longer-term adaptation to lowering of K$^+$ availability.

Since the appearance of polypeptides related to K$^+$-deprivation (as detected by ^{35}S methionine labeling) required at least 12 - 18 h of K$^+$-deprivation, the increase of influx evident after the first hour in low-K$^+$ (Chapter 2) must be accounted for by responses which appear to precede the increased synthesis of these polypeptides. These may be considered as the fine control of the existing carriers; the initial response to an interruption of K$^+$ supply. Schaefer et al. (1975), in a study of the effects of various inhibitors of protein synthesis concluded that in barley roots the half-life of the putative K$^+$ pump responsible for delivering K$^+$ to the stele was 100 min. By contrast, the half-life for the carrier responsible for the absorption of K$^+$ from the external medium was much longer. This early observation is in accord with the present findings and may support the hypothesis

advanced many years ago (Glass, 1976) that K^+ uptake may be regulated allosterically by cytoplasmic $[K^+]$. This idea, once enthusiastically supported became unpopular when detailed analyses of cytoplasmic $[K^+]$ by compartmental analysis established that the $[K^+]$ of the cytoplasm remained essentially constant during deprivation (Leigh and Wyn Jones, 1984). Clearly, the continued transport of K^+ to the shoot following removal of exogenous K^+ (Hooymans, 1978) must inevitably reduce cytoplasmic $[K^+]$ in the short term, even though on a relatively long-term basis (steady state growth prior to EDX analyses and the several days pretreatment involved in compartmental analysis) the analyses appear to indicate constancy. Moreover, an important issue should be appreciated regarding feedback systems: it would seem impossible that constancy can be maintained in a particular compartment without feedback from that compartment. The exclusive role of the vacuole in controlling influx, by virtue of its much larger size, should not be accepted at face value.

VI. EFFECT OF LOW TEMPERATURE UPON K$^+$ TRANSPORT IN BARLEY

1. INTRODUCTION

Low temperature (in the range from 5 - 15°C) decreases the rates of cellular processes (as indicated by their Q_{10} values in this temperature range) including ion transport, which require energy in the form of ATP (Raven and Smith, 1978; Lyon, 1973; White et al., 1987; Petraglia and Poole, 1980). However, plants possess the capacity to adjust to low temperatures (Hale and Orcutt 1987 and references therein). The adaptive changes to chilling and freezing temperatures, both in terms of winter survival and metabolic efficiency are termed acclimation, acclimatization or hardening (Levitt, 1980). For example, it is documented that exposure of plants to low but above freezing temperatures (e.g. 5°C) for extended periods increases the capacity for ion uptake (Clarkson, 1976; Raven and Smith, 1978; Sanders, 1981; Deane-Drummond and Glass, 1983; Siddiqi et al., 1984; White et. al., 1987 and Gunvor et al.,1989). In the case of K$^+$, acclimation to low temperature increased root [K$^+$] concurrent with an elevated K$^+$ influx (Clarkson et al., 1974; Clarkson, 1976; Deane-Drummond and Glass, 1983). Siddiqi et al. (1984) suggested that low temperature had altered the gain between the negative feedback signal of root [K$^+$] and K$^+$ influx (Cram, 1976; Siddiqi et.al., 1984). Later, White et. al. (1987) proposed that K$^+$ influx into rye seedlings exposed to low root temperatures is regulated by the increased demand placed on the root system by a proportionally larger shoot and that acclimation of K$^+$ influx to low temperature may be a result of an increased hydraulic conductivity of the root system. However, although temperature differentials between shoot and root may intensify such responses (Clarkson, 1976; Siddiqi et.al., 1984), this increase is believed to be elicited by root temperatures per se (Siddiqi et al.1984); barley

plants maintained with root and shoot temperature at 5°C still had increased K^+ influx and elevated root $[K^+]$.

The process of acclimation to low temperature is gradual. When plants are first exposed to a lower temperaure, ion fluxes are significantly lowered compared to those measured at higher temperature (Q_{10} ~ 2 or more). The first indication of the adjustment to low temperature, in terms of increased ion fluxes occurs after ~ 6h of continuous exposure to low temperature, with full acclimation occurring by ~ 3 days (Nordin, 1977; Siddiqi et al., 1984). In studying P_i uptake by several wild plant species, Chapin (1974a) derived a parameter which he referred to as "acclimation potential" defined as the ratio of the V_{max} for P_i uptake by roots acclimated at 5°C to the V_{max} for P_i uptake by roots acclimated at 20°C; fluxes being measured at a standard temperature. A high acclimation potential is characteristic of a situation where large compensatory changes in the rates of absorption have occurred in response to changes in the temperature at which the plant was grown (Chapin, 1974a).

It is now believed that these responses are a result of several factors. These may include changes in membrane fluidity due to alterations in lipid composition (Yoshida and Uemura, 1984; Levitt 1980 and references therein; Lynch and Steponkus. 1987). These in turn may cause conformational changes of membrane proteins (Caldwell and Whitman 1987, Lynch et al. 1987; Quinn, 1988). Such changes are considered to occur after relatively short exposures (~ 6 h) to low temperature. Long-term acclimation (over a period of days) has been suggested to result from increased synthesis of specific transport proteins (Clarkson 1976, Siddiqi et al.,1984), by membrane augmentation (Singh et al., 1975) and by changes in the quantitative nature of the feedback effects of accumulated ions (Sidiqi et al., 1984). Membrane augmentation is defined as the increase of lipid

and protein content in the membrane component on a DNA or unit cell basis (Singh et al., 1975).

The molecular mechanisms whereby acclimation to low temperature is achieved are not well understood. However, evidence indicates that membranes, primarily the plasma membrane, are closely involved in cold acclimation as well as in freezing injury (Graham and Patterson and references therein, 1982; Siminovitch et al., 1975; Levitt, 1980; Palta et al., 1982; Steponkus et al., 1983; Quinn, 1988). Guy et al. (1985) have reported a rapid increase of several mRNA populations in spinach leaves after 2 days exposure to 5°C. They proposed that these particular mRNA's may encode for proteins that are directly involved in the development of increased freezing tolerance. Hahn and Walbot (1989) have shown that four polypeptides (molecular weights of 95, 75, 25 and 21 kDa) are induced in rice leaves during cold acclimation. Uemura and Yoshida (1983) and Yoshida (1984) also proposed that changes in protein composition as well as phospholipid enrichment of the plasma membrane may play a dominant role in the development of cold hardiness.

