Electrogenic Proton/L-Glutamate Symport in Isolated *Asparagus sprengeri* Mesophyll Cells

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Abstract

Medium alkalinization occurred upon the addition of L-Glu to mechanically isolated *Asparagus sprengeri* mesophyll cells suspended in 1 mM $CaSO_4$. Alkalinization resulted from the coupled entry of H^+ and L-Glu anion into the cells. This H^+/L-Glu symport did not stimulate K^+ efflux. K^+ efflux has been observed during H^+/amino acid symport in other systems. The stimulation of K^+ efflux by proton coupled symport is regarded as an indicator of a plasma membrane depolarizing electrogenic symport process. H^+/L-Glu symport in *Asparagus sprengeri* mesophyll cells was investigated to determine whether or not the process was electrogenic.

The rate of uptake of 0.25 µM ^{3}H-MTPP$^+$ (Methyltriphenylphosphonium, methyl-^{3}H) is a probe for monitoring changes in the membrane potential. ^{3}H-MTPP$^+$ uptake was reduced by K^+ or CCCP, agents known to depolarize the membrane potential. Uptake of ^{3}H-MTPP$^+$ was also inhibited by L-Glu but not by D-Glu. Conversely, 10 mM external MTPP$^+$ inhibited the uptake of ^{14}C-U-L-Glu. Simultaneous measurements of the rates of ^{14}C-U-L-Glu uptake and L-Glu dependent H^+ influx showed that the molar stoichiometry of H^+/L-Glu symport was 2 to 1.

K^+ or Na^+ stimulated H^+ efflux was completely inhibited by DCCD, DES, oligomycin and antimycin reagents which inhibit ATP driven H^+ efflux. The H^+ efflux was also stimulated by the weak acids, butyric acid and acetic acid, which are known to acidify the cytoplasm. This weak acid stimulated H^+ efflux was also completely inhibited by oligomycin. It was calculated that net L-Glu dependent H^+ influx increased by 100% in the presence of oligomycin and that despite net medium alkalinization H^+/L-Glu symport stimulates ATP dependent H^+ efflux.

The data presented in this study indicate that H^+/L-Glu symport is electrogenic. The data also show that ATP dependent H^+ efflux rather than K^+ efflux is the process compensating for this electrogenic H^+/L-Glu symport.

Introduction

Application of amino acids to plant cells often causes alkalinization of the external medium (Robinson and Beevers, 1981, Wyse and Komor, 1984). This alkalinization is due to coupled H^+ and amino acid symport into the cell. Work with plasma membrane vesicles provided *in vitro* evidence for the existence of this H^+/amino acid symport system (Bush, 1989). The driving force for the uptake and accumulation of amino acids is thought to be a proton electrochemical gradient or proton motive force (pmf) across the plasma membrane of the cell (Poole, 1978). This gradient is believed to be generated by the activity of an outwardly directed H^+-ATPase located in the plasma membrane (Spanswick, 1981, Sze, 1985). The gradient facilitates the reentry of H^+. Reentry of H^+ down this gradient is coupled to and drives the uptake of amino acids. The H^+/amino acid symport process appears to be mediated by a plasma membrane protein carrier (Reinhold and Kaplan, 1984). The H^+/amino acid symport process is thought to be electrogenic involving the movement of positive charge into the cells. This suggestion is supported by depolarization of the membrane potential on the uptake of amino acids (Kinraide and Etherton, 1980), by charge compensating K^+ efflux upon addition of amino acids to the medium (Wyse and Komor, 1984) and by calculations indicating transport of net charge from the stoichiometry of the H^+/amino acid symport process (Kinraide, 1984).

Work with *Asparagus sprengeric* mesophyll cells by McCutchon et al (1987, 1988) showed medium alkalinization upon addition of L-glutamic acid (L-Glu) to the cell suspension medium. This alkalinization response was specific for L-Glu, transient, saturable and inhibited by CCCP and high pH values. A specific H^+/L-Glu symport system located in the plasma membrane of these cells was proposed. It was also found that L-Glu stimulated CO_2 evolution, indicating that L-Glu was

rapidly metabolized inside the cells. Further work demonstrated that ^{14}C-GABA released to the medium was the product of ^{14}C-U-L-Glu decarboxylation and L-Glu decarboxylase activity was identified in these cells. However, the H^+/L-Glu symport process did not stimulate K^+ efflux. This result suggested an electroneutral symport process (1 H^+/L-Glu) rather than an electrogenic process (2 or more H^+/L-Glu).

This study attempts to determine whether H^+/L-Glu symport into *Asparagus sprengeric* mesophyll cells is an electrogenic process. Predictions for an electrogenic process are (1) a transient depolarization of the membrane potential; (2) 2 or more protons coupled to every anion of L-Glu entering the cells; (3) charge compensation in the form of K^+ efflux or H^+ efflux. Experimental procedures to test the these predictions included:

(1) Monitoring changes in the membrane potential upon addition of L-Glu to the suspension medium using a lipophilic cation technique;

(2) Simultaneous measurements of the rates of ^{14}C-U-L-Glu uptake, ^{14}C-GABA release and L-Glu dependent H^+ influx to calculate the molar stoichiometry of H^+/L-Glu symport;

(3) Simultaneous measurements of H^+ and K^+ fluxes during L-Glu transport to see whether K^+ efflux occurs in response to H^+/L-Glu symport;

(4) Investigations into ATP dependent H^+ efflux stimulated by K^+ or Na^+ or weak acids;

(5) Investigations into ATP dependent H^+ efflux during H^+/L-Glu symport.

Literature Review

A. Plasma Membrane H^+ Translocating ATPase

1. Introduction

ATPases are a universal feature of living cells. They facilitate H^+, K^+, Na^+ and Ca^{2+} movement across biological membranes to mediate the transduction of chemical energy into osmotic and electrical energy. The ATPases found to date can be divided into three major categories —" P ", " V ", and " F " type (Pedersen & Carafoli, 1987). This classification is based mainly on:

(a) the reaction mechanism of the ATPase

(b) the location of the ATPase

(b) the structure of the ATPase.

ATPases of the " P " type are found in the plasma membrane of eukaryotic cells and catalyse Na^+/K^+, Ca^{2+} or H^+ transport. In the catalytic reaction cycle, there is a covalent phosphorylated intermediate. They are defined as P-type ATPase to indicate this phosphorylation. ATPases of the " V " type are defined as those located in the membrane of organelles other than the mitochondria, chloroplasts and the endoplasmic or sarcoplasmic reticulums. They are found in the vacuolar tonoplast membrane of higher plant cells, Neurospora and yeast cells (Rudnich, 1986). ATPases of the " F " type are defined as those consisting of F_0 and F_1 moieties. They are found in bacteria, chloroplasts and mitochondria. F_1 is a water soluble moiety involved in catalytic activity and F_0 is a moiety involved in H^+ translocation.

Whereas the P and V types couple ion movement to ATP hydrolysis, the F type use downhill H^+ movement to drive ATP synthesis.

The focus of this section will be the plasma membrane H^+ translocating ATPase found in fungi and plant cells. This ATPase was originally identified by in *vivo* studies on electrical potential and pH measurements within intact cells and studies on active transport. The ATPase activity was later characterized in isolated plasma membrane vesicle (Hodges, 1972, and Sze, 1980). Subsequently, the molecular properties of this ATPase are demonstrated in purified preparation of enzyme solubilized with detergents (O' Neill and Spanswick, 1984).

2. The Plasma Membrane H^+ Translocating ATPase —— in *Vivo* Studies

Evidence for the plasma membrane ATPase was derived from the hyperpolariztion of the plasma membrane beyond the potassium diffusion potential. This hyperpolarization has been observed in fungi (*Neurospora crassa* (Slayman, 1965)), algae (*Acetabularia mediteranea* Satter, 1970), and higher plants (*Beta ualgaris* Mercier and Poole, 1980). Hope (1965) observed a large hyperpolarization in *Chara. australis* on addition of bicarbonate and suggested an active electrogenic pump for anions, HCO_3^- or Cl^-, into the cell. Poole(1966) has also shown that bicarbonate caused a hyperpolarization in beet tissue. However, the work of Spanswick(1970) demonstrated that substitution of other buffers having the same pH as the bicarbonate solution produced a similar hyperpolarization in *Nitella. translucers* and the addition of HCO_3^- at constant pH had no effect on the membrane potential. He concluded that the effect of HCO_3^- on ion transport was due to its ability to control the pH of the external solution. Meanwhile, the relationship between the external pH and the membrane potential was investigated in *Nitella clavata* showing the strong dependence of the membrane potential on

external pH in the range 4-7 (Kitasato, 1968). It was postulated that the effect of external pH indicated a high permeability to H^+ and the large passive influx of H^+ was compensated for by an active electrogenic efflux. An electrogenic H^+ pump located in the plasma membrane of *Nitella* was hypothesized by Kitasato (1968). However, Spanswick(1972) offered another explanation of the effect of external pH on the membrane potential in which the effect of external pH was on the pump electromotive force rather than the diffusion potential. Subsequently, Spanswick(1974) published the data which showed that there was no effect of 1mM ouabain, an inhibitor of the Na^+- K^+ pump (MacRobbie, 1962), and no immediate effect of Cl^--free solution on the membrane potential, indicating that the pump maintaining hyperpolarization (negative inside) was neither a Na^+-K^+ pump nor a Cl^- pump. A correlation between membrane hyperpolariztion and H^+ efflux has also been observed in higher plant tissues. Therefore, a electrogenic H^+ pump located in the plasma membrane of fungi and plant cells causes and maintains a hyperpolarization beyond the potassium diffusion potential.

The energy source for the electrogenic H^+ pump has been investigated. Many studies showed that the hyperpolarization was sensitive to respiratory or photosynthetic inhibitors. DNP, CCCP, and DCCD were found to depolarize membrane potential (Kitasato, 1968, and Spanswick, 1974), suggesting a dependence of the pump on energy metabolism and probably ATP as the energy source for the pump. Further work directly measured the relationship between the membrane potential and the cytoplasmic ATP level. The work with red beet (Mercier and Poole, 1980) showed that in a range from 40 to 60% of the control ATP level mediated by different concentrations of KCN, the membrane potential rose sharply with increase in ATP level. The ATP-dependent potential was about -120 to -130 mV and Km for ATP was about 10 to 30 uM ATP in *Nitellopsis obtusa* (Mimara et al,1983). Meanwhile, DCCD and vanadate were identified as inhibitors to this ATP driven

H^+ pump because they can induce a depolarization without causing a significant change of ATP level. These studies provide *in vivo* evidence for the electrogenic H^+ pump as an electrogenic H^+ translocating ATPase.

3. The Plasma Membrane H^+ Translocating ATPase —— *in Vitro* Studies

The evolution of techniques for the isolation and characterization of the plasma membrane from fungi and plant cells has provided insights for identifying and analyzing the ATP-dependent electrogenic H^+ pump at the biochemical level. Identification of the pump as an ATPase originated from the work of Hodges and colleagues (1972). The ATPase associated with the plasma membrane requires Mg^{2+} and is stimulated by monovalent ions (Hodges et al, 1972), and is specific for ATP as substrate (Briskin and Poole, 1983, Serrano and Peslona, 1985). The pH optimum of the ATPase is 6.5 to 7 (Leonard and Hotchkiss, 1976). The activity of the plasma membrane ATPase is completely inhibited by 100 to 300 μM of DCCD, which inhibits known enzymes involved in H^+ translocation such as the F_0F_1 ATPase of mitochondria (Linnett and Beechey, 1979) and the vacuolar ATPase (Sze, 1984). Thus, the ATPase associated with the plasma membrane has a H^+ translocating function, confirming the suggestion from the *vivo* studies. The plasma membrane ATPase is also inhibited by vanadate (Serrano et al, 1985, and Willsky, 1979) which suggests that there may be a covalent phosphorylated intermediate in the ATPase catalytic reaction cycle. Vanadate has been known to act on phosphate-hydrolysing enzymes forming this kind of intermediate (Serrano et al, 1985 , and Bowman et al, 1978).

Tightly sealed membrane vesicles serve as an ideal system for identifying the physiological role of the plasma membrane ATPase. Independent works by Rasi-Caldogno et al (1981) and Sze and Churchill (1981) showed that ATPase in plasma

membrane vesicle acts as a electrogenic pump. They detected a membrane electrical potential in plasma membrane vesicle generated specifically by Mg·ATP and reduced by DCCD and vanadate. The potential was found to be dissipated by CCCP, indicating that H^+ is the electrogenic ion (Sze and Churchill, 1981). Subsequently, a H^+ gradient across the membrane of vesicle generated by the ATPase was demonstrated by Hagen et al (1980), Benett and Spanswick (1983) and Churchill and Sze (1983). These studies provided in vitro evidence for the existence of an electrogenic H^+ translocating ATPase in plasma membrane of fungal and plant cells and its major physiological role in generating a H^+ electrochemical gradient across the plasma membrane.

The plasma membrane H^+ translocating ATPase purified from fungal(Malpastick and Serrano, 1980) and plant (Serrano, 1987) cells contains a major polypeptide of about 100 kdalton. This purified ATPase showed similar properties to ATPase in isolated plasma membrane with respect to pH optimum (6 to 7) (Bowman et al, 1981) , K_m for ATP (0.3 to 1.8 mM) (Bowman et al, 1981, Vara and Serrano, 1982, and Dufour and Goffeau, 1983), inhibition by vanadate, DCCD and DES, and a requirement for Mg^{2+} (Vara and Serrano, 1982). Kinetic studies showed that Mg·ATP was a substrate and free ATP was a weak inhibitor (Brooker and Slayman, 1983). Studies on the mechanism of the ATPase catalytic reaction provided evidence for the existence of two conformations of plasma membrane ATPase during its phosphorylated reaction cycle (Addison and Scarbrough, 1982, and Amorry et al, 1982).

The reconstitution of the purified plasma membrane H^+ translocating ATPase into liposomes provides a simplified model system to directly examine the physiological role of the plasma membrane ATPase. The system links the plasma membrane ATPase identified *in vitro* in isolated plasma membrane, to the electrogenic H^+ pump operating *in vivo* On the addition of Mg·ATP to the system

consisting of the purified ATPase incorporated into a phospholipid vesicle (reconstituted vesicle), a medium alkalization (Villalobo et al, 1981) and an intravesicular acidification were observed (Dufour et al, 1982, and Vara and Serrano, 1982). This acidification induced by Mg·ATP was reversed by weak bases, such as ammonia, and by H^+ ionophores, such as nigericin and uncouplers , 1799 and FCCP(Malpartila and Serrano. 1981, Vara and Serrano, 1982, and Dufour et al, 1982). Thus the ATPase reconstituted in liposomes has a function in generating a H^+ gradient. It was stimulated by the presence of permeable ions, such as K^+, and ionophores, such as valinomycin(Malpartila and Serrano. 1981, Vara and Serrano, 1982, and Dufour et al, 1982), demonstrating the electrogenic nature of H^+ movement. These studies complement and corroborate the *in vivo* studies.

4. Regulation of the plasma- membrane H^+-ATPase

The cyclic interconversion of enzymes between covalently modified and unmodified forms is a mechanism of regulation. Phosphorylation/ dephosphorylation is one of the most studied post translational mechanisms affecting enzymes. Fig1 shows that a phosphorylation/dephosphorylation regulating system involves the two distinct enzymes.

$$\underset{\text{(native)}}{E} + ATP \longrightarrow \underset{\text{(modified)}}{E\text{-}P_i} + ADP \qquad (1)$$

$$E\text{-}P_i + H_2O \longrightarrow E + P_i \qquad (2)$$

Fig1. Phosphorylation and dephosphorylation reactions.

Reaction 1 is catalyzed by protein kinase(s), and Reaction 2 is catalyzed by phosphoprotein phosphatase(s). For each complete cycle, one equivalent of ATP is hydrolyzed to ADP and P_i. The ATP consumption provides free energy to support such regulating mechanisms.

It has been known that the activity of the plasma membrane H^+-ATPase is stimulated by red light, blue light and hormones. But there is no convincing evidence for confirming that these external regulatory factors directly act on this enzyme. They may play the role of first messenger in a signal chain regulating system. This indicates that there are other regulating factors inside the cell directly or indirectly regulating the H^+-ATPase. The accumulated experimental data has shown that the likely intracellular regulatory factor is Ca^{2+}-stimulated protein kinase which phosporylates the ATPase.

4.1. Protein kinase and phosphatase activities associated with the plasma membrane

Protein kinase activity in plants has been found to occur in both particulate and soluble fractions. Work done by Salemath and Marme (1983) showed that some protein kinase activity was associated with a plasma membrane fraction obtained from dark grown zucchini (*Cucurbita pepo* L., cv. Senator) hypocotyl hooks. This membrane fraction was used as a source for protein kinase and substrate protein and phosphorylation of proteins was measured as ^{32}P incorporation. After phosphorylation, the substrate peptides were separated by SDS-PAGE and autoradiographed.There were at least 15 peptides (relative molecular weights: 14k, 17k, 18k, 26k, 34k, 36k, 45k, 50k, 55k, 60k, 63k, 70k, 80k, 100k and 180k) which were clearly detectable on the autoradiograph and which were all phosphorylated within the first 5 min and showed either no or only slight dephosphorylation. The

results indicate that protein kinase(s) is bound to membrane and may play a role in regulating the physiological functions of the proteins associated with the membrane. The phosphorylation of the M_r=100k polypeptide may have special physiological significance because this peptide is the same size as the plasma membrane H^+-ATPase. Thus, the activity of the plasma membrane H^+-ATPase could be mediated via a protein kinase. Further work with oat root done by Schaller and Sassoman (1988) showed that the M_r=100k phosphorylated polypeptide had the function of the ATPase. This result confirmed the identity of the phosphorylated oat root M_r=100k polypeptide as the ATPase. In order to identify the amino acids phosphorylated in this ATPase, phosphoamino-acid analysis was performed using thin-layer electrophoresis. Both phosphothreonine and phosphoserine were isolated from the M_r=100k phosphoprotein. It seems that plant plasma membrane H^+-ATPase are phosphorylated at multiple sites, threonine and serine residues. These sites may be associated with the regulation of the ATPase activity. Phosphorylated aspartate residues are known as catalytic intermediates involved in coupling ATP hydrolysis to proton transport. To distinguish between phosphoanhydride bond formation, in aspartyl carboxyphosphate, and phosphate ester bond formation, characteristic of protein kinase-mediated phosphorylation, the phosphorylated samples were treated with hydroxylamine. Hydroxylamine has been known to seletively remove phosphate in the anhydride bond. The results showed that hydroxylamine treatment did not change the phosphate labeling of H^+-ATPase (Bidwai and Takemoto, 1987), confirming that the phosphorylation was mediated via protein kinase and occured at the regulating sites of H^+-ATPase.

Protein phosphatase activity is required for a regulatory mechanism of protein phosphorylation and dephosphorylation. It promotes dephosphorylation of the phosphorylated protein and decreases activity. Dephosphorylation associated with the plasma membrane was detected in the work of Salimath and Marme(1983).

They showed one peptide (M_r=63k) became phosphorylated very quickly and it reached its maximum of ^{32}P incorporation after only 1 min, then it became rapidly and specifically dephosphorylated . This time course indicates the presence of a protein kinase and a phosphatase activity. But the amount of ^{32}P incorporated into the polypeptide of M_r100k (the probable H^+-ATPase) remained constant over the total incubation period of 20 min. The turnover of phosphorylation/ dephosphorylation may be smaller in the M_r100k polypeptide. The dephosphorylation of the phosphorylated plasma membrane proteins associated with the plasma membrane was clearly demonstrated by Ladror and Zielinski (1989). After incubation of the medium with $MgATP^{2-}$, ^{32}P- label membrane proteins were transfered to the same medium without ATP. Subsequently, the removal of ^{32}P-label from prelabeled membrane proteins was observed over 60 min. This confirmed that endogenous protein phosphatase exists in the plasma membrane.

4.2. Possible role of Ca^{2+} as second messenger

The activity of the plasma membrane H^+-ATPase in plants is known to be stimulateed by blue light. The time course of blue light stimulation showed that blue light-dependent hyperpolarization of the membrane potential peaked for several minutes after application of a blue light pulse (Assmann et al, 1986). This indicates that blue light functions as an activator, rather than a direct energy source for H^+-ATPase. It seems that there is a receptor for blue light in the plasma membrane, but the relationship between receptor and activation of the plasma membrane H^+-ATPase is not clear. Thus, the stimulation of H^+-ATPase by blue light occurs rapidly enough to indicate regulation by a covalent modification such as phosphorylation/ dephosphorylation. If blue light acts as a first messenger, it is

expected that there is a second messenger in the cytosol to activate phosphorylation. It has also been reported that blue light could stimulate Ca^{2+} channels, suggesting that the likely candidate of intracellular messenger is Ca^{2+}.

In animals, the phosphorylation of proteins via a c-AMP-dependent protein kinase plays a major role in the regulation of many biochemical and physiological processes. Cylic-AMP acts as an intracellular messenger. But free Ca^{2+} also stimulates protein kinases. Some of the Ca^{2+}-dependent protein kinases catalyze the same substrate proteins as the c-AMP-dependent protein kinases. It seems that Ca^{2+} plays a role in a signal chain of phosphorylation/dephosphorytion regulating system. In plants, although there has been convincing evidence for the existence of c-AMP, a stimulatory effect of c-AMP on protein phosphorylation has not yet been detected. In contrast, the accumulated experimental evidences indicate the regulatory effect of Ca^{2+} on plant protein phosphorylation.

The relationship between free Ca^{2+} concentration and phosphorylation of plasma membrane-bound polypeptides was investigated by Salimath and Marme(1983). The result showed that phosphorylation activity was strongly stimulated by Ca^{2+}. Phosphorylation of about 10 membrane-bound proteins, containing M_r100k protein, was enhanced by Ca^{2+}, being maximal in most case at a concentration of about 3 mM free Ca^{2+}. In the presence of 1mM calmodulin, the enhancement of protein phosphorylation by Ca^{2+} became apparent at 0.1 mM, was optimal at 0.3 mM free Ca^{2+} and stayed constant up to 1mM free Ca^{2+} . This indicates that regulation occurs at physiological Ca^{2+} concentrations between 0.1 and 1 mM. However, one peptide of M_r180k exhibits strong inhibition of ^{32}P incorporation at physiological Ca^{2+} concentrations. This may have resulted from an inhibition of a specific protein kinase or activation of a specific phosphoprotein phosphatase. Thus Ca^{2+} may not only have the function of stimulation but also the function of inhibition of protein phosphorylation. Studies on the effect of free-Ca^{2+}

concentration on phosphorylation of the M_r100k polypeptide, identified as H^+-ATPase, of oat plasma membrane vesicles showed that stimulation of phosphorylation occurred at less than 1 mM free Ca^{2+}, reaching a maximum at less than 7 mM (Schaller and Sussman, 1988). This data confirms that Ca^{2+} stimulates the phosphorylation of the plasma membrane H^+- ATPase.

The observation that Ca^+ stimulates the phosphorylation of H^+-ATPase indicates the stimulation of H^+-ATPase activity and ATP-dependent proton transport by Ca^{2+}. Direct evidence that intracellular Ca^{2+} regulates the activity of H^+-ATPase was provided by Lew (1989). In his work, ionophoretic injection of Ca^{2+} to increase intracellular levels demonstrated that intracellular Ca^{2+} stimulates electrogenic H^+ transport at the plasma membrane of *Neurospora* H^+-ATPase activity was measured by current-voltage analysis. The results showed that as intracellular Ca^{2+} was increased by ionophores. There was stimulation of electrogenic H^+ transport resulting in membrane potential hyperpolarlization and increased short circuit current. At higher Ca^{2+} levels (higher than 100 mM), H^+ transport was inhibited (Lew, 1989). The relationship between Ca^{2+} concentrations and the activity of H^+-ATPase in isolated plasma membrane was also investigated. Only the inhibition of the activity of H^+-ATPase by Ca^{2+} was observed although the Ca^{2+} concentration was lower (10^{-7} M) (Lew, 1989). This *in vitro* result did not correspond with that *in vivo* It may be that Ca^{2+}-dependent activation of H^+-ATPase activity *in vivo* requires a protein kinase or other cytosolic factors which are lost during isolation of the plasma membrane. Thus Ca^{2+} may regulate the plasma membrane H^+-ATPase as an intracellular messenger rather than a direct activator. The mechanism of the regulation of the plasma membrasne H^+-ATPase activity via a Ca^{2+}-stimulated protein kinase is not fully understood. Further work should identify and purify this kinase to investigate *in vitro* the relationships

between the kinase activity and H^+ -ATPase activity, ATP-dependent H^+ transport, Ca^{2+} concentration.

4.3. Effects of intracellular pH on a protein kinase mediating phosphorylation of the plasma membrane H^+-ATPase

Both auxin and fusicoccin have been known to stimulate H^+-ATPase activity. They have also been reported to induce rapid intracellular acidification (Brummer, 1985). In addition, artificially induced intracellular acidification has been found to activate H^+-ATPase (Hager and Moser, 1985). After the rapid acidification, a fusicoccin induced intracellular alkalinization occurs while the H^+-ATPase remains activated (Reid et al, 1985). It seems that intracellular acidification activates H^+-ATPase and then results in intracellular alkalinization. The stable activation is the result of the regulating system being sensitive to acidification. This indicates the intracellular acidification not only directly stimulates H^+-ATPase but also plays a role in the signal chain of regulating the H^+-ATPase. The mechanism of intracellular acidification-dependent regulation may involve the protein kinase activity. Phosphorylation of the plasma membrane H^+-ATPase showed a high pH-dependence: at pH value below 5. Above 7.5, there was little ^{32}P incorporation (Schller and Sussman, 1988). It was suggested that the plasma membrane-bound protein kinase has an acidic pH optimum.

Therefore, intracellular pH may serve as an intracellular messenger in a phosphorylation/dephosphorylation regulating system. It may regulate the plasma membrane H^+-ATPase by controlling protein kinase activity. To date, these is no convincing evidence demonstrating the direct effect of H^+ and Ca^{2+} on protein kinase. They may mediate the protein kinase by indirect mechanisms.

B. Plasma membrane potential

In 1961, Mitchell proposed that the energy-transducing intermediate in oxidative phosphorylation was not a chemical compound but a physical form of energy: an electrochemical proton gradient across energy-transducing membranes(Mitchell, 1961). This chemiosmotic theory indicates that ion pumps and currents play an important role in the energetic and regulatory properties of biological membranes. In the case of plants, electrophysiology has provided evidence for the existence of an electrogenic proton-pumping ATPase in the plasma membranes(see above section). Since the proton pumps directly contribute to the inside negative membrane potential, the membrane potential has become one of the important indicators of the physiological condition of plant cells. Therefore, studies on the measurement and regulation of membrane potential in plant cells has also become an important aspect of plant physiology.

1. Measurement of Membrane Potential

The membrane potential has been measured by three main methods, i.e. microelectrode insertion, passive uptake of a lipophilic cation and charged fluorescent probes. In large cells of plants, algae and fungi, the basic tool of electrophysiology is the microelectrode. In the small cells of higher plants, ion distribution is one method which may be suitable for potential measurements. Fluorescent techniques have been used in protoplasts and membrane vesicles.

1.1. Measurement of Membrane Potential by Microelectrode

The membrane potential, ΔE, is defined as the electric potential difference between two compartments separated by a membrane. The value of the membrane

potential is equal to the electric potential difference between two reference electrodes positioned in the compartments at the opposite sides of the membrane. Therefore, the microelectrode insertion technique can provide the most direct and conceptually simple way to measure the potential difference across the membranes.

The microelectrode method was applied to some plant cells such as beet root (Poole, 1966) but has been less successful in the cells of many other plants because microelectrodes are difficult to insert through the hard external walls of plant cells particularly small cells which are less than 10 to 15 µm in diameter. Electrode insertion may cause leaks around the inserted electrode (Serrano, 1985). This method was used to measure membrane potentials of some protoplasts i.e. tobacco protoplast. However, the reported membrane potential of protoplasts vary widely, ranging from expected negative values to positive values. Some results (Pantoja and Willmer, 1986) indicated that zero and positive values may be due to a lack of turgor pressure. When the cell wall is removed, osmotic rupture is prevented by maintaining the cell in high osmotism which result in a lack of turgor pressure. When suction pressure applied to the protoplasts was increased, the membrane potential became more negative. Therefore, measurements of the membrane potential of protoplasts using microelectrode method has problems because removal of the cell wall may change the potential.

1.2. Measurement of Membrane Potential by Lipophilic Cations

A membrane permeable ionic species, whose flow is not coupled to other processes , equilibrates with the membrane potential according to the Nernst equation:

$$\Delta E = \frac{R\,T}{n\,F} \ln \frac{[A^{n+}]_i}{[A^{n+}]_o} \qquad (1)$$

Where ΔE is the membrane potential, F is the Faraday constant, R is gas constant, T is temperature. A^{n+} is permeable cation.

Equation (1) shows that the calculation of ΔE is on the basis of the measurement of the equilibrium distribution of a permeable ion between inner and outer compartments.

The first employed permeable ions were potassium and rubidium in the presence of the specific ionophore valinomycin. However, the existence of physiological transport systems for these cations may cause deviation from the Nernst equation. In addition, valinomycin may be toxic, or ineffective in some systems because it may not cross the cell wall to reach the membrane. Lipophilic cations introduced by the group of Skulachev (Grinius et al. 1970) can obviate these difficulties. These lipophilic cations are not the substrates of cellular transport systems and do not require ionophores to permeate. They are also able to diffuse through phospholipid bilayers without the presence of a lipophilic anion due to their very dispersed positive charge (Grinius et al. 1970). Equilibration of the lipophilic cations such as $MTPP^+$ and TPP^+ across the cell membrane according to the Nerst equation has been used in bacerial, animal and plant cells to estimate the membrane potential.

The passive uptake of [^{3}H]tetraphenylphosphonium (^{3}H-TPP^+) was used to measure the membrane potential of *Chlorella vulgaris* cells and inside negative values were reported (Komor and Tanner, 1976). In their studies, ^{3}H-TPP was concentrated about 300 times over the external level of 15 mM during a 1 to 2

hours incubation period. The proton ionophores, dinitrophenol and FCCP (carbonylcyanide, *R* -trifuoromethyloxy-phenylhdrazone) and high external salt concentrations, 200 mM potassium citrate, both reduced the equilibration. Felle et al (1980) found that the membrane potential of giant E. coli cell calculated from the equilibration of ^{3}H-TPP^{+} compared favourly with that using intracellular microelectrodes under a variety of conditions. Similarly, work with two marine macroalge showed that the values of the membrane potential obtained by the two techniques were close (Reed and Collins , 1981). The results from studies on higher plant cells were also consistent with expectations. Komor et al (1981) demonstrated that a membrane potential of -60 mv at pH6.0 was obtained when sugarcane suspension cells were equilibrated with 5.1 mM MTPP^{+}. A membrane potential of about -62 mv for oat leaf protoplast was obtained using the equilibration of MTPP and was depolarized by azide, CCCP, decreased external pH and increased externl KCl concentrations.

However, the quantitative measurement of the lipophilic cation distributiuon has been faced with some experimental difficulties. These are the organellar accumulation and extracellular binding of lipophilic cations. A predominantly mitochondrial location of MTPP^{+} was reported for isolated hepatocytes (Hoek et al, 1980). Beardall and Raven (1981) also point out that TPP^{+} would be taken up by cell walls and into mitochondria and vacuoles. Failure to account for oranellar accumulation and extracellular binding wound result in overestimation of the membrane potential (Ritchie, 1984).

Although the equilibration of lipophilic cations for the calculation of the membrane potential has problems, the rate of uptake of lipophilic cations seems to be governed by the membrane potential. The presence of high external concentration of K^{+}, uncouplers or inhibitors which generally depolarizes the membrane potential also decreased in the uptake of lipophilic cations. This

indicates that the rate of lipophilic cation uptake can be employed in monitoring changes in the membrane potential during studies on the electrogenic nature of sustrate transport. Komor and Tanner (1976) used this technique to determine that H^+/hexose symport was electrogenic due to inhibition of TPP^+ uptake by this symport. The uptake of ^{3}H-MTPP was also applied to monitor changes in the membrane potential which was caused by the efflux of K^+ from cultured rose cells (Huesta and Murply, 1988).

1.3. Measurement of Membrane Potential by ΔE Dye (Fluorescent Probe)

Some useful indicators of ΔE are dyes which exhibit fluorescent changes after accumulation inside the cells or the membrane vesicles. This fluorescent change results from high concentrations of dye inside cells and is relative to the interaction between dye molecules and absorption of dye to the membrane. Thus dyes are permeant ions whose distribution across the membrane is according to the membrane potential and the fluorescent response of dyes is dependent on the partition of dye molecules between the cells and the medium (Hoffman et al. 1974, Sims et al. 1974). These dyes can be used as indicators of the membrane potential. The membrane potential measurement of protoplasts isolated from *Vgora mungo* hypocotyl using cyanine dye diS-C3-(5) was reported by Oka et al. (1987). In their studies, the relative fluorescence intensity of diS-C3-(5) solution with protoplasts increased with the external potassium concentrations. This results from diS-C3-(5) efflux which is stimulated by K^+ influx induced depolarization of the membrane. They also showed that the membrane potential of the protoplasts satisfies the Nernst equation for K^+ over the range of external K^+ concentration from 10 to 100 mM (Oka et al. 1987). Within this range, the fluorescence intensity linearly increaseed with the external K^+ concentrations, suggesting the

fluorescence intensity change can be used to calibrate the membrane potential of protoplasts.

The fluorescent probe method can be used to easily and quantitatively monitor the negative inside membrane potential and is more convenient than the method using radioactive lipophilic cations (Oka et al. 1987). But dye molecules taken up inside the vesicle will also bind to the inner membrane surface, further contributing to a decrease in the fluorescence. This phenomena may also contribute to changes in the distribution of permeable cationic dyes and therefore it is not possible to obtain absolute values of the membrane potential from the quenching of the fluorescence.

2. Origin of the Membrane Potential

The origin of the membrane potential has been one of the problem raised by electrophysiology. The contribution of differential ion diffusion processes to the membrane potential was established in the 1950s. However, the ion diffusion potential calculated from the Goldman equation did not agree with actual measurements of the membrane potential. This indicated that there was some other mechanism generating the membrane potential. Slayman (1965) proposed the existence of an electrogenic ion pump in the plasma membrane of *Neurospora*. It is now generally accepted that the membrane potential of plant cells is the sum of a passive ion diffusion potential and the potential generated by the electrogenic H^+ ATPase.