Although considerable attention has been directed to the lipid component of membranes during acclimation to low temperatures (Willemot, 1979; see Li and Sakai, 1982 and references therein), less information is available regarding the changes in membrane protein composition (Yoshida and Uemura, 1984; Uemura and Yoshida, 1984) and their activities (Hellegren et al., 1983; Jian et al., 1982; Dupont and Mudd 1985, White, 1987). Jian et al. (1982), using cytochemical methods reported that plasma membrane bound H^+-translocating ATPase activity in plant cells increased during cold acclimation. In these studies, plants were grown under physiologically abnormal conditions (*Vigna radiata* grown in 1 mM $CaSO_4$ in the dark, Yoshida et al., 1986) or under poorly controlled conditions in the field (Yoshida and Yemura, 1984) or by means of cell cultures

(DuPont and Mudd, 1985). Although plants grown under field conditions are suitable for studying the general changes associated with the hardening process, to study the regulation of ion transport it is more appropriate to use intact plants grown under controlled environmental conditions.

In this chapter the changes observed in growth rates, K^+ transport (influx and efflux) and accumulation, acclimation potentials of K^+ fluxes, protein composition and membrane ATPase activities resulting from exposure of barley roots to differential temperature are described. A review by Clarkson et al. (1988) provides a comprehensive introduction to the studies available related to this topic.

2. MATERIALS AND METHODS

2.1. Plant material and growth condition

Seeds were germinated according to the methods described in Chapter 2. After 3 - 4 days of germination, seedlings were transferred to hydroponic tanks containing 0.01 strength Johnson's solutions containing 100 μM K^+ for a further 3-6 days in controlled environment rooms. Air and solutions temperatures were maintained at 15°C (abbreviated to 15/15°C) or at 20°C (20/20°C). Subsequently, seedlings to be subjected to low temperature treatments were transferred to hydroponic growth tanks containing solutions having the same composition. These solutions were maintained at 5°C, while the air temperature was maintained either at 15°C or at 20°C. (These treatments will be referred to later as differential temperature treatments: DT-seedlings, while those grown at 15/15°C or 20/20°C will be referred to as HT-seedlings). Tanks contained 45 l of nutrient solution, which were cooled in the low temperature tanks by immersing stainless steel cooling coils in the mixing chambers of the hydroponic tanks. Water containing antifreeze was circulated in the coils, pumped by a cooling water bath. (Forma refrigerated bath. model 2325). Times of transfer to low-temperature conditions were staggered in order to obtain seedlings at the same chronological age but differing in the durations of exposure to low-temperature. After these treatments, seedlings were used for growth studies, tissue [K^+] determinations, K^+ (^{86}Rb) influx and for membrane isolation. The age of seedlings at harvest was between 10-14 days.

2.2 Growth studies

Fresh weights and dry weights of seedling shoots and roots were recorded at different time intervals as shown in the results section. Relative growth rates were calculated using equation 1 (see Noggle and Fritz, 1983).

$$\text{Relative Growth Rate (RGR)} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1} \qquad (1)$$

where,

W_2 = weight at time 2

W_1 = weight at time 1

t_2 = time 2

t_1 = time 1

2.3 K^+ influx and tissue K^+ concentrations

The methods used for measurements of K^+ influx and tissues $[K^+]$ were identical to those described in Chapter 2. Influx was measured at high (20^oC) and low (5^oC) temperatures for both high and low-temperature-grown plants. Acclimation potentials (AP) were calculated as the ratio of influx at 20^oC of DT-grown plants : influx at 20^oC of HT-grown plants. Temperature coefficients (Q_{10}) which represent the degree of temperature sensitivity of the metabolic reactions were calculated according to equation 2.

$$Q_{10} = \left(\frac{\text{Rate at } t_2}{\text{Rate at } t_1} \right)^{\left(\frac{10}{t_2 - t_1} \right)} \qquad (2)$$

t_1 and t_2 = two temperatures at which the measurements were carried out.

2.4 K^+ efflux determination by compartmental analysis

Determination of half lives of ^{86}Rb efflux to unlabeled growth solutions (0.01X Johnson's) were carried out using the methods for ^{86}Rb efflux from roots labeled with ^{86}RbCl according to Memon et al. (1985). Briefly, seedlings grown at HT or DT for 7, 14 and 21 days were preloaded with ^{86}Rb (0.5 μCi/l) for 18 h at their growth temperatures. These were then transferred to efflux solutions (1 L), (composition and temperature identical to growth and loading solutions except that radiolabel was absent) and 1 ml samples were collected from the external medium every 2 min until the first 10 min followed by sampling at 15, 30, 60, 90, 120, 180, 240 and 300 min. Sampling at each time interval was carried out in duplicate. After 2.5 h from the time of transfer to efflux media, concentrated nutrient stock solutions was added to the solutions to avoid significant depletion of nutrients. Data were analysed using the computer program "EFFANP" used by Memon et al. (1985).

2.5 ^{35}S-Methionine labeling

The methods used were similar to those described in Chapter 5 except that the temperature of the labeling solutions and their inorganic composition were identical to those in which the plants had been grown. Labeling was carried out for a 12 h period. Solutions were replaced after the first 6 h to prevent significant depletion of inorganic nutrients. After this 12 h labeling period, roots were washed in cold distilled water to remove isotope from the Water Free Space. They were then blotted dry with paper towels, weighed and used for isolation of membranes.

2.6 Isolation of membrane fractions

Membrane fractions were isolated as described in Chapter 3 section 2.3.2.

2.7. Protein determination and ATPase assay

ATPase activity and membrane protein levels were determined as described in Chapter 3.

2.8 Electrophoresis, western blotting and autoradiography

Electrophoresis, blotting and autoradiography were as described in Chapter 3.

3 RESULTS AND DISCUSSION

3.1 Effect of differential temperatures on shoot and root growth

Generally, shoot and root growth rates decreased when roots were grown at $5^{o}C$ (Table 14). At the end of the first 7 day period root dry weights were found to be 32% of controls maintained at $15^{o}C$ while no differences were observed in shoot dry weights. The lack of reduction of shoot growth during the first week was unexpected. During the 2nd week, shoot and root dry weights of DT plants decreased by 40 % and 45 % of the controls, respectively. However, during the third week, shoots of DT plants showed a greater reduction (50 % of the control) in dry weights than roots of DT plants (37 % of the control). Lowering of root growth was not unexpected; as Clarkson et al. (1988) have stated "the most evident plant response to temperature is a change in growth rate". Fennel et al. (1990) reported that in spinach, low-root temperature decreased the xylem water potential regardless of shoot temperature. Differential temperatures ($20/5^{o}C$) evoked these differences within the first 6 h of treatment, while low temperature ($5/5^{o}C$) caused only a small change during this period (Fennel et al., 1990). This reduction in xylem water potential may be caused by several factors. Clarkson (1976) has suggested that the hydraulic conductivity of roots exposed to low temperature is lower than that of the controls. Reduction in hydraulic conductivity may also have decreased the apoplastic transfer of nutrients from root parenchyma to stele. Kleinendorst and Brouwer (1970) and Watts (1971) have also demonstrated that reduced water uptake at low temperature is correlated with reduced growth rate.