2.1. Diffusion Potential

If a permeable ion is maintained at different concentrations on both sides of membrane, a diffusion potential given by the Nernst equation is established (see this section, Equation 1). The diffusion potential from the Nernst equation corresponds to the potential exhibited by the membrane when the ion being considered is present alone and no other mechanisms of generating electrical potential are involved. However, there are different ions present in the same system and every one has a diffusion potential. This indicates that the membrane potential in a system containing many ions is dependent on the behavior of every ion. The higher the permeability of a given ion, the more the membrane potential will be close to the diffusion potential of this ion. The quantitative relationship between the membrane potential , the concentration and permeability of 3 different monovalent cations is given by Goldman equation.

$$\Delta E = 0.06 \log \frac{P_K [K^+]_o + P_{Na} [Na^+]_o + P_{Cl} [Cl^-]_i}{P_K [K^+]_i + P_{Na} [Na^+]_i + P_{Cl} [Cl^-]_o}$$

Where P_k, P_{Na}, and P_{Cl} are the permeability coefficients of the ions defined by the equation:

$$J = -P_A 2.3 \left(\frac{n\Delta E}{0.06}\right) \frac{[A^n]_o - [A^n]_i \, 10^{n\Delta E/0.06}}{1 - 10^{n\Delta E/0.06}}$$

Where J_A, is the net flux of the ion A^n (from outside to inside) (Serrano, 1985).

2.2. Potential Generated by An Electrogenic Proton-pumping ATPase

Evidence for the H^+-pumping ATPase is derived from membrane hyperpolarization beyond the potassium diffusion potential. The hyperpolarization is dependent on energy metabolism as demonstrated by the depolarizing effects of respiratory inhibitors. Under standard conditions 1mM azide or dinitrophenol which are respiratory inhibitors depolarized the membrane potentials of *Neurospora crassa* cells from near -200mv to about -30mv and at a maximal rate of 20 mv/second. The internal potential usually recovered within 10 minutes of the inhibitor being removed (Slayman, 1965). It seemed that an electrogenic pump, utilizing ATP, was located at the plasma membrane and pumped H^+ out resulting in membrane hyperpolarization beyond the diffusion potential. Mercier and Poole (1980) carried out experiments to demonstrate the relationships between the membrane potential, ATP level, proton efflux and cation uptake. At low ATP levels the potential rises sharply with increases in ATP, but above an ATP level of about 50% of the normal level the potential changes very little with ATP concentrations. This result confirms suggestions for the existence for a proton-pumping ATPase located in the plasma membrane and the ATP dependent membrane potential. The maximum ATP dependent membrane potential measured by the microelectrode method in tonoplast-free cells of *Nitellopsis obtusa* was about -120 to-130 mv (Mimura et al. 1983). A vanadate-sensitive H^+-translocating ATPase isolated from red beet plasma membrane has been solubilized in active form and successfully reconstituted into artificial proteoliposomes (O' Neill and Spanswick, 1984a). The characteristics of this ATPase such as substrates specificity, pH optimum, inhibitor sensitivity, and cation stimulation are similar to those of the ATPase associated with native plasma membranes (O' Neill and Spanswick, 1984b).

3. Regulation of Plasma Membrane Potential

Since the membrane potential consists of the diffusion potential and the potential generated by an electrogenic H^+-pumping ATPase, factors affecting ion diffusion and ATPase activity , i.e. ATP level inside the cell, metabolic inhibitors, K^+ , turgor pressure and pH, may regulate the membrane potential.

3.1. K^+

Potassium is more permeable than other ions so that the membrane potential is mainly dependent on the potassium diffusion potential. This means that passive K^+ movement directly contributes to the regulation of the membrane potential. Potassium influx causes the depolarization of the membrane potential (Briskin and Leonard, 1979). When 0.1 mM KCl was presented to suspensions of tobacco cells or protoplasts, the depolarization represented a potential change from -41 to -31 mv and for protoplasts, -40 to -5 mv. When the membrane potential changes, potassium flux is often observed. The depolarization of the membrane potential induced by Ca^{2+} or boron uptake stimulates potassium efflux which results in repolarization (Ketchum and Poole ,1989). Conversely, the hyperpolarization of the membrane potential induced by H^+ efflux causes potassium uptake (Mercier and Poole, 1980).

3.2. ATP level

Since the H^+-pumping ATPase was considered to contribute to the membrane potential, the relationship between potential and cellular ATP level has been investigated. In red beet, the membrane potential was hyperpolarized from about -

75 to -200 mv while ATP levels were increased 40 to 60% of the control level (treatment with different concentrations of inhibitors). No significant change in the membrane potential was obtained above 62% of control level which is 0.7 to 0.9 mM ATP (Mercier and Poole, 1980). Similar results to those obtained by Mercier and Poole were found in *Nitellopsis obtusa* by Mimura (1983). The membrane potential was dependent on the ATP concentration inside cells, showing a saturation curve with a maximum ATP-dependent membrane potential value of about -120 mv. ATP serves as an energy source for pumping cations i.e. H^+ out to maintain the membrane potential negative inside.

3.3. pH

The effect of intracellular and extracellular pH on the membrane potential can be predicted. The membrane potential generated by the H^+ pump in the absence of potassium movements is defined as the pump electromotive force, E_p, or the potential of the H^+ pump, Δm_{H^+}. It is dependent on the free energy of the ATP/ ADP system and can be calculated as follows:

$$ATP + n\,H^+_i + H_2O \longrightarrow ADP + n\,H^+_o + P_i$$

Where n indicates the stoichiometry of the pump.

Defining ΔG_{ATP} as the change in free energy for the hydrolysis of 1 mole of ATP. We can write

$$\Delta G_{ATP} = n\,\Delta\mu_{H^+}$$

$$\Delta\mu_{H^+} = 96.5\,\Delta E_{H^+} - 5.8\,\Delta pH \qquad (KJ/mol)$$

$$\Delta G_{ATP} = n (96.5 \Delta E_{H^+} - 5.8 \Delta pH)$$

$$\Delta E_{H^+} = \frac{\Delta G_{ATP}}{96.5\, n} + 0.06 (pH_i - pH_o) \qquad (4)$$

From equation 4, lowering the external pH (pH_o) will make ΔE_{H^+} more positive (depolarization) and lowering the intracellular pH (pH_i) will make ΔE_{H^+} more negative (hyperpolarization). As predicted, when the pH values of the standard bathing solution decrease from 8.0 to 6.0 , mean intracellular potentials are depolarized from -206 to -167 mv(Mercier and Poole, 1980). Lowering the intracellular pH with butyric acid in *Maize* root segment results in hyperpolarization (Marre, 1983).

C. Symport

1. Introduction

Solute movement requires both a pathway and a driving force for transport. Solutes may directly cross the phospholipid bilayer or via a protein carrier inserted into the membrane. The driving force can be chemical concentration gradients, membrane potential, ATP energy, or a combination of these. Solute transports are divided into three catereries based on the involvement of another solute:

(i) Uniport, a transport process involving a single solute,

(ii) Symport, a transport process involving the coupled movement of two or more solutes in the same direction,

(iii) Antiport, a transport process involving the coupled movement of two solutes in the opposite direction.

The transport of the major nutrient, sugars and amino acids, into fungal and plant cells has been regarded as a proton symport. This section will deal with proton symports, especially H^+/sugar and H^+/amino acid symports.

Metabolic energy may be directly or indirectly coupled to membrane transport processes . The direct mechanism demonstrates the coupling of the "uphill" movement of H^+ with the hydrolysis of ATP. The indirect mechanism couples the transport of other solutes to the "downhill" movement of H^+ by means of a carrier system. The transport of sugars and amino acids in plant cells has been considered to be coupled to the back-flux of H^+ down its electrochemical gradient. Therefore, the H^+ electrochemical gradient is the driving force or so-called proton-motive force (pmf) for the proton symport of sugars and amino acids. (Poole, 1978)

The accumulated in *vivo* or in *vitro* evidence based on the measurement of changes in the membrane potential and pH on the addition of sugars and amino acids to the external medium supports the suggestion of the proton symport of sugars and amino acids into the cells. Two models to explain the symport mechanism have been proposed by Reinhold and Kaplan(1984). However, the mechanism of the symport process is not fully understood and the carrier proteins have not been isolated and characterized.

2. The evidence for H^+/sugar and H^+/amino acid symport

In the case of proton symport, the uptake of substrate should be accompanied by transmembrane proton influx. Thus substrate uptake should result in an

alkalinization of the medium. Therefore, medium alkalinization has been considered as an indicator of proton symport. The work done by Komor and his coworkers(1977) with sucrose transport in *Ricinus communis* showed that alkalinization of the medium occurred on addition of sucrose to cotyledons and yielded a change of about 0.1 pH units. However, when a-methylglucoside was added in the same concentration as sucrose, no change in pH was detected. It was suggested that the alkalinization induced by sucrose was specific and resulted from H^+/sucrose cotransport rather than sucrose metabolism. Similar data has been presented to demonstrate a H^+/sugar symport system in other plant cells (Novaky, 1980, Komor, 1981, Kennedy and Stewart, 1982, and Hutchings, 1978).

In plants, evidence for H^+/amino acid symport is much less extensive than for H^+/sugar symport. It should be possible to observe transient pH changes in the medium if proton influx with an amino acid exceeds the rate of proton efflux due to H^+-ATPase. Amino acid induced alkalinization with tomato internode section has been reported (VanBel and Van der Schoot, 1980). But since these pH changes occured over a period of hours or even days, it is difficult to explain them as support for H^+/amino acid symport. These long time alkalinizations may result from the metabolism of amino acids and the release of basic metabolites. However, work done by Robinson and Beevers (1981) showed a typical transient alkalinization in the medium following addition of 5.5 mM L-glutamine to castor-bean cotyledons. The alkalinization peaked after10 min and its rate was 4.9 umol g^{-1} FW colyledon^{-1}. No changes in pH of the medium were observed on addition of D-glutamine. In the presence of inhibitors or uncouplers, such as sodium azide, 2,4-dinitrophenol, trifluoromethoxy carbonyl cynide phenylhydrazone (FCCP) or N-ethylmaleimide, no pH changes following addition of L-glutamine were detectable and L-glutamine uptake was inhibited. These results indicate that L-glutamine transport into cells was coupled to H^+ influx and that the driving force was the

proton electrochemical gradient. They further studied the kinetics of the alkalinization response. The result showed that the rate of H^+ influx was dependent on the L-glutamine concentration and it was saturable above 10-20 mM L-glutamine. The Km and V_{max} of alkalinization response were 4.8 mM and 7.2 umole $H^+ g^{-1}$ FWcotyledons^{-1}, respectively. These results suggested that H^+ /L-Gln symport was mediated by a saturable carrier and that the symport system was highly specific.

In plant cells, another indicator for H^+/substrate symport is a depolarization of the membrane potential induced by substrate transport. Sugar uptake has been considered as proton symport because although sugar is a neutral molecule its uptake resulted in depolarization. A transient depolarization of about 70 mV occurred within the first half minute on addition of both 6-deoxyglucose or glucose and after 1 minute the membrane potential recovered to a new level about 25 mV lower than the original level before sugar was added (Komor and Tanner 1976). In the case of amino acid uptake, the situation seems complicated. The application of neutral or acidic amino acids to oat coleoptiles induced a transient depolarization of the membrane potential then repolarization. The basic amino acids depolarized the membrane potential strongly, but the repolarizations were weak or absent. Depolarization induced by basic amino acids were less sensitive to changes in both extracellular and intracellular pH, but the depolarization induced by other amino acids were strongly affected by pH changes. It was suggested that the neutral and acidic amino acids are cotransported with a single H^+, plus a cation. Basic amino acids seem not to be cotransported with an additional proton (Kinraide and Etheron, 1980).

Studies on proton symport into plasma membrane vesicles provided *in vitro* evidence for H^+/sugar and H^+/amino acids symport. Bush (1989) showed that isolated vesicles from sugar beet (*Beta vulgaris* L. cv Great Westerm) leaves

concentrated sucrose fivefold in the presence of an imposed pH gradient (basic interior), but the presence of carbonyl cyanide m-chloraphenylhydrazone (CCCP), a protonophore, inhibited this sucrose accumulation. This indicated that sucrose transport into the plasmalemma vesicles was H^+ gradient-dependent. The kinetic analysis showed that sucrose transport exhibited saturation with an apparent K_m of 1.2 mM. The sucrose transport was inhibited not only by protein modifiers, such as diethyl pynocarbonate (PEPC) and p-chloromercuribenzenesulfonic acid, but also by the analogues of sucrose, such as glucose, fructose, raffinose or maltose, suggesting that the sucrose transport was protein carrier-mediated and the transport system was specific for sucrose. The H^+/amino acid symport into the plasma membrane-vesicle was also demonstrated by Bush and Langston-Unkefer (1988). An imposed pH gradient (pH 6.0 in medium and pH 8.0 inside vesicle) was used to energize membrane vesicles and to drive amino acid transport. The transport of the amino acids, alanine, glutamate, glutamine and leucine, was protonophore sensitive. Alanine transport studied in detail exhibited saturation kinetics and was also inhibited by inhibitors of proteins involved in proton translocation. These results indicate that amino acid transport is mediated by a H^+/amino acid symport.

3. The stoichiometry of H^+/sugar or H^+/amino acid symport

In the case of proton symport, the measurement of the stoichiometry is more difficult because the unidirectional influx of H^+ can not be observed with isotopes because H^+ are in rapid equlibrium with a molar excess of H_2O. Net pH changes in the medium after addition of substrate have been used in the measurement of stoichiometry. Some measured stoichiometries of H^+/sugar or H^+/amino acid have

been reported. The stoichiometry of H^+/sugar symport was determined as 0.8 H^+ per hexose molecule in sugarcane suspension cells by Komor and his colleagues (1981), as 0.3 H^+ per 3-o-methylglucose molecule in soybean suspension cells by de Klerk-Kiebert and coworkers (1983). These data suggested that the stoichiometry of H^+/sugar was 1 H^+ per sugar molecule. The measured stoichiometries of H^+/amino acid were 0.2-0.3 H^+ per amino acid transport in Ricinus cotyledos (Robinson and Beevers, 1981). The stoichiometry of H^+/L-Glu was 2 in *Staphylococcus* aureus cells (Mitchell et al, 1979).

To date, the measured stoichiometries are not satisfactorily accurate. Problems may be associated with the measurement of H^+ flux. The H^+ influx considered to be coupled to substrate transport has been shown through net pH changes of the medium. The value from this measurement may be underestimated due to CO_2 release, leakage of organic acids or stimulation of H^+ efflux by the plasma membrane H^+-ATPase in response to internal acidification. Therefore, a corrected stoichiometry should take into account process influencing the pH of the medium other than the symport process.

D. GABA Production

GABA (g-Aminobutyric acid), a non-protein amino acid, is very widely distributed and has been found in algae, fungi, mosses, ferns and flowering plants (Fowden, 1958). It is present at high concentrations that often exceed those of many of normal protein amino acids (Thompson et al, 1953, Fowden, 1964). Some data indicated that GABA is a temporary storage product in nitrogen metabolism (Selman and Cooper, 1978) or has a role in the regulation of nitrogen nutrition (Desmaison and Tixier, 1986). Accumulation of GABA in plants has been reported in response to environmental stress conditions such as anaerobiosis (Streeter and

Thompson, 1972), low temperature (Wallace et al, 1984), γ-radiation (Satyanarayan and Nair, 1986), low pH (Lane and Stiller, 1970) and addition of NH_4^+, or glutamine (Kishinami and Ojima, 1980) and auxin (Kishinami, 1988). However, the mechanisms responsible for GABA accumulation under stress conditions are not clear.

In many cases, GABA is produced in higher plants by a-decarboxylation catalysed by L-glutamate 1-decarboxylyase EC 4.1.1.15 (GAD).

$$\text{L-Glu} + H^+ \xrightarrow{\text{GAD}} \text{GABA} + CO_2$$

Convincing evidences has shown rapid conversion of added ^{14}C-U-Lglutamate to ^{14}C-U- GABA in germinating seeds (Vandewalle and Olsson, 1983), intact leaves (Streeter and Thompson, 1972) and roots (Guinn and Brinberholf, 1978), intact plant cells (Bown et al, 1989). GAD activity, like GABA, has been observed in many plants (Kulkarni and Schonie, 1956). Subcelllular distribution studies indicated that plant GAD is a soluble, cytosolic enzyme (Wallace et al, 1984, Streeter and Thompson, 1972). The information on the properties of plant GAD in crude extracts showed that the optimum pH was within a low narrow pH range of 5.7 to 6.2 (Kulkarni and Schonie, 1956). The K_m value for L-glutamate is between 3 and 9 mM for mature tissue (Smith and Waygood, 1961) and between 22 and 25 mM for embryonic tissues (Inatomic and Slaughter, 1975).

In plants, the optimum pH of GAD is quite low and there is sharp increase in activity when the pH decreases from physiological value. Under anaerobic conditions, lowered cytoplasmic pH (Roberts et al, 1984) was correlated with an increase in GAD activity and the accumulation of GABA. This suggests that acidic conditions could favour GAD activity. As a result of GAD activity, a decrease in pH

may be reduced because H^+ is consumed during decarboxlation. Thus it seems that GAD plays a role in the the regulation of cytoplasmic pH in plant cells. This metabolic pH-stat role has been suggested by Reggiani et al. (1988)and Bown et al.(1988).