Lowering of water and nutrient transport would be expected to affect the shoot growth rates by affecting metabolic activities (Carey and Berry, 1978). However, under such unfavourable conditions plants may lessen the dependence on external supplies (Clarkson, 1988) and maximize the utilization of internal resources; young seedlings could use seed reserves while older plants might

compensate for the lack of availability of resources by internal redistribution of nutrients and by lowering of metabolic activities.

The relative growth rates of shoots of DT seedlings were typically lower than those of HT seedlings (Table 15). The highest relative growth rate (an order of magnitude higher) was shown by shoots of HT seedlings during the second week of growth while those of DT seedlings showed the lowest relative growth rate. This implies that the reduction of root temperature has imposed growth limitation during this initial time period. Roots showed similar trends but the magnitude of differences between HT and DT seedlings were smaller than those of shoots; roots of the HT seedlings had relative growth rates which were 3X those of DT seedlings. However, during the third week, shoots of DT seedlings appeared to show some measure of acclimation and grew more rapidly; their relative growth rates (0.03 d^{-1}) were only 57% lower than those of shoots of HT seedlings (0.071 d^{-1}). By contrast to the shoots, roots of DT seedlings showed a higher (2X) relative growth rate than those of HT seedlings during week 3.

These observations demonstrate that the initial slow growth rates exhibited by roots of seedlings exposed to low temperature may soon be compensated for by subsequent rapid growth rates. Such compensation may be a result of allocation of more biomass to the root system under such growth conditions. This agrees well with Clarkson's statement " The physiological and morphological changes observed in the root system ensure that root function is not the limiting factor for survival and growth of plants" (Clarkson et al., 1988).

During the study period, the shoot:root dry weight ratio dropped in the DT seedlings while that of HT seedlings increased with time (Table 16). This supports the findings of Macduff et al. (1987) who reported an increase of shoot dry matter with increasing temperature. Usually, shoot/root ratios decrease when plants are transferred to a cooler environment (Berry and Raison 1981 and

references therein). I have previously reported such changes in growth rates and root:shoot weight ratios (De Silva 1984). Wild plants growing in cooler temperatures tend to have a larger root system (Chapin, 1974). Partitioning of dry matter in favour of root growth can be seen as an acclimatory or adaptive response; the increase in relative root size may compensate, in part, for the decrease in the rate of nutrient uptake per unit root mass observed in short-term measurements (Clarkson, 1988). However, interpreting the increased dry weights of root systems should be done with caution since it has been shown that in chilled plants accumulation of reserve carbohydrates also contributes to the increased dry weight (Ap Rees et al., 1982). Although, for many plants (e.g. rape, barley, oat etc.) increases in shoot/root ratios have been reported with increasing temperature, in some plants (e.g. ryegrass) shoot/root ratios decrease with increasing temperature (Clarkson, 1986; Cumbus and Nye, 1982).

The most apparent changes associated with the first few days of exposure to low temperature in DT seedlings were in the morphology of the root system. DT seedlings had shorter, thicker roots than those of HT seedlings and had no root hairs. It has been reported that exposure to low temperature for several days is necessary for acclimatory changes to occur in cells or in organisms (Siddiqi et al., 1984; Clarkson, 1988). Some of these changes occur at the membrane level (Quinn, 1988 and references therein), while others involve changes in relative size and morphology, indicating modifications at the whole plant level (Clarkson, 1988. and references therein; MacDuff et al., 1987).

Table 14 Changes in dry weight (mg) ± SE per plant

Control (15°C) and low-temperature (5°C) grown seedlings. Seedlings were at the higher temperature during the first 3 days of germination. Aerial temperature for both treatment and control was 15°C.

Age	5°C		15°C	
Days	Root	Shoot	Root	Shoot
7	7.4±.28	30.9±1.5	11.0±.86	31.2±.64
14	8.1±.13	32.7±2.2	15.2±.82	55.2±1.7
21	12.1±.41	40.1±1.8	19.3±1.3	90.8±2.4

Table 15 Changes in shoot and root relative growth rates (d^{-1}) in relation to low root temperature.

Seedlings were grown at control (15/15°C) and differential temperatures (15/5°C). All seedlings were at the higher temperature (15/15°C) during the first 3 days of germination. The seedlings to be treated were then exposed to DT conditions. Aerial temperature for both treatment and control was 15°C. All the values had standard errors below 10% of the mean.

Age	Shoot		Root	
	5°C	15°C	5°C	15°C
7-14	0.008	0.081	0.013	0.046
14-21	0.030	0.071	0.057	0.034

Table 16 Shoot/root ratios of seedlings grown at HT and DT.

Seedlings were at the higher temperature during the first 3 days of germination after which they were transferred to either 15°C (Control) or to low-temperature (5°C) tanks. Aerial temperature for both treatment and control were 15°C.

Age	5°C	15°C
7	4.2	2.8
14	4.0	3.6
21	3.3	4.7

The physiological and morphological changes observed in the root system appear to compensate for reduced root function in such a way as to minimize the deleterious effects of low temperature (Clarkson et al. 1988). The acclimation of uptake and transport of nutrients (being one of the major functions of the root system) can be assumed to be an important component of acclimation of the root system. Several hypotheses have been advanced regarding the mechanisms by which plants acclimate to low temperature. However, detailed information is unavailable.

The next section will focus on changes in rates of K^+ uptake and accumulation in barley as a response to low temperature.

3.2 Effect of differential temperatures on K^+ fluxes and tissue [K^+]

3.2.1 K^+ influx

K^+ (^{86}Rb) influx measured at 5°C and 20°C increased with time of exposure to differential temperature (Fig 31A). These results are slightly different from those of Raven and Smith (1978) and White et al. (1987). Raven and Smith (1978) found that in *Chara*, Cl⁻ influx was at least two fold lower at 5°C than at 15°C in the first 24 h after the temperature was changed, but after acclimation for 96 h rates of influx recovered to control values. White et al. (1987) reported an initial lowering of influx in roots of rye seedlings within the first 2-3 days of exposure to low temperature which was restored to control values during the next few days. The differences observed in these two species may be the result of genotypic variations in the ability to acclimate to low temperature. Chabot and Billings (1972) reported that the degree of acclimation is genetically controlled and therefore, as Chapin (1974) reported, different species may have different acclimation potentials (AP). Table 17 reports the AP values for K^+ uptake of DT seedlings. These were calculated as the ratio of influx of DT seedlings at 20°C (or

5°C) : influx of HT seedlings measured at 20°C (or 5°C). The AP values calculated for the rye seedllings from data from White et al. (1987) are also given in Table 17. Barley had higher values for acclimation potential than rye. Since barley used in the present study was a winter variety, this cultivar may have a better ability to acclimate to low temperature. Usually, the $[K^+]$ of the roots of seedlings grown at low temperature were equal to or even higher than those of HT seedlings (Fig 31B). Under such conditions the increased influx reveals an alteration of the negative feedback by $[K^+]_{int}$ on its influx, as suggested by Siddiqi et al. (1984).

The temperature sensitivity remained unchanged during prolonged exposure to low temperature except for the first day (Q_{10} = 2.7). Many years ago, Hoagland et al. (1926) also observed phenomena similar to those described in the previous paragraph. They found that when *Nitella* was cooled to 10°C during the first 6 h, Br^- uptake showed a high temperature sensitivity (Q_{10} =3.5). However, extended exposure to low temperature decreased the value for Q_{10} indicating a lowered sensitivity to low temperature resulting from acclimation. Similar behavior for roots of barley and rye (Clarkson, 1976; White et al., 1987) has been reported. Likewise, the pattern of K^+ influx of plants exposed to differential temperatures observed in the present study is in accord with those discussed above and support the general conclusion reached by Siddiqi et al. (1984), and Macduff et al. (1987), that full adjustment of ion fluxes to a change in root temperature requires 3-4 days for completion.
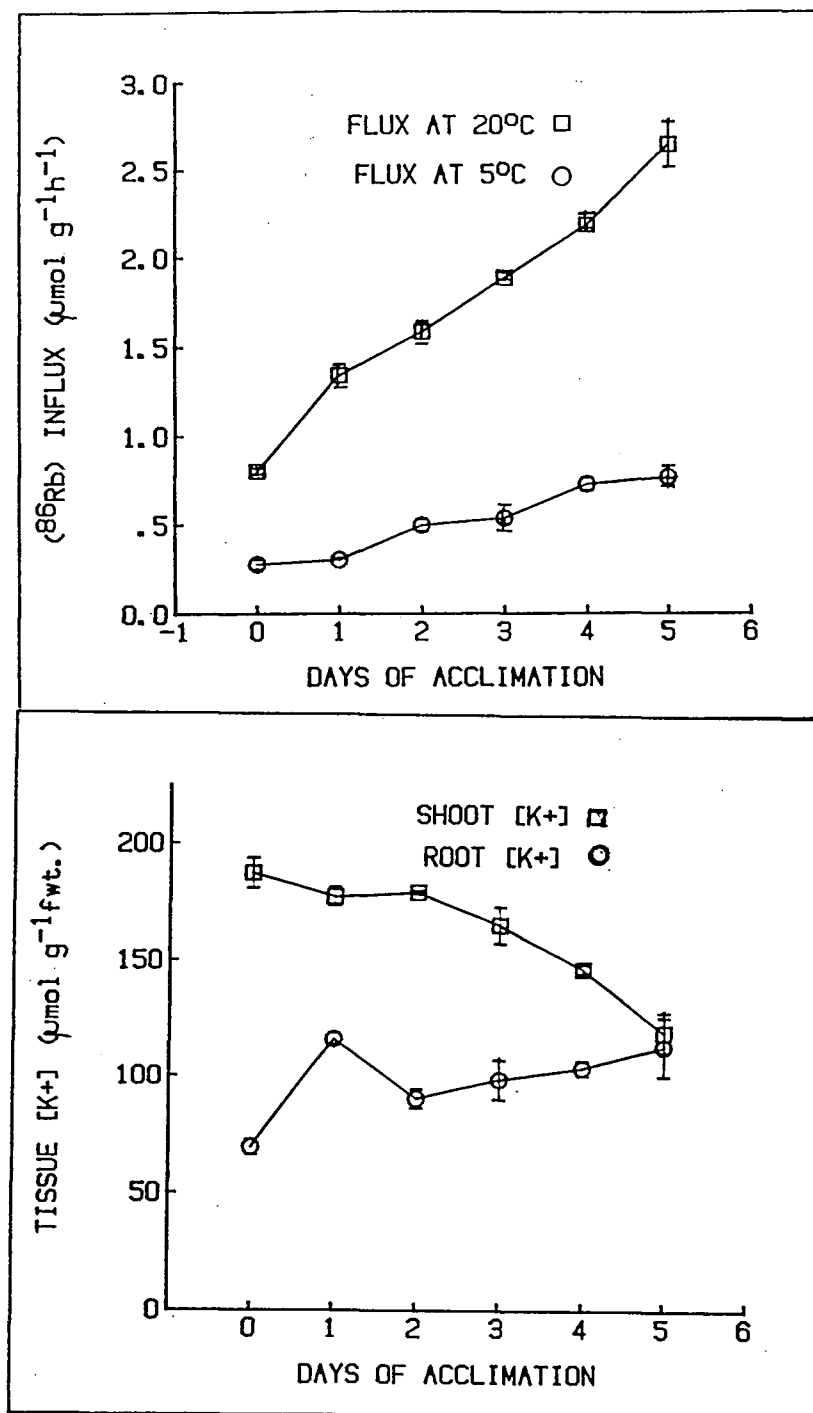
Fig 31   The effect of duration of DT upon subsequent K^+ *(* ^{86}Rb *) influx (A) and tissue [*K^+*] (B).*

K^+ (^{86}Rb) influx (A) at 20°C and 5°C and tissue [K^+] of roots and shoots (B) of seedlings, grown under control (20/20°C) (0 days) and differential (20/5°C) (1-6 days) temperature conditions.

Table 17 Acclimation potentials (AP) and Q_{10} values

AP were calculated as the ratio of influx of DT seedlings at 20°C (or at 5°C) : influx of HT seedlings at 20°C (or at 5°C). The ratio of fluxes measured at 5°C was also determined. Both of the ratios therefore represent perturbation fluxes for the DT and HT-grown seedlings respectively. AP calculated for rye using data of White et al. (1987) are given in parentheses. Q_{10} values were calculated according to equation 2 given in the methods section (section 6.2.3)

Days of Acclimation	Acclimation Potentials		Q_{10}
	5°C	20°C	
0	--	----	2.0
1	1.09	1.66 (0.50)	2.7
2	1.78	1.99 (0.59)	2.2
3	1.93	2.36 (0.91)	2.3
4	2.61	2.73 (0.96)	2.1
5	2.76	3.30 (1.04)	2.1

3.2.2. Compartmental analysis

The results of compartmental analysis performed according to Memon et al. (1985b) are shown in Table 18. It is interesting to note that the vacuolar half lives ($t_{1/2}$) of the HT seedlings measured within 3 weeks, increased (70% in week 2 and 44% during week 3), while that of DT seedlings increased only during week 2 (35%) and decreased (27%) during the third week to a value equivalent to that shown in the first week. Increasing vacuolar $t_{1/2}$ indicates a slower turnover of vacuolar K^+. Therefore, efflux from the vacuole appears to be reduced in HT roots indicating retention of more vacuolar K^+. By contrast, DT roots adjusted tonoplast K^+ fluxes in order to maintain cytoplasmic $[K^+]$ constant. Cytoplasmic $t_{1/2}$ values of HT seedlings gradually decreased while those of DT seedlings increased with prolonged exposure to low temperature. This phenomenon also indicates a slower turnover of cytoplasm K^+ of DT seedlings. Therefore, the $t_{1/2}$ values for both vacuole and cytoplasm favour the maintenance of constant cytoplasmic $[K^+]$. This strategy seems to play an important role in the process of acclimation of K^+ transport and accumulation to low temperature. Cell wall $t_{1/2}$ values which represent the exchange of ions within the cell wall matrix, showed low rates in the both HT and DT-seedlings (ion transport through the cell wall usually take place by diffussion and bulk flow). As expected, HT seedlings had lower $t_{1/2}$ values for cell wall exchange than DT seedlings, indicating faster exchange of ions in this compartment.