Materials and Methods

A. Materials

1. Chemicals

Chemicals	Supplier
KP_i Standard Buffer (pH 6.86)	BDH Chemical Co.
KP_i Standard Buffer (pH 6.40)	"
$CaSO_4$	"
KCl	"
NaCl	"
$Ca(OH)_2$	"
NaOH	"
HCl	"
H_2SO_4	"
Phenol	"
Methanol	"
Butyric acid	Sigma Chemical Co.
D-glutamic acid (D-Glu)	"
L-glutamic acid (L-Glu)	"

L-methionine, L-sulfoximine (L-MSO)	Sigma Chemical Co.
Dicycloherylcarbodiimide (DCCD)	"
Diethylstilbestrol (DES)	"
Oligomycin	"
Antimycin	"
Carbonyl cyanide-*R* -trichloromethoxyphenyl hydrazon (CCCP)	"
2 [N-Morpholino] ethane sulfonic acid (MES)	"
Fusicoccin (FC)	Italchemia S. A.
Silica-gel G	Macherey Nagel
^{14}C-U-L-glutamic acid (^{14}C-U-L-Glu)	New England Nuclear
Methyltriphenylphosphoniumiodide, methyl-^{3}H (^{3}H-MTTP)	"
Aqueous Counting Solution (ACS)	Amersham

All chemicals were of analytical grade. All of the compounds were dissolved in distilled water with the exception of DCCD, CCCP, DES, oligomycin, antimycin and FC which were dissolved in 80% of ethanol. When needed,the pH of the solutions was adjusted by adding H_2SO_4 or $Ca(OH)_2$.

2. Plants

In this study, the mesophyll cells were isolated from cladophylls of *Asparagus sprengeri* The plants were grown in a greenhouse under natural sunlight at ambient temperature. The plants were watered daily, fertilized and the greenhouse fumigated regularly to prevent infestation by parasites.

B. Methods

1. Isolation of Mesophyll Cells

Asparagus sprengeri mesophyll cells were isolated following the mechanical disruption method described by Colman, Mawson and Espie (Colman et al, 1979). Mature fronds with dark green cladophylls were harvested from greenhouse grown plants. Approximately 10 g of cladophylls were removed to a Buchner funnel then rinsed in distilled water. The washed cladophylls were cut into section approximately 0.5 cm long, then placed into a 100 ml beaker with distilled water and vacuum infiltrated for 20 min, with aspiration full on. Susequently, air was allowed back into the vacuum chamber slowly and intracellular air spaces in the cladophylls were replaced with water. This step permitted easy isolation of the cladophyll cells. The sections were collected in a Buchner funnel then transfered to a smooth mortar. Ten ml of 1.0 mM $CaSO_4$ was added to the mortar and the sections were very gently ground with a pestle for about 5 min until the suspension in the mortar was dark green. The suspension was removed with a Pasteur pipette and filtered though two layers of cheescloth into 30 ml glass centrifuge tubes kept on ice. This procedure was repeated 4 more times with 10 ml of 1 mM $CaSO_4$. The green suspension was centrifuged in a Clinical centrifuger at setting 2 for 1 minute. The supernatant fluid was discarded and the dark green pellet resuspended in appoximated 50 mls of 1.0 mM $CaSO_4$. This final cell suspension was kept on ice in the dark, prior to cell counting and subsequent experiments. Cells were prepared daily.

2. Cell Counting

Before any experiment, the number of cells in the cell suspension was determined as the number of cells per ml of cell suspension. This determination used a Spencer " Bright Line " hemocytometer and a Wild light microscope. A small amount of cell suspention was added to hemocytometer with a Pasteur pipette and covered with a cover slip. The volume of one grid square was 4.0×10^{-6} ml. The cells occupying 5 grid squares, one located in the centre and the others at four corners, were counted and averaged. This procedure was repeated 7 times and the 8 counts were summed and averaged to produce the number of cells per grid square. The number of cells per ml of cell suspension was calculated by dividing by 4.0×10^{-6} ml:

$$\text{number of cells/ml of cell suspension} = \frac{\text{number of cells/per grid square}}{4.0 \times 10^{-6}\ \text{ml}}$$

In order to examine the intactness of the plasma membrane, the cell suspension was treated with a 20% (w/v) solution of Evans Blue. The mixture was allowed to sit for 5 min before the cells were counted. Blue cells which resulted from taking up dye were considered as not having an intact plasma membrane (Taylor & West, 1980). In this study, the percent of cells with an intact plasma membrane exceeded 85%.

3. General Conditions for Transport Experiments

All of the experiments were performed using cell suspension in cylindrical glass reaction vessels. The temperature within the reaction in a vessel was regulated by a water jacket connected to a recirculating 30 ^{0}C water bath.

Aeration was supplied by inserting the tip of a metal needle into the cell suspension medium. The needle was connected by a tube to an air flow system.

Stirring of the cell suspension was accomplished by placing a micro magnetic spinbar in the reaction vessel on a Corning PC-351 stirrer.

Light was provided by a 300W light source. Light was eliminated by placing 2 layers of thick dark cloth over the reaction vessel.

4. Determination of Rates of Net H^+ Flux

Rates of net alkalinization or acidification were determined using the pH vs time method. In all experiments, the cell suspension, 10 x 10^6 cells in 10 ml of 1mM $CaSO_4$, was contained in an open glass reaction vessel, stirred and aerated (500ml/min). The illumination conditions were dark or light as described earlier.

Measurements of pH were performed with a Fisher combination pH electrode (G202c) containing 4mM KCl saturated with AgCl. The electrode was attached to a Fisher Model 310 Accumet pH meter which was in turn connected to a Cole-Parmer model 8371-20 double pen chart recorder.

In most cases, the initial pH of the cell suspension was about 6.1. In other cases of lower or higher values, the pH was adjusted to 6.1 with H_2SO_4 or $Ca(OH)_2$. The pH of solutions added the cell suspension was also adjusted to 6.1 before use.

At the end of the experiments, the contents of the reaction were back-titrated Pwith 1 mM H_2SO_4 or NaOH over the pH range just covered by the reaction in order to determine the buffering capacity of the cell suspension in that pH range.

The net rates of H^+ flux was calculated as follows,

$$\frac{A\ (cm/min)\ \ X\ \ B\ (nmoleH^+/cm)}{10\ (\ 10^6\ cells\ )}$$

$$= 0.1\ A\ X\ B\ (\ nmole\ H^+/10^6\ cells \cdot min\ )$$

—— A: the rate of pen movement

—— B: pen movement in response to known nmole additions of H^+

—— The number of cells in the cell suspension medium was 10×10^6.

5. Simultaneous Recording of Net H^+ and K^+ Flux

All experiments were performed in a glass reaction vessel under the dark with stirring but no aeration. These conditions were chosen because light absorption by the black K^+ electrode interfered with recordings and aeration appeared to produce a noisy signal from the reference electrode. The cell suspension consisted of 10 ml of medium containing 1 mM $CaSO_4$ and 0.5 mM K_2SO_4. The number of cells in the cell suspension was 10×10^6.

Simultaneous measurements of the flux of H^+ and K^+ were made using a 3 electrodes system consisting of G202c glass pH electrode, F2312 K^+ ion selectrode and a common K701 reference electrode. The K^+ electrode was filled with 200 ml of 10 mM KCl while the reference electrode had a secondary bridge filled with 4 M NH_4NO_3 so that K^+ was prevented from leaking out of the electrode into the cell suspension medium. The H^+ and K^+ electrodes were connected via a Fisher Accumet meter and a Radiometer Copenhagen PHM64 meter respectively to a Cole-Parmer model8371-20 double pen chart recorder.

The rates of H^+ flux were calculated as described earlier. The rates of K^+ flux was calculated as follows,

$$\frac{A' \text{ (cm/min)} \quad X \quad B' \text{ (nmole}K^+\text{/cm)}}{10 \ (\neg 10^6 \text{ cells})}$$

$$= 0.1 \ A \ X \ B \ (\text{nmole } K^+/10^6 \text{ cells} \cdot \text{min})$$

—— A': the rate of pen movement

—— B': pen movement in response to known nmole additions of K^+

—— The number of cells in the cell suspension medium was 10×10^6.

6. Determination of the rate of ^{14}C-U-L-Glu uptake

Measurements of ^{14}C-U-L-Glu uptake were conducted in a vessel containing 11 X 10^6 cells in 2.2 ml of 5 mM MES/1mM $CaSO_4$ buffer adjusted to pH 6.0 with $Ca(OH)_2$. The cell suspension was stirred, never aerated and exposed to light.

Before addition of ^{14}C-U-L-Glu, a 0.2 ml aliquot of cell suspension was removed and collected on a Millipore fitter (HA type, 0.45 mM size). This was called the blank. Once ^{14}C-U-L-Glu solution of known concentration and specific radioactivity was added, a 0.2 ml aliquot was removed immediately at time 0 and every 2 minutes for 10 minutes. Once removed, the cells were mixed with 5 ml of cold cell suspension medium (5 mM MES/1mM $CaSO_4$ with pH 6.0) collected on a millipore filters and then washed twice with 10 ml of the same medium. At the end of the experiment, a 0.2 ml aliquot was directly transfered from the cell suspension into a scintillation vial containing 1ml of 100% methanol. This sample was called the total. A 0.6 ml aliquot was in turn transfered into a centrifuge tube for measuring the amount of ^{14}C-GABA in the medium. The Millipore filters with collected cells were added to scintillation vials containing 1ml of 100% methanol and allowed to sit for 5 minutes. The methanol caused cell disruption and the filters to disintegrate creating an homogeneous mixture. Fourteen ml of Aqueous Counting Scintillant (ACS) was then added to each of the vials. The contents of the vials were then subjected to a Vari-whirl mixer, shaking and placed in a Beckman LS1800 Scintillation system for radioisotope determination.

The 0.6 ml of cell suspension was centrifuged at top speed (set to 7) in a centrifuge (Iec clinical centrifuge). The supernatants were stored in a freezer, prior to analysis.

7. Determination of the Rates of ^{14}C-GABA Release from Cells Incubated with ^{14}C-U-L-Glu

After feeding ^{14}C-U-L-Glu, ^{14}C-GABA in the medium was separated from the cells by centrifugation and identified by thin layer chromatography (TLC). Determination of the amount of radioactivity in ^{14}C-GABA was made using liquid scintillation counting. Determinations were made simultaneously with those for ^{14}C-L-U-Glu uptake (Method 2.6).

Before addition of ^{14}C-L-U-Glu, a 100 µl aliquot of cell suspension was transferred to a microcentrifuge tube. The sample was centrifuged for 1 min and 80 µl of supernatant fluid was then transferred to another microcentrifuge tube for analysis by TLC. This sample was called the blank. Once ^{14}C-L-U-Glu solution was added (concentration and specific radioactivity are indicated in the results), a 100 µl of aliquot was immediately transferred (time 0 sample)and every 2 minutes from 1 min to 9 min. Supernatant fluids from each of these sample was obtained with the same procedure as for the blank. Fifteen g powder of silica gel G and 30 ml distiled water were mixed in a flask by shaking for 1 min. The mixture was in turn poured into a spreader and spread over glass plates (20 cm X 20 cm) to give a layer 0.25 mm thick. The layers of gel on the plates were dried for 5 min at room temperature and were then moved into an oven at 90 ^{0}C for 1.5 hours.

Twenty ml of supernatant for each of 7 samples termed blank, 0, 1, 3, 5, 7, 9, was spotted on the dried thin layer of gel. After spotting of, 3-5 µl of 0.2 mM cold GABA was added to each spot to identify the GABA position after developing. The plates were placed in a closed glass chamber with a solvent consisting of 75g phenol dissolved in 25 ml distiled water. The samples were developed for 4 hours under dark condition. After developing, the plates were placed in a fumehood for one night (about 15 hours) to allow phenol to volatilize.

The chromatograms were sprayed with 0.5 % ninhydrin and were then placed in an oven at 60^0C for 15 min.

The powder from the GABA area was scraped onto a paper and then transferred to a scintillation vial containing 1 ml of 100% methanol. Fourteen ml of ACS was also added to the vial . The vial contents were then mixed and placed in a Beckman LS1800 Scintillation System for radioisotope counting.

8. Determination of Rates of [^{3}H] MTPP Uptake

All experiments were conducted in the fumehood and performed in a glass reaction vessel identical to that described earlier. The cell suspension was stirred, illuminated but not aerated. The cell suspension volume was 1.2 ml of a medium consisting of 5mM MES at pH 6.0. The number of cells in the cell suspension medium was 18 x 10^{6}.

Before the addition of any reagent, a 100 µl aliquot of cell suspension containing 1.5 x 10^{6} cells was moved and collected on a Millipore filter (HA type, 0.65 mm). This was called the blank. Once [^{3}H]MTPP was added (concentration and specific radioactivity varied with the experiment), a 100 µl aliquot was immediately removed and every 3 min for 15 min. The cells were collected on a Millpore filter with 5 ml of 1mM cold MTPP and then washed with 10 ml of 1mM MTPP and 10 ml of distiled water. At the end of the experiment, a 100 µl aliquot of the suspension was transferred directly into a scintillation vial containing 1ml of 100% methanol. The Millipore filters with collected cells were also added to scintillation vials containing 1ml of 100% methanol. After 5 min, 14 ml of ACS was added to each of the vials. The vials were then placed into a Beckman LS1800 Scintillation System for radioisotope counting. This procedure was modified from that described by Murphy et al (1975).

Results

A. Is H^+/L-Glu Symport Electrogenic ?

In this section are described experiments designed to determine whether or not the H^+/L-Glu symport system is electrogenic. Electrogenic uptake would result from the symport of 2 or more H^+ for every anion of L-Glu entering the cells. This process would be electroneutral if the molar stoichiometry of the symport was 1 H^+/L-Glu. An electrogenic symport may be driven by the membrane potential and inhibited by depolarization of the membrane potential caused by the diffusion of permeable cations such as K^+, Na^+ and lipophinic cations.

1. The Influence of K^+ or Na^+ on the Rate of L-Glu Dependent medium alkalinization

Electrogenic symport may be driven by the membrane potential. Thus, depolarizing the membrane potential, by addition of K^+ or Na^+ to the external medium, may inhibit the rate of the symport. In this experiment, the rate of the symport was indicated by the rate of L-Glu dependent medium alkalinization.

The influence of K^+ or Na^+ on the rate of L-Glu dependent medium alkalinization was studied using the pH vs time method. Oligomycin, an inhibitor of the ATP synthesis, was applied to inhibit ATP-dependent H^+ efflux.

When cells were added to the cell suspension medium, a rapid alkalinization was followed by a slow acidification or H^+ efflux process. This normally continued for 10 min. When 10 μg/ml oligomycin was applied to the cell suspension medium. H^+ efflux was rapidly inhibited (Fig. 1). This normally continued for 8 min. Subsequently, 2.5 mM L-Glu was added and alkalinization of the medium occurred without any apparent lag period (Fig. 1). After 4 min of this L-Glu dependent medium alkalinization, K^+ or Na^+ solutions as K_2SO_4 or Na_2SO_4 were added to the cell suspension medium. Any inhibition of L-Glu dependent medium alkalinization by K^+ or Na^+ was then observed.

Mean rates of L-Glu dependent medium alkalinization in the dark are shown in Table 1 were approximately 0.65 nmole H^+/min $\cdot 10^6$ cells. After addition of K^+ at a final concentrations of 2 mM, 10mM and 20 mM, the rates of alkalinization decreased to 0.37, 0.20 and 0.06 (Table 1). The percent inhibition was 42, 69 and 92 with addition of 2 mMK^+, 10 mM K^+ and 20 mM K^+, respectively (Table 1). The results in the light were similar to those in the dark (Table 2). Although an n of 3 for each K^+ concentration does not allow the calculation of significant difference, it should be noted that K^+ inhibited the rate of L-Glu dependent alkalinization consistently, rapidly and by a large precentage value (Fig. 1 and Table 1, 2).

The effect of Na^+ on L-Glu dependent alkalinization is shown in Table 3 (in the dark) and Table 4 (in the light). The percent inhibitions were 29, 28 and 56 in the dark and 13, 17 and 40 in the light by addition of 20, 40 and 80 mM Na^+, respectively (Tables 3 and 4). Thus the inhibition by Na^+ at 20 mM was a third or less of that found for K^+.

The results from this experiment showed the addition of K^+ or Na^+ in the cell suspension medium inhibited L-Glu dependent medium alkalinization and that inhibition was greater with increasing concentrations of K^+ or Na^+. It indicated that H^+/Glu symport may be driven by the plasma membrane potential which was depolarized with the presence of K^+ or Na^+. Despite the addition of oligomycin to inhibit ATP dependent H^+ efflux, it is posible that the decrease in net alkalinization may result from some stimulated mechanism of H^+ efflux after addition of K^+ or Na^+.

Fig. 1 Inhibition of L-Glu Dependent Medium Alkalinization by K^+

These are pH vs time tracings showing the inhibition of L-Glu dependent medium alkalinization by varying K^+ concentrations in non-illuminated cells. To a reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ was added 10 µg/ml oligomycin, followed by 2.5 mM L-Glu. Concentrations of K^+ as K_2SO_4 were added 4 min after addition of L-Glu. At the end of the experiment, the contents of the reaction vessel were backtitrated with 1 mM H_2SO_4.

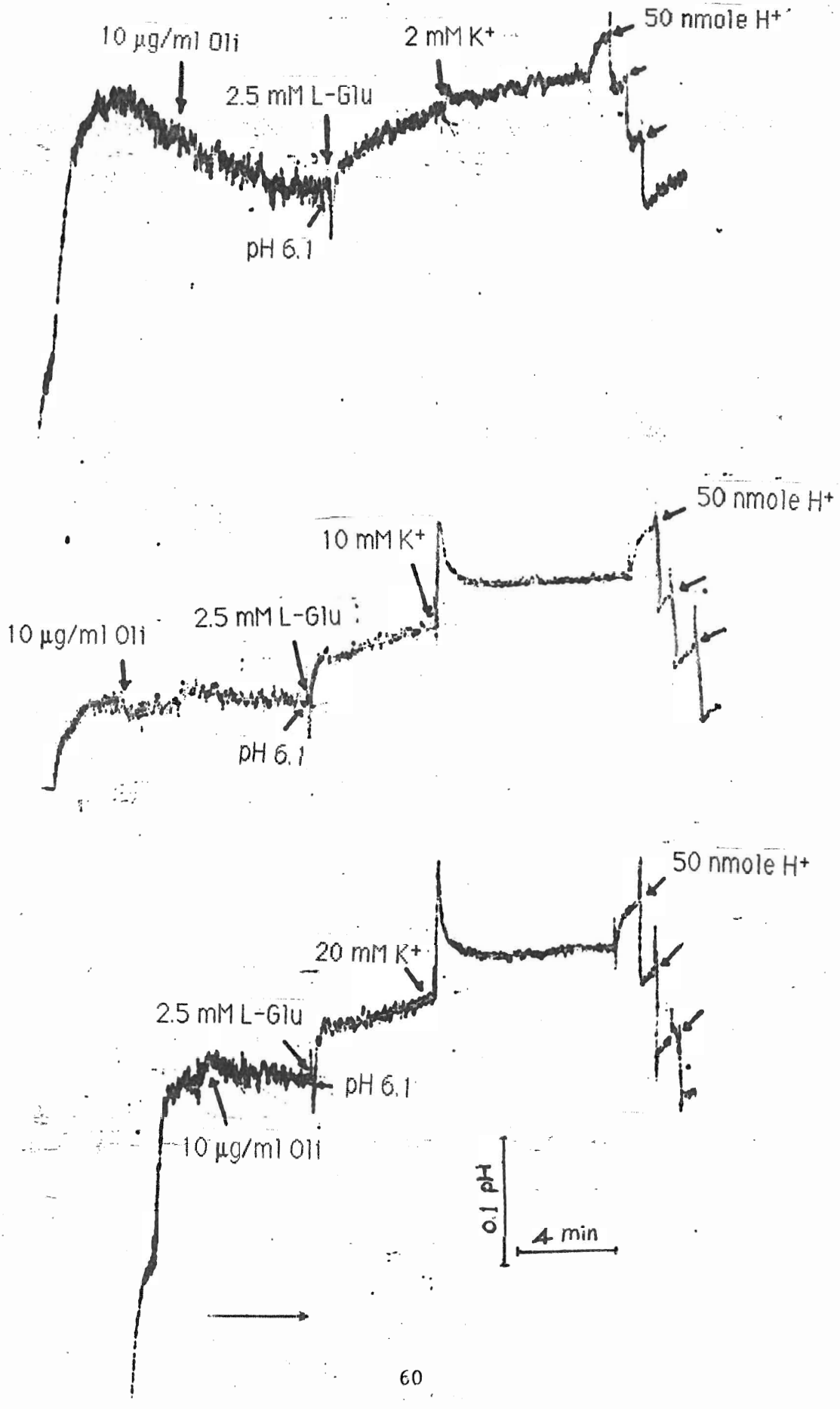

10 µg/ml Oli
2.5 mM L-Glu
2 mM K+
50 nmole H+
pH 6.1
10 µg/ml Oli
2.5 mM L-Glu
10 mM K+
50 nmole H+
pH 6.1
10 µg/ml Oli
2.5 mM L-Glu
20 mM K+
50 nmole H+
pH 6.1
0.1 pH
4 min

Table 1. Inhibition of L-Glu Dependent Medium Alkalization by K^+ in the Dark

(- With 10 µg/ml Oligomycin)

	Rate of H^+ Flux		
	2mM K^+	10mM K^+	20mM K^+
	nmole H^+/ min· 10^6 cells		
After the addition of 2.5 mM L-Glu	-0.64 (0.34)	-0.65 (0.12)	-0.72 (0.24)
Subsequent the addition of K^+	-0.37 (0.10)	-0.20 (0.03)	-0.06 (0.07)
% Inhibition	42	69	92

—— Each value is the mean of 3 trials (with S.D. in brackets)

—— Negative value indicates H^+ influx

—— Rates of alkalinization are expressed in nmole H^+/min · 10^6 cells

—— The reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$

—— The cell suspension was stirred and aerated in the dark

—— The concentration of L-Glu added was 2.5 mM

—— K^+ added as K_2SO_4

Table 2. Inhibition of L-Glu Dependent Medium Alkalization by K^+ in the Light

(- With 10 µg/ml Oligomycin)

	Rate of H^+ Flux		
	2mM K^+	10mM K^+	20mM K^+
	nmole H^+/ min· 10^6 cells		
After the addition of 2.5 mM L-Glu	-0.75 (0.18)	-0.81 (0.25)	-0.76 (0.14)
Subsequent the addition of K^+	-0.45 (0.13)	-0.25 (0.16)	-0.15 (0.17)
% Inhibition	33	73	80

Conditions as for Table 1.

Table 3. Inhibition of L-Glu Dependent Medium Alkalization by Na^+ in the Dark

(With 10 µg/ml Oligomycin)

	Rate of H^+ Flux		
	20mM Na^+	40mM Na^+	80mM Na^+
	nmole H^+/ min· 10^6 cells		
After the addition of 2.5 mM L-Glu	-0.64 (0.05)	-0.59 (0.01)	-0.60 (0.14)
Subsequent the addition of Na^+	-0.45 (0.01)	-0.42 (0.02)	-0.27 (0.17)
% Inhibition	29	28	56

—— Each value is the mean of 3 trials (with S.D. in brackets)

—— Negative value indicates H^+ influx

—— Rates of alkalinization are expressed in nmole H^+/min · 10^6 cells

—— The reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$

—— The cell suspension was stirred and aerated in the dark

—— The concentration of L-Glu added was 2.5 mM

—— Na^+ added as Na_2SO_4

Table 4. Inhibition of L-Glu Dependent Medium Alkalization by Na^+ in the Light

(With 10 µg/ml Oligomycin)

	Rate of H^+ Flux		
	20mM Na^+	40mM Na^+	80mM Na^+
	nmole H^+/ 10^6 cells · min		
After the addition of 2.5 mM L-Glu	-1.03 (0.27)	-1.05 (0.13)	-1.07 (0.14)
Subsequent the addition of Na^+	-0.90 (o.14)	-0.87 (0.21)	-0.64 (0.19)
% Inhibition	13	17	40

Conditions as for Table 3.

2. The Influence of K^+ and Na^+ on the Rate of L-MSO Dependent Medium Alkalinization

L-MSO has a similar molecular structure to L-Glu. It has been suggested that L-MSO transport into *Asparagus* mesophyll cells occurs via the same proton symport as L-Glu (McCutcheon & Bown, 1987). The purpose of this experiment was to examine the effects of $K^+$and Na^+ on the L-MSO dependent medium alkalinization.

The effects of K^+ and Na^+ on the rates of L-MSO dependent medium alkalinization was studied using the pH vs time method. The conditions of this experiment were the same as that for the similar L-Glu experiment but only 20 mM K^+ or Na^+ concentrations were studied.

In the presence of 20 mM K^+, the mean rate of L-MSO dependent medium alkalinization in the dark decreased from 1.43 (nmole H^+/min· 10^6 cells) to 0.36, and from 1.24 to 0.42 in the light. The calculated percent inhibitions were 75.8 in the dark and 65.7 in the light (Table 5).

In the presence of 20 mM Na^+, the mean rate of L-MSO dependent medium alkalinization decreased from 0.91 (nmole H^+/min· 10^6 cells) to 0.58 in the dark, and from 1.19 to 0.74 in the light. The calculated percent inhibitions were 36.3 in the dark and 37.5 in the light (Table 6).

The result showed that the presence of K^+ or Na^+ to the cell suspension medium inhibited the medium alkalinization induced by L-MSO. These results are similar to the inhibition of L-Glu dependent alkalinization; and also indicate a greater inhibition by K^+ over Na^+.

Table 5. Inhibition of L-MSO Dependent Medium Alkalinization by 20 mM K^+

(With 10 ug/ml Oligomycin)

Condition:

——— Negative value indicates H^+ influx

——— Rates of alkalinization are expressed in nmole H^+/min · 10^6 cells

——— The reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$

——— The cell suspension was stirred and aerated in the dark or light

——— The concentration of L-MSO added was 2.5 mM

——— The concentration of K^+ as K_2SO_4 added was 20 mM

——— The concentration of oligomycin added was 10 µg/ml

——— The cell suspension was incubated with oligomycin for 6 min before addition of L-MSO

——— K^+ was added 4 min after addition of L-MSO

——— At the end of the experiment, the contents of the reaction vessel were backtitrated with 1 mM H_2SO_4.

		Rate of H^+ Flux (nmole H^+ / 10^6 cells · min)		
		MSO	MSO + K^+	% Inhibition
Dark				
15 07 88		-1.04	-0.30	
18 07 88		-1.57	-0.72	
19 07 88		-1.67	-0.71	
	Mean	-1.43	-0.36	75.8
	SD	0.40	0.33	
Light				
15 07 88		-1.56	-0.69	
18 07 88		-1.26	-0.52	
19 07 88		-0.88	-0.06	
	Mean	-1.24	-0.42	65.7
	SD	0.34	0.33	

Table 6. Inhibition of L-MSO Dependent Medium Alkalinization by 20 mM Na^+

(With 10 mg/ml Oligomycin)

		Rate of H^+ Flux (nmole H^+ / 10^6 cells · min)		
		MSO	MSO + Na^+	% Inhibition
Dark				
15 07 88		-0.74	-0.49	
18 07 88		-1.56	-1.19	
19 07 88		-0.42	-0.06	
	Mean	-0.91	-0.58	36.3
	SD	0.72	0.57	
Light				
15 07 88		-1.69	-0.92	
18 07 88		-1.08	-0.59	
19 07 88		-0.81	-0.71	
	Mean	-1.19	-0.74	37.8
	SD	0.45	0.16	

Conditions as for Table 5: except that Na_2SO_4 was added to give 20 mM Na^+

3. Effect of K^+ or Oligomycin on the Rates of ^{14}C-U-L-Glu Uptake

The purpose of this experiment was to determine the effect of 40 mM K^+ on the uptake of ^{14}C-U-L-Glu. The ^{14}C-U-L-Glu uptake method was applied in this study (Method 2.6). To a reaction vessel containing 10 x 10^6 cells in 2 ml of 1mM $CaSO_4$ was added 30 µl of 33.6 mM ^{14}C-U-L-Glu (specific activity 4400 dpm/nmole) to give 0.5 mM L-Glu. When oligomycin or K^+ was added, they were preincubated with the cells suspension for 4 or 2 min, respectively, before addition of ^{14}C-U-L-Glu. Subsequently, a 200 µl aliquots of the cell suspension was removed every 2 minutes for 10 minutes to determine the ^{14}C-U-L-Glu uptake . The time course of ^{14}C-U-L-Glu uptake is shown on Fig. 2 and the rates of ^{14}C-U-L-Glu uptake were calculated (Table 7) .

When neither K^+ nor oligomycin was added, the mean rate of ^{14}C-U-L-Glu uptake was 0.034 nmole L-Glu/min · 10 x 10^6 cells. In the absence of oligomycin, K^+ had no effect on the rate of ^{14}C-U-L-Glu uptake. Oligomycin reduced the rate of the ^{14}C-U-L-Glu uptake by 30%. When K^+ was presented to the cell suspension with preincubation of oligomycin, the rate was reduced to 56% of that in the control or 79 % of that in the presence of oligomycin. Thus, it appears that addition of K^+ to the cell suspension had no effect on the rate of the ^{14}C-U-L-Glu uptake in the absence of oligomycin but inhibited the ^{14}C-U-L-Glu uptake in the presence of oligomycin.

Table 7. Influence of K^+ or Oligomycin on the Uptake of ^{14}C-U-L-Glu

	Rate of ^{14}C-U-L-Glu uptake			
	control	+ K^+	+ oli	+ oli+K^+
	nmole L-Glu/min· 10^6 cells			
18 01 89	0.022	0.024	0.012	0.009
15 02 89	0.067	0.065	0.053	0.043
08 03 89	0.013	0.018	0.006	0.006
Mean	0.034	0.035	0.024	0.019
% of control	100	104	70	56

——— Rates Of ^{14}C-U-L-Glu uptake are expressed in units of nmole L-Glu/min · 10^6 cell

——— The reaction vessel contained 2.2 X 10^6 cells in 2.2 ml of 1 mM $CaSO_4$

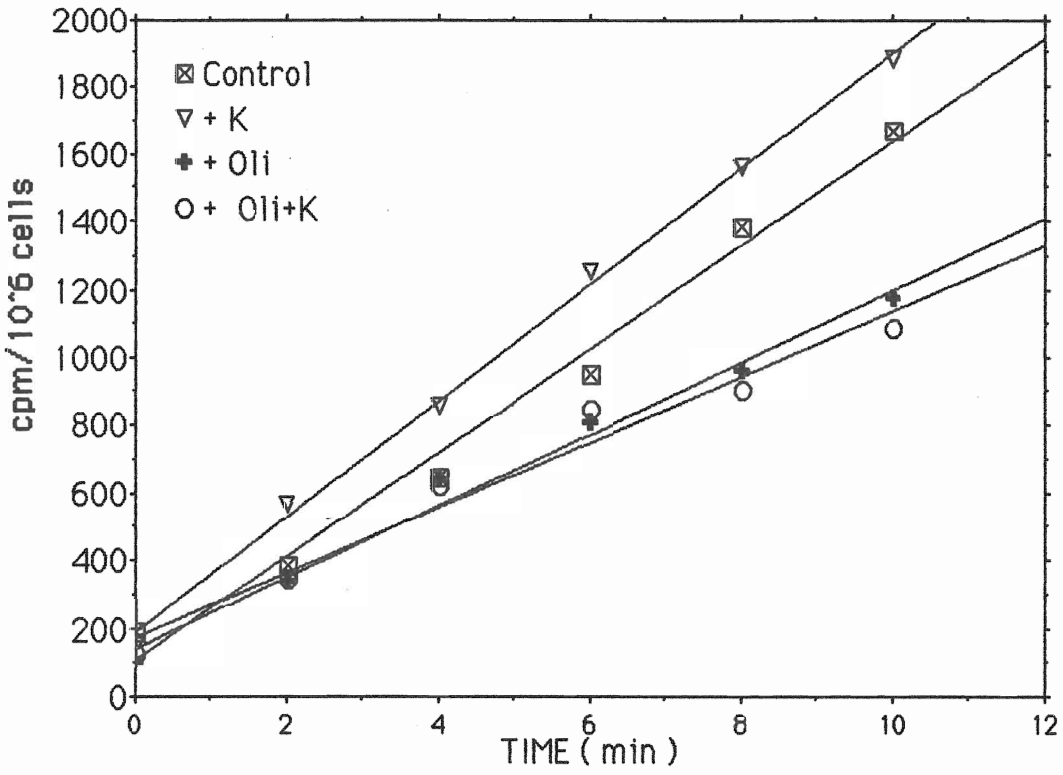
——— The cell suspension was stirred in ambient light

——— The cell suspension was incubated with 10 µg/ml oligomycin for 4 min before addition of ^{14}C-U-L-Glu

——— K^+ as K_2SO_4 was added 1 min before addition of ^{14}C-U-L-Glu to give 40 mM K^+ in the reaction vessel

——— The concentration of ^{14}C-U-L-Glu added was 0.5 mM (4400 dpm/nmole)

Fig. 2. Influence of K^+ or Oligomycin on the Uptake of ^{14}C-U-L-Glu



Conditions as for Table 7.

4. Effect of K^+ or Oligomycin on the Rates of ^{14}C-GABA Release

^{14}C-GABA in the medium was produced from ^{14}C-U-L-Glu which was taken up by the cells. Thus, to calculate the total uptake of ^{14}C-U-L-Glu, the amount of ^{14}C-GABA in the medium should be measured. The samples were taken 10 minutes after the addition of ^{14}C-U-L-Glu in the ^{14}C-U-L-Glu uptake experiment. The purpose of this experiment was to measure the amount of ^{14}C-GABA in the medium in order to calculate the total uptake of ^{14}C-U-L-Glu. ^{14}C-GABA was separated from the cells and identified by TLC (Method 2.7). The amount of ^{14}C-GABA was in turn determined using a Beckman1800 Scintillation System. The measured amount of ^{14}C-GABA in each sample was divided by 10 minutes to give an average rate of ^{14}C-GABA release which was expressed in units of nmole GABA/min $\cdot 10^6$ cells. The effects of K^+ or oligomycin on the rates of ^{14}C-GABA release were examined. These rates were shown in Table 8.