3.2.3 Tissue $[K^+]$ concentrations

HT shoots and roots demonstrated their highest relative growth rates (0.081 d^{-1} for shoot; 0.046 d^{-1} for root) in week 2 (Table 15). K^+ was accumulated at its highest rate during this period; 56% increase in shoots and 40% increase in roots compared to shoot and root $[K^+]$ in the previous week (Table 19). DT plants, by

contrast, showed lower RGR for both shoots and roots (0.008 d^{-1} for shoot and 0.013 d^{-1} for root), with shoot showing 36% increased K^+ accumulation and roots showing 71% increased K^+ accumulation during week 2. This appears to indicate that the slower growth rates of DT shoots has lowered the demand for K^+, resulting in the accumulation of most of the absorbed K^+ in the roots. However, it may also be due to an initial lowering of the translocation of K^+ to shoots (De Silva, 1984) by low root temperature. Increments of K^+ accumulated during the third week were similar in shoots and roots of DT-grown plants, despite a 2X higher relative growth rate shown by these roots during this period. Ion uptake in general has been found to be highly correlated with both shoot relative growth rate (Pitman, 1972) and shoot/root ratio (Jeschke, 1984; Cheeseman & Wickens, 1986). The RGR and K^+ accumulation pattern in 3 week old plants grown at DT agree well the results of Pitman and Cram (1975).

During week 3, shoots and roots of these plants showed increased relative growth rates compared to those of week 2. However, the shoot/root ratios decreased with extended exposure to DT-conditions. Therefore, results of the present work contradict those of Jeschke (1984) and Cheeseman & Wickens (1986). In spite of the very high RGR shown by HT shoot (0.071 d^{-1}), there was no significant increase in the shoot [K^+] during week 3.

The influence of temperature on nutrient accumulation, especially in long-term acclimation, appears to correlate better with its effect on plant growth than its direct effect on the root influx parameters (see White et al., 1987).

Clarkson (1976) and Sanders (1981) have shown that following several days of growth at low temperature there is increased transport capacity which is especially apparent when these plants are returned to high temperatures.

Table 18 Half-lives ($t_{1/2}$) ± S.E. for K$^+$ (^{86}Rb) exchange in three compartments of roots as functions of the duration of exposure to low temperature.

Compartmental analysis of control (15°C) and DT (5°C) barley seedlings. Efflux of K$^+$ to the external media was determined from the loss of ^{86}Rb from the seedlings (preloaded with ^{86}Rb) to the incubation media. Efflux was determined at the same temperatures as the plants had been exposed to during the previous growth periods. Units for vacuolar $t_{1/2}$ for are h, cytoplasm and cell wall $t_{1/2}$ are min. Results are the means of two independent experiments.

Compartment	Duration at low-T	Growth temperature	
	Days	15°C	5°C
Vacuole (h)	7	23.16 ± 0.53	24.30± 0.49
	14	39.06 ± 0.61	36.64± 1.37
	21	56.94 ± 3.10	26.86± 1.97
Cytoplasm (min)	7	31.66 ± 9.67	23.17± 1.34
	14	30.62 ± 0.61	25.67± 1.37
	21	23.96 ± 0.31	27.14± 0.85
Cell wall (min)	7	1.39 ± 0.36	1.68± 0.39
	14	0.29 ± 0.04	1.01± 0.38
	21	0.39 ± 0.15	1.38± 0.14

Considering the time course of the changes of K^+ influx associated with low temperature, Siddiqi et al.(1984) suggested the operation of two mechanisms. They proposed that long-term adaptations may be achieved by an increase in the number of transporters and by alteration in the quantitative basis of the feedback between cellular K^+ and influx. The second hypothesis indicates an alteration of an allosteric type of control of K^+ influx by tissue $[K^+]$. It has been reported that in animal cells, temperature affects the strength of binding of activators and inhibitors which are important in the allosteric control of many enzymes of glycolysis and the TCA cycle (Horwitz et al. 1979). White et al. (1987) have suggested that this would only be important at limiting external concentrations. However, $[K^+]$ concentrations in the growth media used by Siddiqi et al. (1984) and in the present study are far from limiting. Both differential growth temperature and lowering of K^+ supply in the growth media increased K^+ uptake capacity. Therefore, it may be speculated that a single mechanism operates under those conditions. However, unless proper comparisons of the membrane composition and their activities from plants exposed to these two types of stresses are performed, a hypothesis can not be put forward.

In the following section, the effect of exposure to low temperature on protein content, polypeptide profiles, plasma membrane H^+-ATPase activities and ^{35}S-methionine labeling patterns will be discussed.

Table 19 Changes in tissue [K$^+$] ± SE as a function of the duration of exposure to low-temperature

Shoot and root [K$^+$] (μmol. g^{-1} fwt) of seedlings of control (15/15°C) and those exposed to differential-temperature (5/15°C) for 7, 14 and 21 days.

Age	shoot		Root	
Days	5°C	15°C	5°C	15°C
7	53.14 ±2.39	115.5 ±7.55	44.9 ±2.39	67.1 ±3.59
14	71.5 ±6.40	180.8 ±2.17	77.1 ±2.88	93.6 ±6.47
21	105.9 ±3.29	174.7 ±5.35	102.8 ±3.29	75.4 ±2.61

3.3 Effect of differential temperature on protein content, H^+-ATPase activities and membrane polypeptide profiles

The topic of acclimation to low temperature by higher plants species has been extensively reviewed by several authors (Clarkson, 1988; Graham and Patterson, 1982). However, studies available on qualitative and quantitative changes in proteins in relation to low temperature are sparse.

Graham and Petterson (1982) have reviewed the work available on the changes of proteins and metabolic activities in relation to low temperature. It is believed that the characteristic spectrum of proteins produced by a particular organ is often altered at temperatures close to or below the limit for growth, and that this may be the general tendency among different organisms outside the range of temperature to which they can usually adapt. For instance, in plants there is a general increase in soluble proteins at low temperature. However, in these reports, except for the changes in disulphide bonds (Levitt, 1980) the type of alterations in structure or activity shown by membrane proteins during cold acclimation is not clear.

3.3.1 Changes in root protein content in relation to low temperature

Soluble and microsomal protein content increased by 65% and 25%, respectively, during 3 days of exposure to low temperature, indicating an increase in total protein per unit fresh weight (Table 20). These results agree with those of Gusta and Weiser (1972) and Siminovitch et. al.(1968), who reported an increase in RNA (mainly rRNA), and in soluble and membrane bound proteins in the frost resistant Korean boxwood in response to low temperature exposure. Increases of soluble proteins during cold acclimation appear to be a common observation (Kacperska-Palacz, 1978). However, Rosinger et. al. (1984), reported a lowering of the soluble protein content in leaves of pea, tomato, french bean and Mung bean. The extent of this lowering of protein content appeared to depend on the chilling

sensitivity of each species. However, these same authors demonstrated that the reduction of protein content was caused by water stress since it was not observed when the relative humidity was increased to 100%. In the present work barley seedlings were grown at 75% RH in hydroponic solutions. Under these conditions it is unlikely that our plants experienced water stress and therefore, our results may be considered similar to those obtained for plants grown at 100% RH in soil.

The amount of protein in plasma membrane preparations form barley roots expressed on a fresh weight basis, agrees well with values reported for tobacco callus by Sze (1980), for Jeruselum artichoke tuber by Ishikawa (1985) and for *Vigna* hypocotyls by Yoshida et al. (1986). While the microsomal protein content of DT plants failed to show significant differences from those of controls (Table 21), plasma membrane protein content declined with longer exposure to low temperature. By contrast endomembrane fractions (tonoplast- and golgi- enriched) revealed an increased protein content with extended exposure to low temperature. Similar results have been reported for membrane fractions obtained from rye (White et al., unpublished data in Clarkson, 1988). The latter authors also reported that tonoplast preparations experienced the highest increase in protein content. Plasma membrane fractions in the present work showed much lower quantities. This may be due to the fact that these plasma membranes were purer due to sequential fractionation by two different protocols. On the other hand, the tonoplast + golgi-enriched fraction in the present work may not be as clean as the tonoplast fraction of White et al. (unpublished data in Clarkson, 1988).

3.3.2 *Plasma membrane H^+-ATPase activities of DT-grown roots.*

Vanadate sensitive, Mg^{2+},K^+ ATPase activities of plasma membrane fractions derived from roots of HT and DT seedlings are given in Table 22. The specific activities observed for controls are in the range of those reported by

Gallerger and Leonard (1982) for corn root cells and DuPont and Mudd (1985) for tomato cell cultures. The stimulation of activity by K^+ was relatively low (25 % in HT and 3 days DT seedlings and 17 % in 6 days DT plants). Uemura and Yoshida (1985) also demonstrated that K^+ stimulation of ATPase in winter rye was quite low. Vanadate inhibition of these activities was in the range of 50-60%, which agrees well with values reported by the other authors (Dupont and Hurkman, 1985; Poole et al., 1984).

It is interesting to note that basal activities, which may represent the activity of non-specific phosphatases or of Ca^{2+} stimulated ATPase activities (Caldwell and Haug, 1980), also decreased initially and increased with prolonged exposure to low temperature. The latter authors demonstrated that, unlike other plants, barley ATP-hydrolysing activity was stimulated by millimolar concentrations of Ca^{2+}. More recently Dupont and Hurkman (1985) suggested that the Ca^{2+} stimulated activity was due to contamination of the plasma membrane by Ca^{2+}-phosphatases which tended to adhere to the plasma membrane fraction even after washing with KCl or EDTA. The increased level of non-specific activities may also reflect an increased breakdown of phospholipids which are known to increase in plant membranes as a response to low temperature, by phospholiphase and lipooxygenase activities (Yoshida, 1984). In calculating specific activities, basal activities (Mg^{2+},-K^+) were subtracted from all other activities to correct for inorganic phosphates released as a result of above mentioned causes.

An overall decline of ATPase activities of DT seedlings was observed during the first 3 days of exposure to low temperature (Table 20). Vanadate sensitive H^+-ATPase activity had declined by 28% of control values during the first 3 days of exposure to low temperature.

Table 20   Effect of growth temperature upon total and soluble protein contents.

Total protein content was obtained by summing values for the 1K pellet (cell wall & debris), the 10K pellet (nuclei & mitochondria), the 80K supernatant (soluble proteins) and the 80K pellet (microsomes). The age of low temperature acclimated seedlings is the same as that of controls. After growing at 20°C initially, low temperature treatment was given for the indicated time periods.

Growth Temperature. ^{o}C	Duration HT + LT days	Protein content Soluble mg.g^{-1}fwt.	Total
20	11 + 0	09.2±.08	11.04
20/5	11 + 3	14.7±.20	16.70
20/5	06 + 5	14.1±.55	16.07

*Table 21 Effect of growth temperature upon protein content of various membrane
fractions.*

Tonoplast + golgi fractions (15-34% interphase) were separated from plasma
membrane-enriched fractions using a 15-34-45% sucrose step gradient. The 34-45%
interphase (plasma membrane-enriched fraction) was further purified on a
PEG/DEX two phase system. Mic: Microsomal fractions, TG: Tonoplast + golgi
fractions, PM: plasma membrane fractions EM: membrane partitioned into Dextran
lower phase of the phase sysytem. SE for all the fractions were below 10% of the
mean. (nd; not detected).

Growth temperature °C	Duration HT + LT Days	Protein content Mic.	TG	PM	EM
			mg.g^{-1}.fwt.		
20/20	11 + 0	0.516	0.136	0.021	0.33
20/5	10 + 1	0.523	0.107	0.026	0.27
20/5	9 + 2	0.403	0.145	0.022	0.36
20/5	8 + 3	0.496	0.144	0.015	nd
20/5	7 + 4	0.510	0.138	0.016	0.26

Table 22   Vanadate sensitive H^+-ATPase activities

H^+-ATPase specific activities (μmol.mg^{-1} protein.h^{-1}) $\pm$ SE (within parentheses) in plasma membranes obtained from roots of control (20°C) and acclimated seedlings (5°C) for the indicated time periods. H^+-ATPase activity, vanadate inhibition and K$^+$ stimulation were calculated after subtracting the basal (non-specific) activities. Values are from a representative experiment, and are the means of triplicate determinations.

	Duration at low temperature (Days)		
Treatment	0	3	6
-Mg^{2+}, -K$^+$	4.7±1.6	10.8±0.5	39.5±2.9
+Mg^{2+},	26.7±3.7	18.2±1.2	63.8±0.86
+Mg^{2+},+K$^+$	31.0±0.33	20.7±0.36	68.7±0.01
+Mg^{2+},+K$^+$+Van	23.6±0.34	15.3±3.0	50.2±0.02
H^+-ATPase	7.45	5.34	18.43
% K$^+$ stimul.	25	25	17
% Van inhib.	48	54	63

This was not unexpected since there is a general tendency of plants to lower their metabolic activities as a response to low temperature exposure. However, with prolonged exposure to low temperature (a further 3 days), H^+-ATPase activity has dramatically increased (147% of HT values). It is noteworthy that, vanadate sensitive H^+-ATPase activity of the plasma membrane has increased inspite of the decline of total protein content in plasma membrane (Table 21). Therefore, H^+-ATPases seem to account for a greater amount of the plasma membrane proteins in plants exposed to low temperature for prolonged time periods. This result agrees well with observed quantitative increases of H^+-ATPase shown by immunological techniques (see below).