The mean rate of ^{14}C-GABA release in the control was 1.95 nmole GABA/min $\cdot$ 10^6 cells. It was increased to 2.60 when 40 mM K^+ was added but decreased to 1.18 with 10 µl/ml oligomycin. The addition of 40 mM K^+ to the cell suspension with 10 µl/ml oligomycin preincubation had no significant effect on the rate of ^{14}C-GABA release . The results, however, showed that there was a very dynamic situation ' in which' the rate of GABA production was diminished by oligomycin but enhanced K^+. The net effect on GABA production was not very different from the control, but, the means by which this was achieved certainly was different.

Table 8. Influence of K^+ and Oligomycin on the release of ^{14}C-U-GABA

	^{14}C-GABA in medium			
	control	+ K^+	+ oli	+ oli+K^+
	nmole GABA/min· 10^6 cells			
18 01 89	1.88	3.82	1.03	1.32
15 02 89	3.43	3.17	2.08	4.34
08 03 89	0.54	0.82	0.44	0.59
Mean	1.95	2.60	1.18	2.08
% of control	100	133	61	107

Conditions as for Table 7.

5. Effect of K^+ or Oligomycin on the Total Uptake of ^{14}C-U-L-Glu
(^{14}C-U-L-Glu inside cells plus ^{14}C-GABA in medium)

^{14}C-GABA was released to the medium from the cells after the decarboxlation of ^{14}C-U-L-Glu. This indicated that the total uptake of ^{14}C-U-L-Glu included both the amount of ^{14}C-U-L-Glu inside the cells and the amount of ^{14}C-GABA in the medium. The effect of K^+ or oligomycin on the ^{14}C-U-L-Glu transport should be correctly expressed as the effect of K^+ or oligomycin on the total uptake of ^{14}C-U-L-Glu. The data shown on Table 9 was taken from Table 7 and Table 8. However, the units were changed to nmole Glu/min $\cdot 10^6$ cells.

Table 9. Influence of K^+ or Oligomycin on the Total Uptake of ^{14}C-U-L-Gl(Glu inside Cells plus GABA in Medium)

	Rate of ^{14}C-U-L-Glu total uptake			
	control	$+K^+$	+ Oli	+ Oli+K^+
	nmole L-Glu/min· 10^6 cells			
Mean	0.23	0.30	0.14	0.23
% of control	100	130	62	100

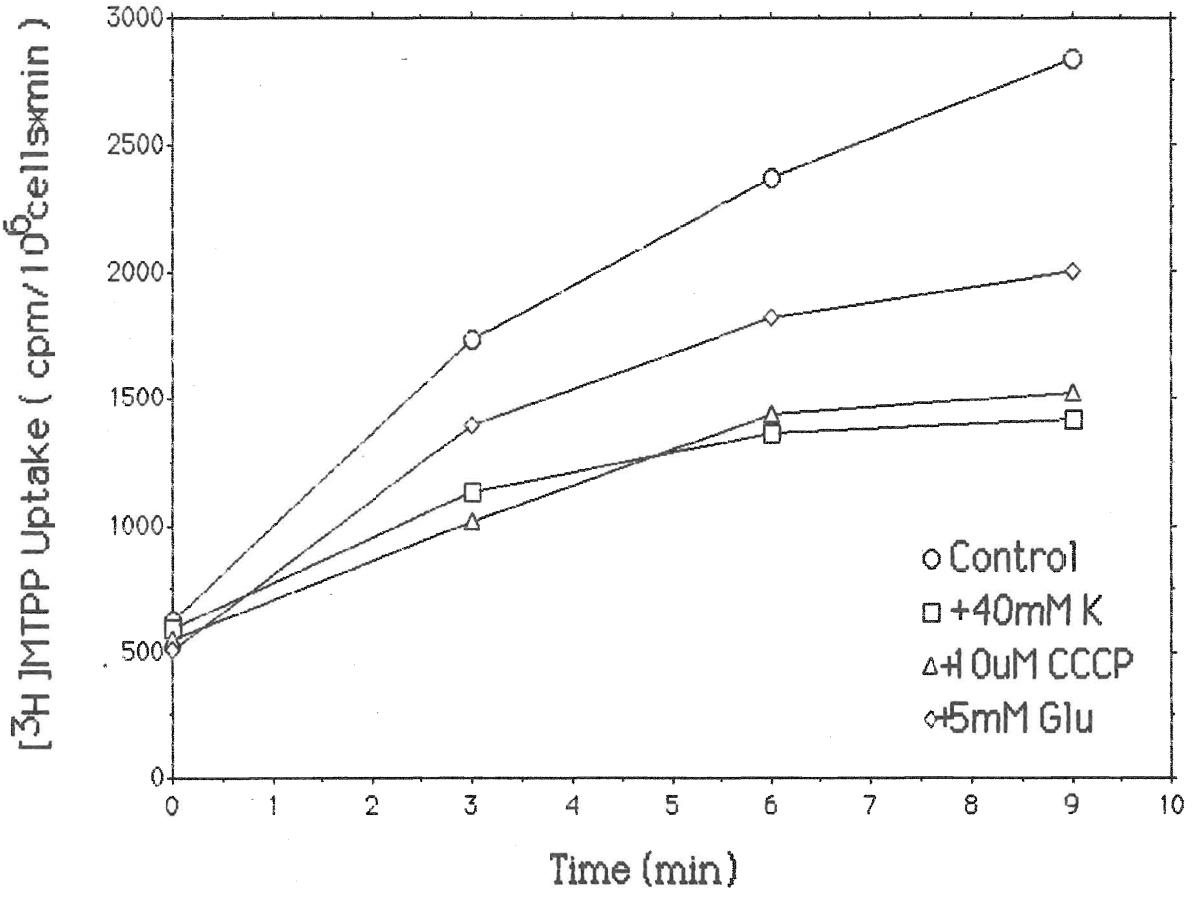
Conditions as for Table 7.

6. Influence of L-Glu, K^+ or CCCP on ^3H-MTPP Uptake

MTPP is a lipophilic cation whose uptake is driven by the membrane potential. The rate of MTPP uptake was used to monitor the changes in the membrane potential. If a process is electrogenic, it should affect MTPP uptake. The purpose of this experiment was to see whether the presence of L-Glu has an effect on ^3H-MTPP uptake. The presences of both K^+ and CCCP, a protonophore, have been known to depolarize the membrane potential. They were applied in this experiment to determine whether the rate of [^3H]MTPP uptake was sensitive to the changes in the membrane potential. The study was made using the ^3H-MTPP uptake method (Method 2.8).

The data in Fig. 3 show that the ^3H-MTPP uptake was inhibited by the presence of 5 mM L-Glu, 40 mM K^+ and 10 μM CCCP. Three minutes after the addition of ^3H-MTPP, ^3H-MTPP uptake was 1735 cpm/10^6 cells in the control, 1394 in the presence of 5mM L-Glu, 1136 in the presence of 40 mM K^+ and 1020 in the presence of 10 μM CCCP (Table 10). Together the data in Fig. 3 and 4 show the influence of L-Glu addition on MTPP uptake in a total of six experiments (n=6). Statistical analysis using the Wilcoxon's signed-rank test indicated the inhibition of ^3H-MTPP uptake by 5 mM was significant at the 0.025 level. The inhibition induced by 5 mM L-Glu was smaller than that due to 40mM K^+ or 10 μM CCCP. The results indicate that ^3H-MTPP uptake is inhibited by a reduction in the membrane potential and that L-Glu transport is an electrogenic depolarizing process.

Fig. 3. The Influence of Glu, K^+ or CCCP on ^3H-MTPP Uptake



These are curves showing the inhibitions of ^3H-MTPP uptake by L-Glu, K^+ or CCCP. To a reaction vessel containing 15 x 10^6 cells in 1ml of 5 mM MES pH 6.0 was added 2.5 X 10^{-4} mM ^3H-MTPP (5.55 x 10^7 dpm). The cell suspension was stirred in ambient light and was incubated with 5 mM L-Glu, 40 mM K^+ or 10 µM CCCP for 2 minutes before addtion of ^3H-MTPP. Each value is the mean of 3 trials.

Table 10. The Influence of Glu, K or CCCP on ^{3}H-MTPP Uptake

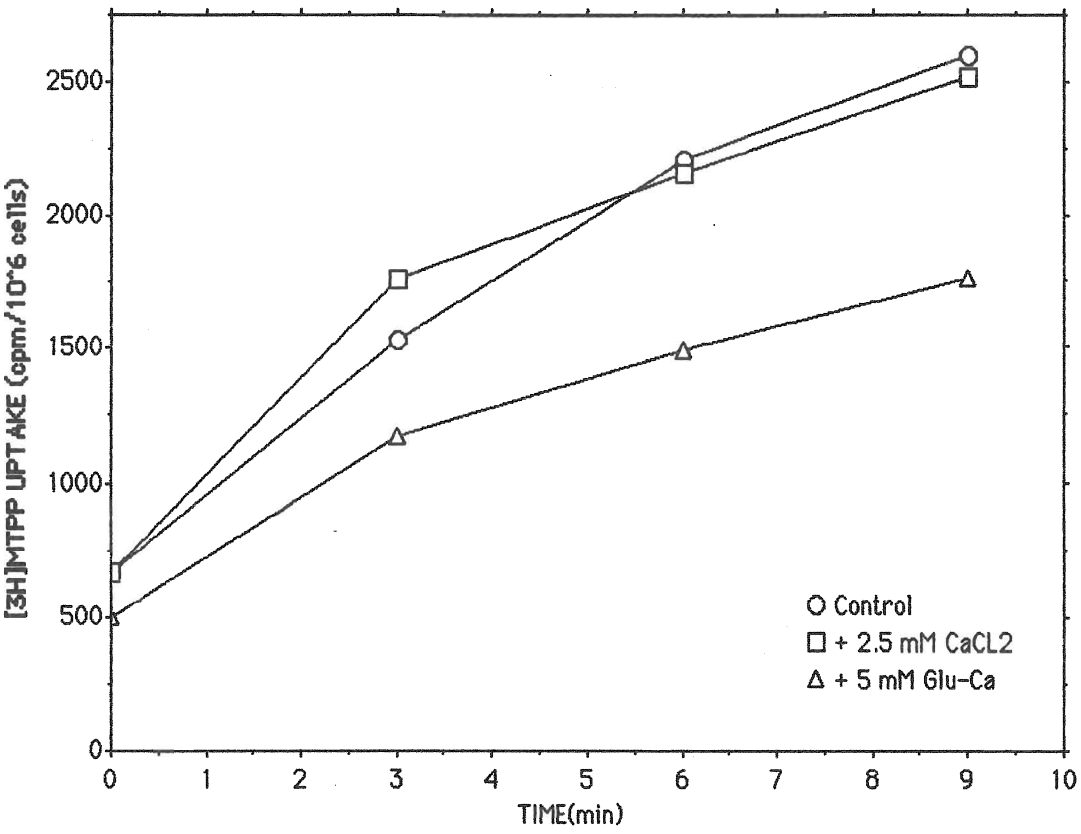
Time (min)	^{3}H-MTPP Uptake (cpm/10^{6} cells)			
	control	+ L-Glu	+ CCCP	+ K^{+}
0	623	507	557	596
3	1736	1394	1020	1136
6	2375	1821	1440	1370
9	2840	2002	1525	1417

Conditions as for Fig. 3.

7. The effect of Ca^{2+} on ^3H-MTPP Uptake

L-Glu solution applied in the [^3H]MTPP uptake experiment was the Ca^{2+} salt. To determine whether Ca^{2+} contributed significantly to the effect of L-Glu on ^3H-MTPP uptake, the effect of Ca^{+} on ^3H-MTPP uptake was studied. Ca^{2+} was added as $CaCl_2$. The final concentration of Ca^{2+} as $CaCl_2$ in the cell suspension was 2.5 mM which is the same as that as Ca^{2+} added with L-Glu.

Fig. 4. The Influence of Ca^{2+} on ^3H-MTTP uptake



These are curves showing the influence of $CaCl_2$ and the calcium salt of L-Glu on ^3H-MTPP uptake. To a reaction vessel containing 15 x 10^6 cells in 1ml of 5 mM MES, pH 6.0 was added 1.6 X 10^{-4} mM ^3H-MTPP . The cell suspension was stirred in ambient light and was incubated with 2.5 mM $CaCl_2$ or the calcium salt of 5 mM L-Glu for 2 minutes before addtion of ^3H-MTPP. Each value is the mean of 3 trials.

8. Inhibition of ^{3}H-MTPP Uptake and Total ^{14}C -U-L-Glu Uptake by Oligomycin

Oligomycin is a respiratory inhibitor which can inhibit ATP synthesis and therefore collapse the membrane potential. ^{3}H-MTPP uptake is known to be driven by the membrane potential and ^{14}C-U-L-Glu uptake, if it is electrogenic, should be inhibited by the presence of oligomycin. The purpose of this experiment was to determine the effect of oligomycin on ^{3}H-MTPP uptake and ^{14}C-U-L-Glu uptake.

In the presence of 10 μg/ml oligomycin, the mean rate of ^{3}H-MTPP uptake was 164 cpm/min · 10^{6} cells. Relative to the control, the percent inhibition was calculated to be 32 % (Table 11). The mean rate of ^{14}C-U-L-Glu uptake was 0.26 nmole L-Glu/min ·10^{6} cells in the control and 0.16 on the addition of oligomycin, an inhibition was 38% (Table 11). This data is consistent with other data indicating that L-Glu uptake is electrogenic.

Table 11. Inhibition of ^{3}H-MTPP Uptake and Total ^{14}C-U-L-Glu Uptake by Oligomycin

	Uptake Rate		
	control	+oligomycin	%Inhibition
^{3}H-MTPP (cpm/10^{6}cells·min)	243	164	32%
^{14}C-U-L-Glu (nmole/10^{6}cells·min)	0.26	0.16	38%

—— Each value is the mean of 3 trials

—— The conditions of ^{3}H-MTPP uptake are as in previous Fig.3. Oligomycin was added 2 min before ^{3}H-MTPP.

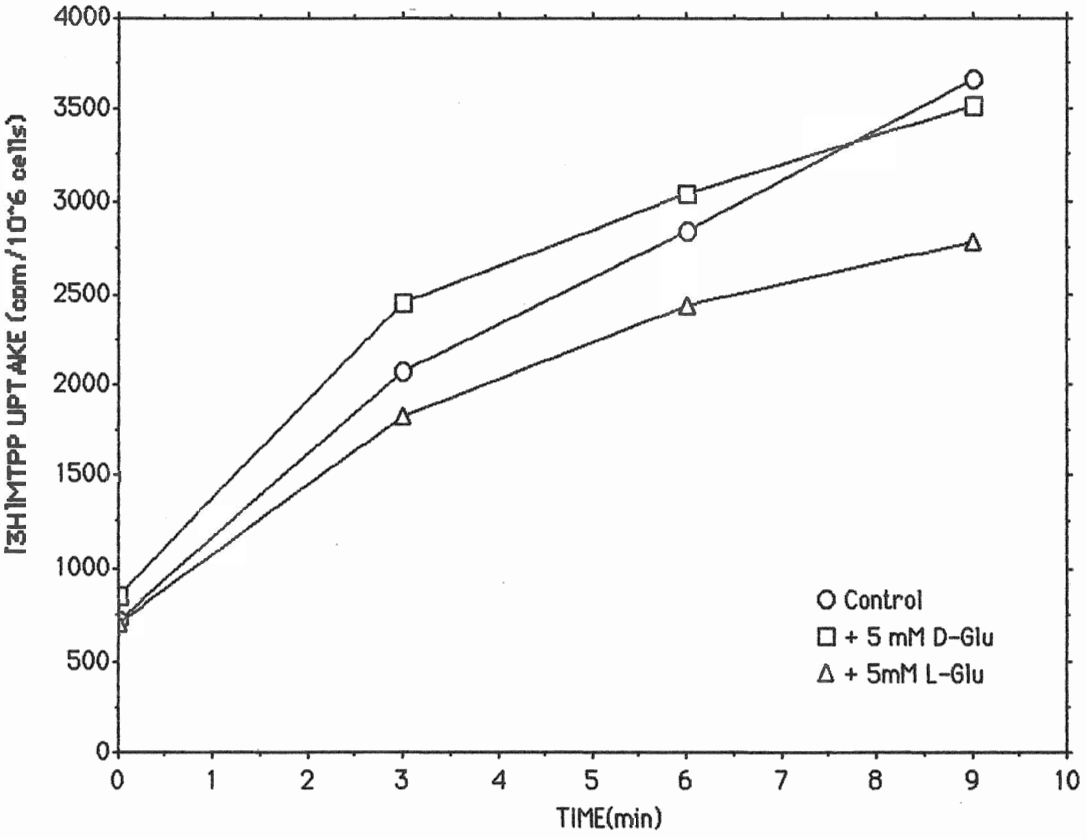
—— The conditions of ^{14}C-U-L-Glu uptake are as in previous Table 7.