The initial lowering of specific activities may well indicate a lowering of the amount of H^+-ATPase enzyme in these membrane preparations. Decrease of carrier protein can occur by inactivation of existing carriers due to physical changes in the membranes (e.g. phase transition) and/or lowering of the synthesis and incorporation of new carriers. The dramatic increase of H^+-ATPase activities during prolonged exposure to DT, may indicate the extent of acclimation. The pattern of K^+ influx with time did not agree with that of the ATPase activity, the latter initially showed a decrease (Table 22) while the influx showed a steady increase after transferring to low temperature (Fig 31A). Therefore, the results reported in the present study agree well with the suggestions of Clarkson et al. (1988) that, increased transport of solutes may not be the result of increased ATPase activity. As suggested by Siddiqi et al. (1984) the observed changes may reflect alterations in the negative feedback mechanism. The increased influx may, therefore, be due to alterations in the "fine control" of the existing machinery in the cell which may be followed in the long term by "coarse control" adjustments. i.e. synthesis of carriers (Raven, 1984).

Figure 32 shows Western blots of plasma membrane polypeptides obtained from 11 days old barley roots, incubated with the antibodies raised against plasma membranes of corn roots. Equal amounts of protein (70 μg) was loaded on to each lane. The cross reactivity of plasma membranes obtained from DT-grown roots was significantly greater than that obtained from HT-grown roots. This clearly indicates an increased amount of H^+-ATPase in the plasma membrane, and agrees well with the above reported *in vitro* ATPase activities (Table 19).

It is interesting to note that the increased H^+-ATPase activity in response to low temperature acclimation was not observed in seedlings grown in low-K^+ (Chapter 4), although both showed increased K^+ uptake rates. It may be concluded that DT seedlings have achieved the same increased solute uptake using a different mechanism(s). The acclimative mechanism(s) for response to low-K^+ conditions must be quite specific for K^+. By contrast, acclimation to low temperature must involve more general responses of the transport machinery.

Findings of the present study agree well with those reported by other workers. Increased H^+-ATPases activity in isolated membrane fractions from plants exposed to low temperature has been reported previously. Dupont and Mudd (1985) reported that the tonoplast of tomato cells can adapt to low temperature and maintain higher rates of proton transport than those of unadapted cells. They suggested that the increase in initial rate of proton transport by the tonoplast may reflect alterations in the enzyme itself. Also, Hellegran et al. (1983) reported an increase in the specific activity of Mg^{2+}-ATPase activity in plasma membrane fractions prepared from cold-hardened pine needles. Using cytochemical studies, Jing et al. (1982) demonstrated increased plasma membrane ATPase activity in cold-hardened wheat seedlings. The latter authors also suggested that the adaptive changes in ATPase activity associated with a particular membrane may be responsible for the development of frost resistance in winter wheat. A very

informative analysis of temperature dependent factors influencing nutrient uptake at different levels of organization is given by Clarkson et al. (1988 and references therein). The latter author stated that, acclimatory changes occur on extended exposure of a cell or an organism to a reduced growth temperature. Some of these changes occur at the membrane level and are related to lipid composition and modulation of carrier activity. Others involve changes in the relative size and sometimes the morphology of the root system. These processes lessen the temperature dependence of ion transport and ensure that the intake of nutrients does not limit growth at low temperatures. These acclimatory changes are seen as part of the general process of regulation of nutrient uptake.

The present findings, along with those of others, demonstrate that the time scale of alterations in membranes varies among species (see Ougham and Howarth, 1988 and references therein), indicating an involvement of regulation of the adaptive mechanisms at the gene level caused by signals from the environmental factors.
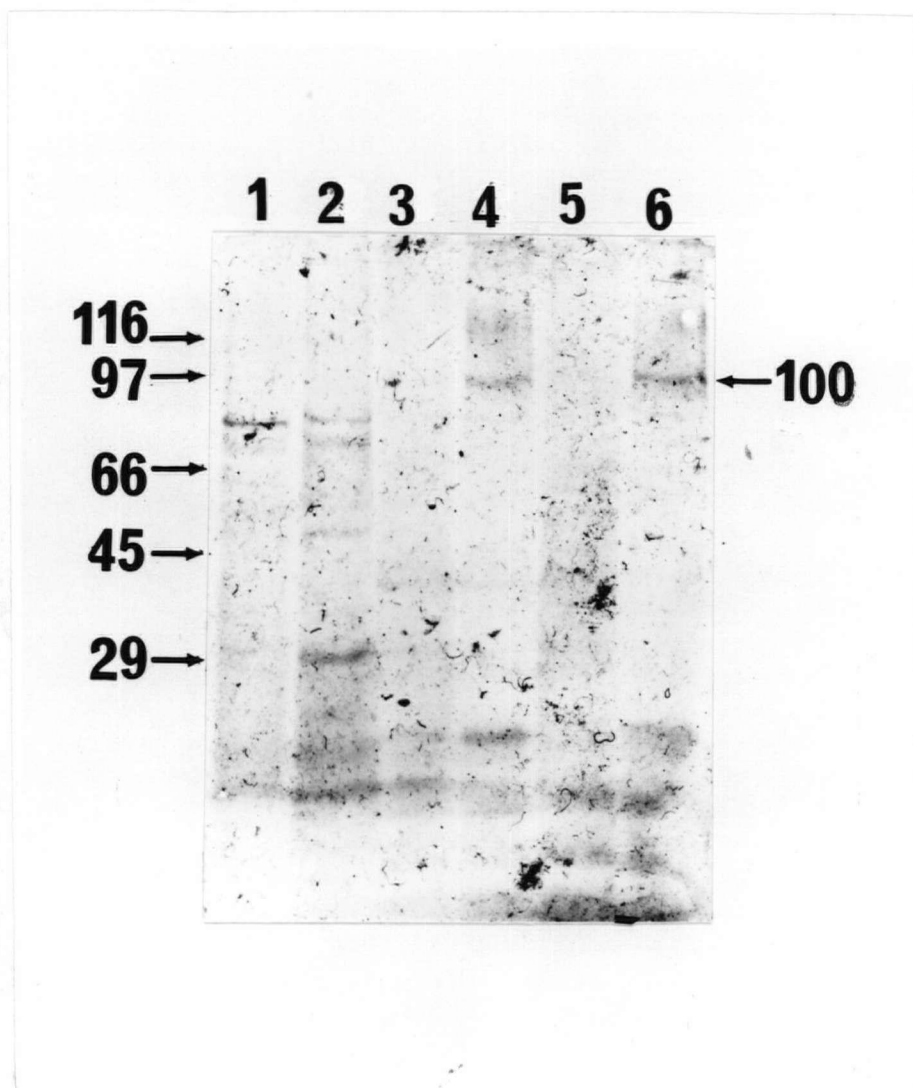
Fig 32. Western blots of microsomal proteins obtained from 11 days old barley roots grown under DT and HT conditions..