9. Comparision between the Influence of L-Glu and D-Glu on ^{3}H-MTPP Uptake

To examine the stereospecific nature of the Glu transport system, the effects of L-Glu and D-Glu on ^{3}H-MTPP uptake were studied. The determination of ^{3}H-MTPP uptake was made using the ^{3}H-MTPP uptake method.

L-Glu was shown to inhibit ^{3}H-MTPP uptake during the 9 minute incubation period. But D-Glu appeared to stimulate ^{3}H-MTPP uptake, especially during the first 6 minutes(Fig. 5). Three minutes after addition of ^{3}H-MTPP, the mean uptake of ^{3}H-MTPP was 2068 cpm/10^{6} cells in the control, 1813 in the presence of L-Glu and 2446 in the presence of D-Glu (Table 12). At six minutes, the mean uptake of ^{3}H-MTPP was 2839 cpm/10^{6} cells in the control, 2430 in the presence of L-Glu and 3042 in the presence of D-Glu (Table 12). This indicates that ^{3}H-MTPP uptake is inhibited by naturally ocurring L-Glu, but not by its stereospecific isomer D-Glu.

Fig. 5. Comparision between the Influence of L-Glu and D-Glu on ^{3}H-MTPP Uptake



These are curves showing the influence of L-Glu and D-Glu on ^{3}H-MTPP uptake. To a reaction vessel containing 15 x 10^{6} cells in 1ml of 5 mM MES · pH 6.0 was added 2.5 X 10^{-4} mM ^{3}H-MTPP (5.55 x 10^{7} dpm). The cell suspension was stirred in ambient light and was preincubated with 5 mM L-Glu, or 5 mM D-Glu for 2 minutes before addtion of ^{3}H-MTPP. Each value is the mean of 3 trials.

Table 12. Comparision between the Influence of L-Glu and D-Glu on ^{3}H-MTPP Uptake

Time (min)	^{3}H-MTPP Uptake (cpm/10^{6} cells)		
	control	+ D-Glu	+ L-Glu
0	727 (225)	849 (58)	715 (160)
3	2068 (390)	2446 (436)	1831 (448)
6	2839 (740)	3042 (906)	2430 (962)
9	3662 (729)	3528 (1215)	2785 (956)

—— Each value is the mean of 3 trials (with S.D. in brackets) .

—— To a reaction vessel containing 15 x 10^{6} cells in 1 ml of 5 mM MES pH 6.0 was added 2.5 X 10^{-4} mM ^{3}H-MTPP (5.55 x 10^{6} dpm).

—— The cell suspension was stirred in ambient light and was preincubated with D-LGlu or L-Glu for 2 minutes before addtion of ^{3}H-MTPP.

—— The concentration of D-Glu added was 5 mM

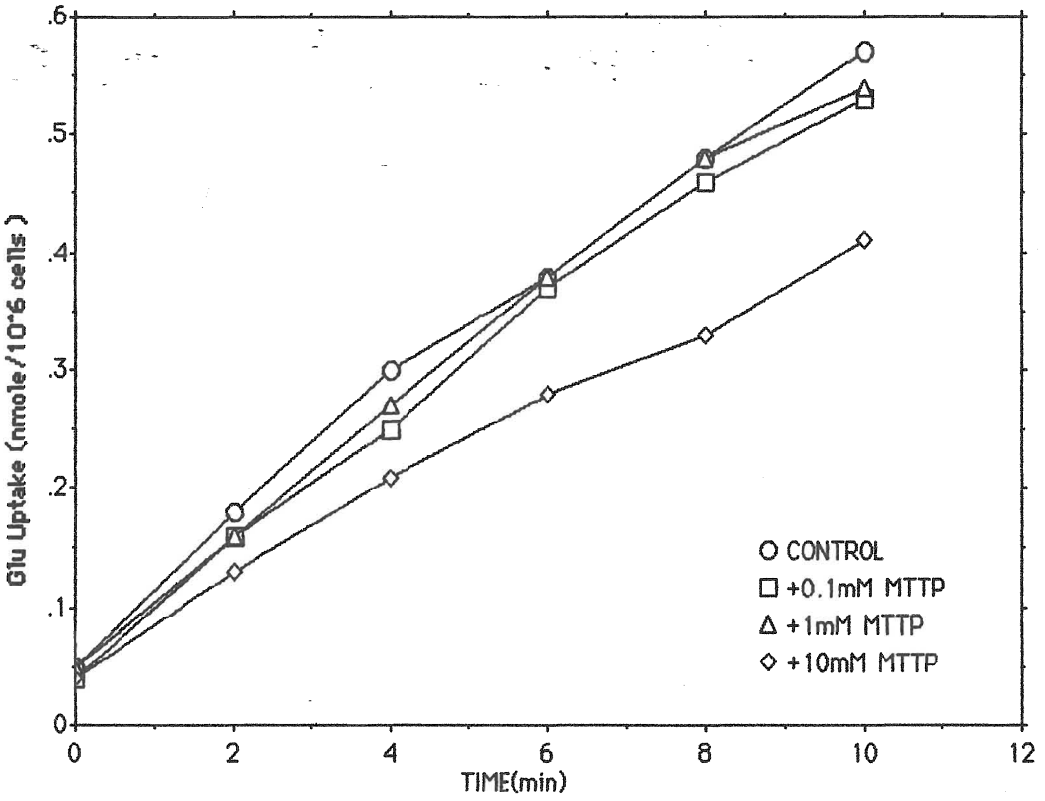
—— The concentration of D-Glu added was 5 mM

10. Influence of MTPP on Total ^{14}C-U-L-Glu Uptake

MTPP is a lipophilic cation whose uptake can depolarize the membrane. If the ^{14}C-U-L-Glu uptake is an electrogenic process, it may be inhibited by the presence of MTPP. To test this hypothesis, the effect of varying MTPP concentrations on total ^{14}C-U-L-Glu uptake was studied using the ^{14}C-U-L-Glu uptake and ^{14}C-GABA release methods. Thirty µl of 33.6 mM ^{14}C-U-L-Glu (4400 dpm/nmole) was added to the cell suspension containing 10 x 10^6 cells in 2 ml of 5 mM MES/1mM $CaSO_4$ pH 6.0 to give 0.5 mM L-Glu. One minute before addition of ^{14}C-U-L-Glu, 0.1mM, 1mM and 10 mM MTPP solutions were added. At zero minute and every two minutes for 10 minutes 100 µl aliquots of the cell suspension were removed and collected on Millipore filters to determine the ^{14}C-U-L-Glu uptake. At 1 minute and every two minutes for 9 minutes 100 µl aliquots of the cell suspension were removed to determine ^{14}C-GABA in the medium.

The data showed that the presence of 0.1 mM or 1 mM MTPP had no appreciable effect on the rate of ^{14}C-U-L-Glu uptake but the presence of 10 mM MTPP inhibited ^{14}C-U-L-Glu uptake from o.o5 (+/- 0.01) to 0.04 (0.00)nmole/10^6 cells· min (Fig. 6 & Table 13). Similarly, GABA efflux was inhibited by 10 mM MTPP from 0.64 (+/- 0.13) to 0.43 (+/- 0.05) nmole/10^6 cells·min (Table 14). When Glu uptake data and GABA release data were combined , inhibition of total ^{14}C-U-L-Glu uptake was 9% and 32% by 1 mM and 10 mM MTPP, respectively (Table 15). The data are consistent with electrogenic uptake of L-Glu; and are complemented by the inhibition of MTPP uptake by L-Glu (Fig. 3). In both cases the results can be interpreted as the inhibition of one electrogenic process by another electrogenic process also moving positive charge into the cells.

Fig. 6. Influence of MTPP concentrations on ^{14}C-U-L-Glu Uptake



These are curves showing the effect of varying MTPP concentrations on ^{14}C-U-L-Glu uptake. To a reaction vessel containing 10 X 10^6 cells in 2 ml of 5 mM MES pH 6.0 was added 0.5 mM ^{14}C-U-L-Glu (5480 dpm/nmole). The cell suspension was stirred in ambient light and was preincubated with 0.1, 1.0 or 10.0 mM MTPP for 1 min before addition of ^{14}C-U-L-Glu. Each value is the mean of 3 trials.

Table 13. Influence of MTTP concentrations on ^{14}C-L-Glu Uptake

	Rate of ^{14}C-L-Glu Uptake			
	control	+ 0.1mM MTPP	+ 1mM MTPP	+ 10mM MTPP
	nmole Glu/min· 10^6 cells			
26 06 89	0.05	0.05	0.05	0.04
27 06 89	0.06	0.05	0.05	0.04
03 07 89	0.05	0.05	0.06	0.04
Mean	0.05	0.05	0.05	0.04
SD	0.01	0.00	0.01	0.00

Conditions as for Fig. 6.

Table 14. Influence of MTPP concentrations on ^{14}C-GABA Release

	^{14}C-GABA in medium			
	control	+ 0.1mM MTPP	+ 1mM MTPP	+ 10mM MTPP
	nmole GABA/min· 10^6 cells			
26 06 89	0.79	0.49	0.56	0.38
27 06 89	0.58	0.56	0.58	0.46
03 07 89	0.55	0.59	0.61	0.46
Mean	0.64	0.55	0.58	0.43
SD	0.13	0.05	0.03	0.05

Conditions as for Fig. 6.

Table 15. The effect of MTPP on the Rates of Total ^{14}C-U-L-Glu Uptake

	Rate of Total ^{14}C-U-L-Glu Uptake			
	control	+ 0.1mM MTPP	+ 1mM MTPP	+ 10mM MTPP
	nmole L-Glu/min· 10^6 cells			
	0.69	0.60	0.63	0.47
% of control	100	87	91	68

Conditions as for Fig. 6.

11. Stoichiometry of H^+/L-Glu Symport

Electrogenic H^+/L-Glu Symport would result from 2 or more H^+ coupled to the transport of each anion of L-Glu entering the cells. Thus an electroneutral process would result from the symport of 1 H^+/L-Glu. The purpose of this experiment was to measure the stoichiometry of H^+/L-Glu symport to determine whether the symport is electrogenic. The rate of ^{14}C-U-L-Glu uptake, the rate of ^{14}C-GABA release and the rate of L-Glu dependent H^+ influx were measured simultaneously. The reaction system consisted of 16 x 10^6 cells in 3.2 ml of 1mM $CaSO_4$ placed in a reaction vessel and a Fisher pH electrode immersed in the cell suspension to trace the changes in pH of the medium. The pH electrode was in turn connected to a pH meter and recorder (Method 2.9). At zero minute and every two minutes for 10 minutes 100 µl aliquots of the cell suspension were removed and collected on Millipore filters to determine the ^{14}C-U-L-Glu uptake. At 1 minute and every two minutes for 9 minutes 100 µl aliquots of the cell suspension were removed to determine ^{14}C-GABA in the medium. The rate of net alkalinization was calculated from the trace on the pH recording chart. Removing 0.5 x 10^6 cells every minute changes the buffering capacity of the system and interferes with the calculation of the rate of net alkalinization. To compensate, the buffering capacity of 0.5 x 10^6 cells was determined at the beginning of the experiment. Rates of all three processes were calculated as nmole/10^6 cells · min.

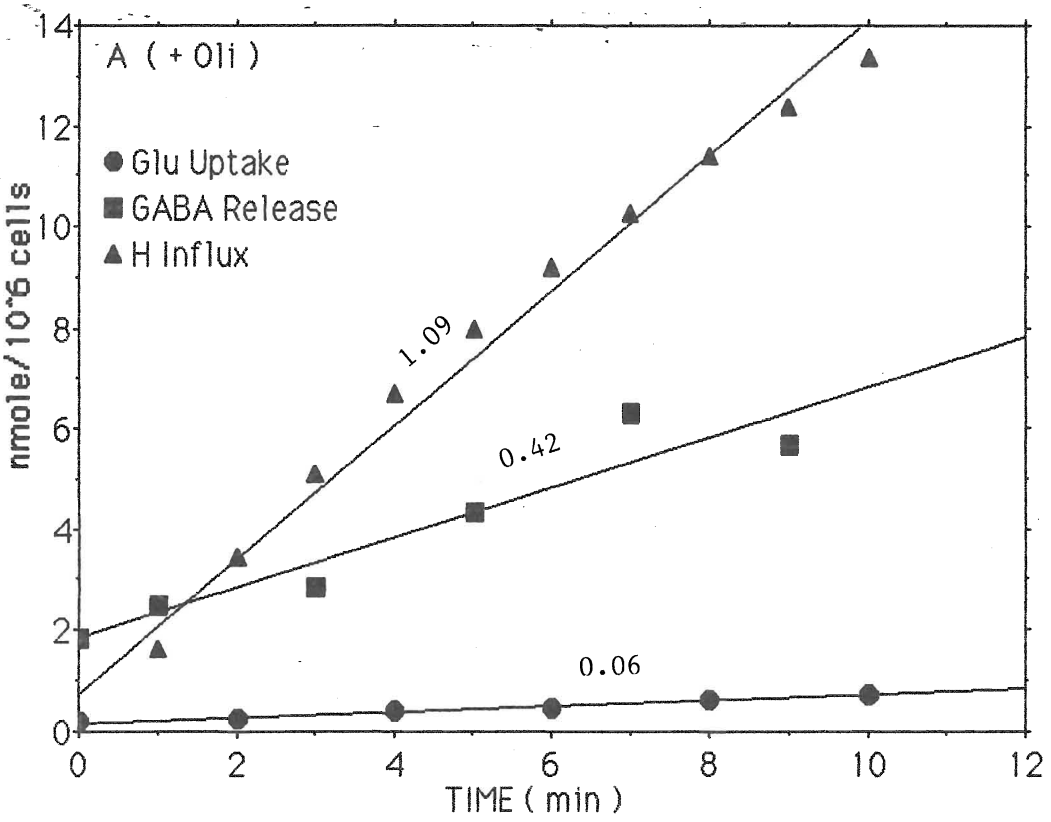
Because H^+/L-Glu symport may cause acidification of the cytoplasm, it may stimulate the activity of the plasma membrane ATPase pumping H^+ out. Therefore, the experiment was conducted in the presence and absence of oligomycin.

In the absence of oligomycin , rates of L-Glu uptake , GABA release and L-Glu dependent H^+ influx were 0.07, 0.52 and 0.69 nmole/10^6 cells · min, respectively. However, in the presence of oligomycin, the corresponding rates were 0.06, 0.42

and 1.09 nmole/10^6 cells · min (Fig. 7 and Table 16, 17, 18). When the three rates were used to calculate the stoichiometry of H^+/L-Glu symport , the values obtained were approximately 2.4 /1 and 1.2 / 1 in the the presence and absence of oligomycin, respectively (Table 19). Thus, when H^+ efflux is inhibited with oligomycin, there is an approximate 100% increase in the measured stoichiometry of the H^+/L-Glu symport system. This suggests a molar stoichiometry of 2 protons for every anion of L-Glu transported. In which case, the symport would be electrogenic.

Fig. 7. Stoichiometry of H^+/Glu Symport
(with and without oligomycin)

These are curves showing the relationship between ^{14}C-U-L-Glu uptake, ^{14}C-GABA release and L-Glu dependent H^+ influx. To a reaction vessel containing 15 X 10^6 cells in 3 ml of 1mM $CaSO_4$ was added 1 mM ^{14}C-U-L-Glu (3311 dpm/nmole). The cell suspension was stirred in ambient light. When 10 μg/ml oligomycin was used, it was added 5 min before addition of ^{14}C-U-L-Glu. At indicated times, 100 μl of the cell suspension containing 0.5 X 10^6 cells was removed to measure ^{14}C-U-L-Glu uptake or ^{14}C-GABA release. Before and after addition of ^{14}C-U-L-Glu, the contents of the reaction vessel were titrated with 1mM H_2SO_4 or 1 mM NaOH to measure the buffering capacity of the medium and to calculate the rate of net medium alkalinization. A. With oligomycuin, B. without oligomycin. Each experiment was repeated three times. The units indicated for the slope are nmole/10^6 cells min.