Blots incubated with the antibodies raised against plasma membranes of corn roots. Equal amounts of protein (70 μg) were loaded onto each lane. Lanes 1, 3 and 5 are from seedlings grown at HT (control). Lanes 2, 4 and 6 are from those grown at DT. Proteins in 1K pellet (Lane 1 and 2), those of unwashed microsomes (lanes 3 and 4) and washed microsomes (lanes 5 and 6). Masses of molecular weight markers are shown at right.

VII PERSPECTIVES

It is now almost 50 years since Van Den Honert first proposed the existence of carrier-like molecules responsible for the absorption of inorganic phosphate by sugar cane roots. Yet, despite spectacular progress in defining the nature of the proton translocating ATPase of the plasma membrane and tonoplast, we are woefully ignorant about the molecules responsible for ion absorption by higher plant roots. I am unaware of any other system in higher plants which has been biochemically defined. Thus, the present study represents the first biochemical work on the potassium transport system.

The tedium involved in isolation and biochemical characterization of various types of membranes and the lack of a suitable system (for example, K^+ transport mutants for higher plants) appears to have hindered studies of the K^+ uptake system at the molecular level. In this study, the well known increase of K^+ influx which results when K^+ is withheld for periods of up to 3 or more days was used to investigate the K^+ uptake mechanism.

Results obtained from kinetic studies in the present work agree well with those of other workers. Typically, lowering of K^+ supply resulted in lowering of root and shoot $[K^+]$ and in an increase in K^+ uptake rate. This trend could be reversed by resupplying K^+ to the growth media. Increase of K^+ influx in K^+ deprived roots after time periods as short as 30 min appeared to be too rapid to be attributed to the synthesis and incorporation of new membrane proteins. This conclusion was supported by the cycloheximide study which established that K^+ influx increased significantly during K^+ deprivation even in the presence of cycloheximide. Therefore, the initial increase in uptake rates may involve factors other than synthesis of membrane polypeptides, for example allosteric regulation (Glass, 1976).

Since there was no unique protocol for isolation and characterization of plant plasma membranes, this study involved an extensive survey and use of available techniques for isolation and characterization of membranes from seedlings grown under different experimental conditions. Fractionation of microsomal membranes on sucrose step gradient was found to be the most effective technique to use in studies of this sort where several treatments had to be handled simultaneously. Aqueous two polymer phase partitioning, although yielding pure plasma membrane enriched fractions, appears to be useful to provide only confirmatory evidence.

In vitro assays revealed that withholding K^+ for several days significantly reduced total protein content and ATPase activity (expressed on a per mg protein basis) of microsomal preparations from barley roots. Vanadate-sensitive ATPase activity was somewhat greater in low-K^+ seedlings. Purified plasma membrane preparations showed less marked differences. There was certainly no indication that the level of vanadate sensitive ATPase activity had increased during K^+ deprivation. The Western blots of microsomal and plasma membrane proteins probed with the antibodies (raised against the 100 kDa polypeptide of corn root plasma membrane ATPase) confirmed this conclusion. Direct measurement of H^+ fluxes associated with K^+ uptake in intact roots have revealed that K^+ deprivation causes the H^+ to K^+ ratio to decline from 1 to 0.5 in barley roots (Glass and Siddiqi, 1982). Serrano (1989) has reported that in yeast the level of this enzyme is tightly regulated and not increased even under nutrient stress. Therefore, the findings of the present study along with those of others (Glass and Siddiqi, 1982; Serrano, 1989), appear to provide evidence against any model which represent dependence of high affinity K^+ uptake system exclusively on H^+ translocating ATPase activity. Therefore, only the models shown in Figure 1c, 1e and 1f may represent the putative high affinity K^+ uptake system in barley roots.

The lack of cross reactivity of barley membranes with antibodies raised against the Kdp ATPase of *E.coli* indicates that barley membranes do not have a K^+ ATPase homologous with the bacterial enzyme. However, based only on this finding, the existence of a K^+ ATPase similar in function but evolutionarily different in structure from that of *E. coli* cannot be ruled out.

The appearance and disappearance of a Coomassie blue-stainable, 45 kDa polypeptide (highly specific for K^+), in the microsomes in parallel with the lowering or resupply, respectively, of K^+ was quite intriguing. Since the 45 kDa polypeptide was not found in the soluble protein fraction it is considered to be membrane associated. Its loss in the presence of 1 mM EDTA in the resuspension buffers indicates that divalent cations are required for its association with the membrane. It may be that the divalent cations act as a bridge between the 45 kDa polypeptide and the microsomal membranes. The characteristic features of this polypeptide may be compared to an amphitropic (Burn, 1988) protein or to an "apocarrier" (Skulachev, 1977) in *E. coli*. This polypeptide appears to be very labile, especially under *in vitro* conditions. Dissociated from its native membrane, it appears to be highly succeptible to proteolytic degradation. Therefore further efforts to determine the location and the role of this polypeptide were unsuccessful.

^{35}S-methionine labeling studies revealed that K^+ deprivation caused synthesis of several novel polypeptides, most notably a 43 kDa polypeptide, integral in nature, in plasma membrane and tonoplast. This polypeptide has properties consistent with a role in K^+ transport. Synthesis of this polypeptide occurs most rapidly during the early stages of K^+ deprivation (within 12 h of removing K^+). When compared with the results of the cycloheximide experiments, it is possible to assume the synthesis of the 43 kDa polypeptide may have been initiated as early as 1 to 2 h after the onset of K^+ deprivation. However, the methods of detection (even using radiolabeling) may not be sensitive enough to detect the very small

amounts of the polypeptides synthesized at this time. The most appropriate approach therefore will be to investigate changes in mRNA populations within the first hours of K^+ deprivation or to employ the techniques of subtractive hybridization with a view to isolating the cDNA clone responsible for this polypeptide.

Regardless of the nature of the environmental stress (K^+ deprivation or low temperature), the plant ultimately acclimates so that the deleterious effects of the imposed stress are compensated. The adaptive mechanism(s) for response to low-K^+ conditions must be quite specific for K^+. By contrast, adaptation to low temperature must involve more general responses of the transport machinery.

We are currently intent on purification of the 43 kDa polypeptide in sufficient quantity to allow for sequencing and oligonucleotide synthesis. Ammonium sulfate fractionation has revealed that the protein is concentrated in the 60-70% fraction. This indicates the more hydrophobic nature of this polypeptide. This polypeptide was eluted with 150 mM KCl from a DEAE Sephadex ion exchange column chromatography (J. Mehroke, unpublished data).

Direct proof of a role for this polypeptide in K^+ transport will await reconstitution experiments in which the protein is incorporated into liposomes. These studies are for the future.

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