A (+ Oli)
Glu Uptake
GABA Release
H Influx
1.09
0.42
0.06
nmole/10^6 cells
TIME (min)
0
2
4
6
8
10
12
14

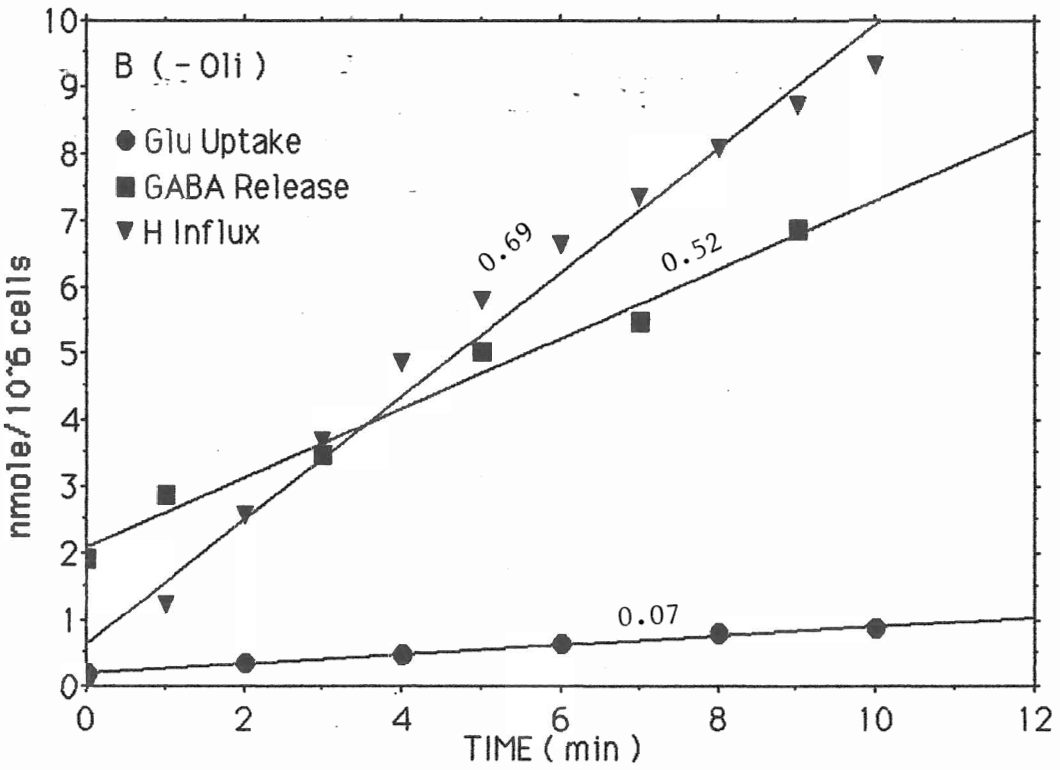

B (- Oli)
Glu Uptake
GABA Release
H Influx
0.69
0.52
0.07
nmole/10^6 cells
TIME (min)
0
2
4
6
8
10
12
1
3
5
7
9

Table 16. ^{14}C-U-L-Glu Uptake, ^{14}C-U-GABA Release and L-Glu Dependent H^+ Influx (with oligomycin)

Time (min)	nmole/10^6 cells		
	^{14}C-L-Glu Uptake	^{14}C-GABA Release	H^+ Influx
0	0.20 (o.o3)	1.88 (0.31)	0.00 (0.00)
1	—	2.48 (0.35)	1.64 (0.39)
2	0.25 (0.02)	—	3.44 (0.78)
3	—	2.89 (0.10)	5.11 (1.17)
4	0.40 (0.04)	—	6.70 (1.48)
5	—	4.35 (0.66)	7.99 (1.54)
6	0.49 (0.02)	—	9.23 (1.73)
7	—	6.34 (0.56)	10.30 (1.76)
8	0.65 (0.11)	—	11.41 (1.85)
9	—	5.72 (1.19)	12.41 (1.94)
10	0.75 (0.05)	—	13.35 (1.93)

Conditions as for Fig. 7. values indicated (+/- SD)

Table 17. ^{14}C-U-L-Glu Uptake, ^{14}C-U-GABA Release and L-Glu Dependent H^+ Influx (without oligomycin)

Time (min)	nmole/10^6 cells		
	^{14}C-L-Glu Uptake	^{14}C-GABA Release	H^+ Influx
0	0.20 (0.01)	1.90 (0.72)	0.00 (0.00)
1	—	2.88 (0.53)	1.25 (0.19)
2	0.35 (0.06)	—	2.55 (0.41)
3	—	3.49 (0.72)	3.70 (0.71)
4	0.49 (0.07)	—	4.83 (1.16)
5	—	5.05 (2.01)	5.80 (1.38)
6	0.63 (0.06)	—	6.62 (1.29)
7	—	5.47 (0.81)	7.38 (1.76)
8	0.78 (0.04)	—	8.08 (1.81)
9	—	6.87 (1.45)	8.71 (1.88)
10	0.90 (0.08)	—	9.34 (1.88)

Conditions as for Fig. 7. Values indicated (+/- SD).

Table 18. Mean Rates of L-Glu Uptake, GABA Release and L-Glu Dependent H^+ Influx

	Rate (nmole/min · 10^6 cells)		
	^{14}C-L-Glu Uptake	^{14}C-GABA Release	H^+ Influx
- Oligomycin			
Mean	0.07	0.52	0.69
SD	0.01	0.10	0.11
+Oligomycin			
Mean	0.06	0.42	1.09
SD	0.01	0.11	0.15

Conditions as fop Fig. 7.

Note that the stoichiometry of uptake can be calculated from these values.

Table 19. Stoichiometry of H^+/Glu Symport

$$n = \frac{Rate_{H^+}}{Rate_{Glu} + Rate_{GABA}}$$

	- Oligomycin	+ Oligomycin
Expt. 21-11-89	1.15	2.17
Expt. 22-11-89	1.14	1.92
Expt. 23-11-89	1.25	2.87
Mean	1.18	2.39
SD	0.16	0.28

Conditions as for in Fig. 7.

B. Does K^+ Efflux compensate for Electrogenic H^+/L-Glu Symport?

The activity of an electrogenic proton L-glutamate symport process would result in the collapse of the driving forces, the membrane potential ($\Delta\Psi$), the proton chemical gradient (ΔpH) and the glutamate concentration gradient, across the plasma membrane. To maintain the membrane potential, there should be a process to compensate for the electrogenic symport. K^+ efflux may be a compensating process and act as a repolarizing current to help reestablish and maintain the membrane potential. In this section are described experiments designed to determine the relationship between L-Glu dependent H^+ influx and K^+ efflux.

1. The Influence of L-Glu on H^+ and K^+ Flux (with Oligomycin)

The purpose of this experiment was to determine the response of K^+ flux to the introduction of L-Glu to the cell suspension medium when ATP dependent H^+ efflux was inhibited by oligomycin. The determination was made using a double pen recorder for H^+ and K^+. Oligomycin was introduced to the cell suspension medium 6 minutes before addition of L-Glu.

Before addition of oligomycin, K^+ in the medium slowly increased. This indicated that the cells released K^+ to the medium when they were added to the medium (Fig. 8). The rate of K^+ efflux was not affected by the presence of oligomycin (Fig. 8). When L-Glu was in turn added to the medium, the rate of K^+ efflux did not change although an L-Glu dependent medium alkalinization occurred (Fig. 8).

The rates of both H^+ influx and K^+ efflux before and after the addition of L-Glu are shown on Table 20. The mean rate of H^+ influx increased from 0.59 nmole H^+/min · 10 x 10^6 cells to 2.14 upon addition of L-Glu, indicating that the percent stimulation of H^+ influx by L-Glu was 263% (Table 20). Because L-Glu increases the buffering capacity, the rates of H^+ influx before and after L-Glu addition have to be calculated using the different buffering capacity obtained by titration at the beginning and end of the experiment (Fig. 8). Meanwhile, the rate of K^+ efflux only increased from 2.22 to 2.63 (Table 20). The calculated percent stimulation of K^+ efflux was 18%. Thus, whereas H^+ influx increased by 2.73 nmole/10 x 10^6 · min, the corresponding increase for K^+ efflux was only 0.41, indicating that K^+ efflux could only account for a minor portion of charge entering the cell.

Fig. 8. The Influence of L-Glu on Flux of H^+ and K^+

These are tracings showing the flux of H^+ and K^+ in response to addition of L-Glu. To a reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ and 0.05 mM K_2SO_4 was added 10 μg/ml oligomycin. Six min after addition of oligomycin, 5 mM L-Glu was added. The cell suspension was stirred and aerated in the dark. At the end of the experiment, the contents of the reaction vessel were titrated with 1 mM H_2SO_4 and 10 mM K_2SO_4. Rates of alkalinization are expressed in nmole H^+/min · 10^6 cells and rates of K^+ efflux are expressed in nmole K^+/min · 10^6 cells.

L-Glu Dependent H+ Influx and K+ Efflux

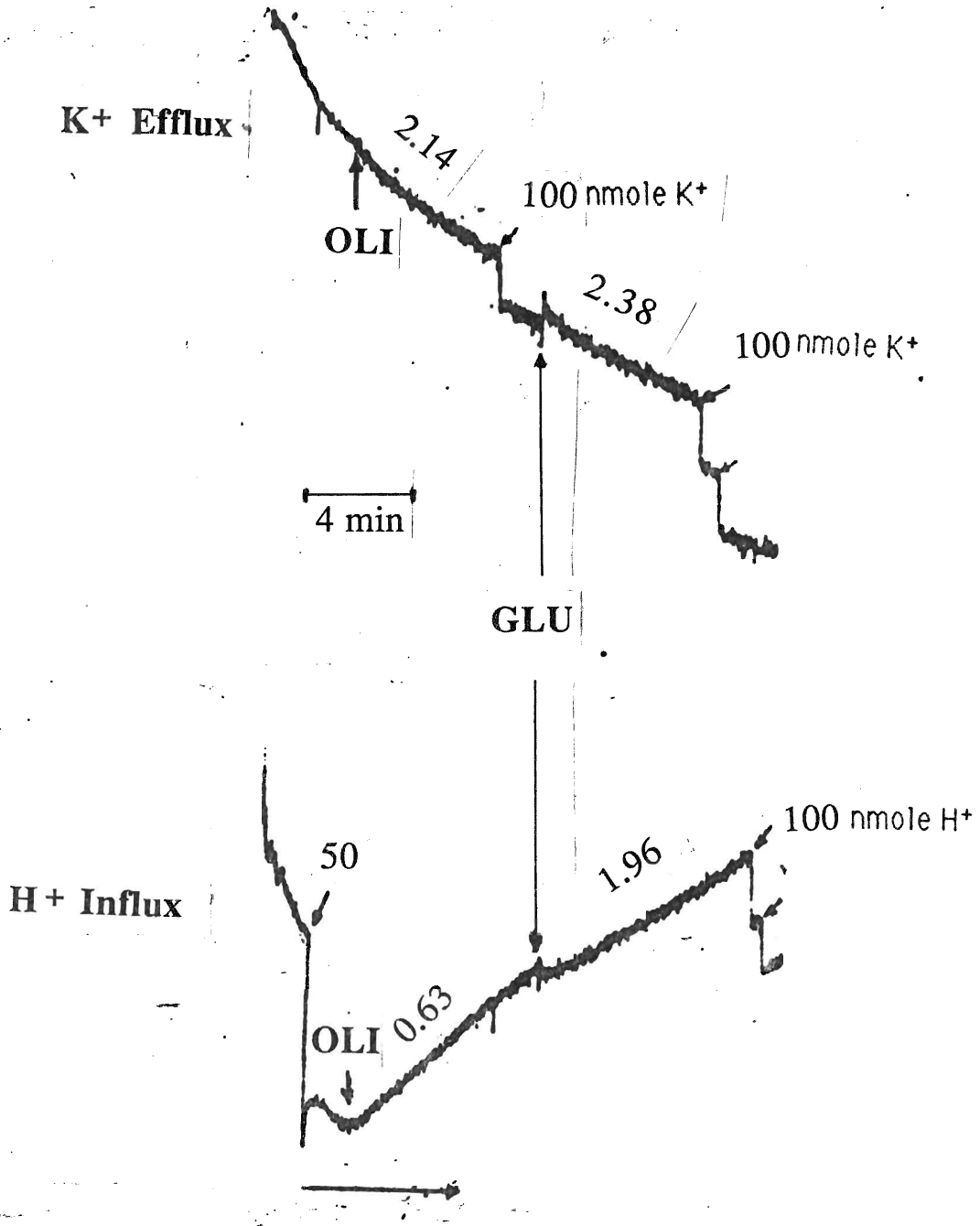


Table 20. The Influence of L-Glu on Flux of H^+ and K^+
(with 10 µg/ml oligomycin)

Conditions:

——— Rates of H^+ influx are expressed in nmole H^+/min · 10^6 cells.

——— Rates of K^+ efflux are expressed in nmole K^+/min · 10^6 cells.

——— The reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ and 0.05 mM K_2SO_4.

——— The cell suspension was stirred in the dark.

——— The concentration of oligomycin in the reaction vessel was 10 µg/ml and was added to the cell suspension medium 6 min before addition of L-Glu

——— The concentration of L-Glu in the reaction vessel was 5 mM.

——— At the end of the experiment, the contents of the reaction vessel were titrated with known volume of 1mM H_2SO_4 and 10 mM K_2SO_4 in order calibrate the system prior to calculation of rates of flux.

	Rate (nmole /min $\cdot 10^6$ cells)		
	Before adding L-Glu	After adding L-Glu	% Stimulation
		H^+ influx	
Expt. 1	0.63	1.96	
Expt. 2	0.87	2.08	
Expt. 3	0.28	2.38	
Mean	0.59	2.14	263
SD	0.30	0.21	
		K^+ efflux	
Expt. 1	2.14	2.38	
Expt. 2	3.13	2.78	
Expt. 3	1.39	2.08	
Mean	2.22	2.41	9
SD	0.87	0.35	

2. The Influence of Na^+ on H^+ and K^+ Flux (with Oligomycin)

The purpose of this experiment was to determine the effect of Na^+ on the flux of H^+ and K^+. H^+ efflux was inhibited by oligomycin.

The mean rate of H^+ influx decreased from 0.65 to 0.25 nmole H^+/min · 10^6 cells (Table 21). Meanwhile, the mean rate of K^+ efflux increased from 2.26 to 3.98 nmoles K^+/min · 10^6 cells(Table 21). This showed that the presence of Na^+ stimulated K^+ efflux by 76%. Thus, although addition of depolarizing L-glutamate did not cause K^+ efflux, Na^+ addition did. This result is consistent with a depolarization in response to Na^+ being compensated to some extent by a K^+ efflux process.

Table 21. The Influence of Na^+ on Flux of H^+ and K^+ (with 10 ug/ml oligomycin)

Conditions:

——— Rates of H^+ influx are expressed in nmole H^+/min · 10^6 cells.

——— Rates of K^+ efflux are expressed in nmole K^+/min · 10^6 cells.

——— The reaction vessel containing 10 X 10^6 cells in 10 ml of 1 mM $CaSO_4$ and 0.05 mM K_2SO_4.

——— The cell suspension was stirred in the dark.

——— The concentration of oligomycin in the reaction vessel was 10 µg/ml and was added to the cell suspension medium 6 min before addition of Na_2SO_4

——— The concentration of Na^+ in the reaction vessel was 40 mM.

——— At the end of the experiment, the contents of the reaction vessel were titrated with known volume of 1 mM H_2SO_4 and 10 mM K_2SO_4 in order calibrate the system prior to calculation of rates of flux.

	Rate (nmole /10^6 cells·min)		% Control
	Before adding Na_2SO_4	After adding Na_2SO_4	
		H^+ influx	
Expt. 1	0.87	0.25	
Expt. 2	0.78	0.27	
Expt. 3	0.29	0.24	
Mean	0.65	0.25	38.5
SD	0.31	0.02	
		K^+ efflux	
Expt. 1	1.85	4.35	
Expt. 2	3.06	5.00	
Expt. 3	1.85	2.60	
Mean	2.26	3.98	176.1
SD	0.70	1.24	

3. The Influence of DES, Erythrocin B, Oligomycin and Fusicoccin on the Fluxes of H^+ and K^+

In the absence of a significant K^+ efflux in response to L-Glu dependent H^+ influx, the relationship between these two fluxes was investigated to clarify the negative results obtained with L-Glu. The flux of H^+ and K^+ in response to the addition of DES, erythrocin B, oligomycin and fusicoccin was measured. The experiments were performed using the double pen recorder method in a cell suspension medium consisting of 1 mM $CaSO_4$ and 0.05 mM K_2SO_4. Upon addition of 100 μM DES, H^+ efflux was changed to H^+ influx and K^+ efflux was stimulated (Fig. 9). The mean rates of the net $H^+$and K^+ fluxes are shown in Table 23. The presence of DES caused a rate of alkalinization of 2.07 nmole H^+/min · 10^6 cells, and stimulated the rate of K^+efflux from 2.52 to 19.33 nmole K^+/min · 10^6 cells.

In other experiments, oligomycin, erythrocin B and fusicoccin were added. The data show that the addition of oligomycin caused a rate of alkalinization of 2.35 nmole H^+/min · 10^6 cells but did not stimulate K^+ efflux. The addition of erythrocin B caused a rate of alkalinization of 3.78 nmole H^+/min · 10^6 cells and increased the rates of K^+ efflux from 1.25 to 5.03 nmole K^+/min · 10^6 cells. The addition of fusicoccin caused a rate of acidification of 1.48 nmole H^+/min · 10^6 cells and decreased the rate of K^+ efflux from 3.35 to 2.35 nmole K^+/min · 10^6 cells (Table 23).

Thus, the use of DES and erythrocin B indicated that H^+ influx was correlated with K^+ efflux. Similarly, fusicoccin which stimulated H^+ efflux inhibited the rate of K^+ efflux (Table 22, 23). Thus in other treatments, there appears to a relationship between the flux of H^+ and K^+. However, this relationship was not found with L-Glu.

Fig. 9 The Influence of DES on the Fluxes of H^+ and K^+

These are tracings showing the flux of $H^+$and K^+ in response to the addition of DES. To a reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ and 0.05 mM K_2SO_4 was added 100 µM DES. The cell suspension was stirred and aerated in the dark. At the end of the experiment, the contents of the reaction vessel were titrated with known volumes of 1 mM H_2SO_4 and 10 mM K_2SO_4. Rates of alkalinization are expressed as nmole H^+/min · 10^6 cells and rates of K^+ efflux are expressed as nmole K^+/min · 10^6 cells.

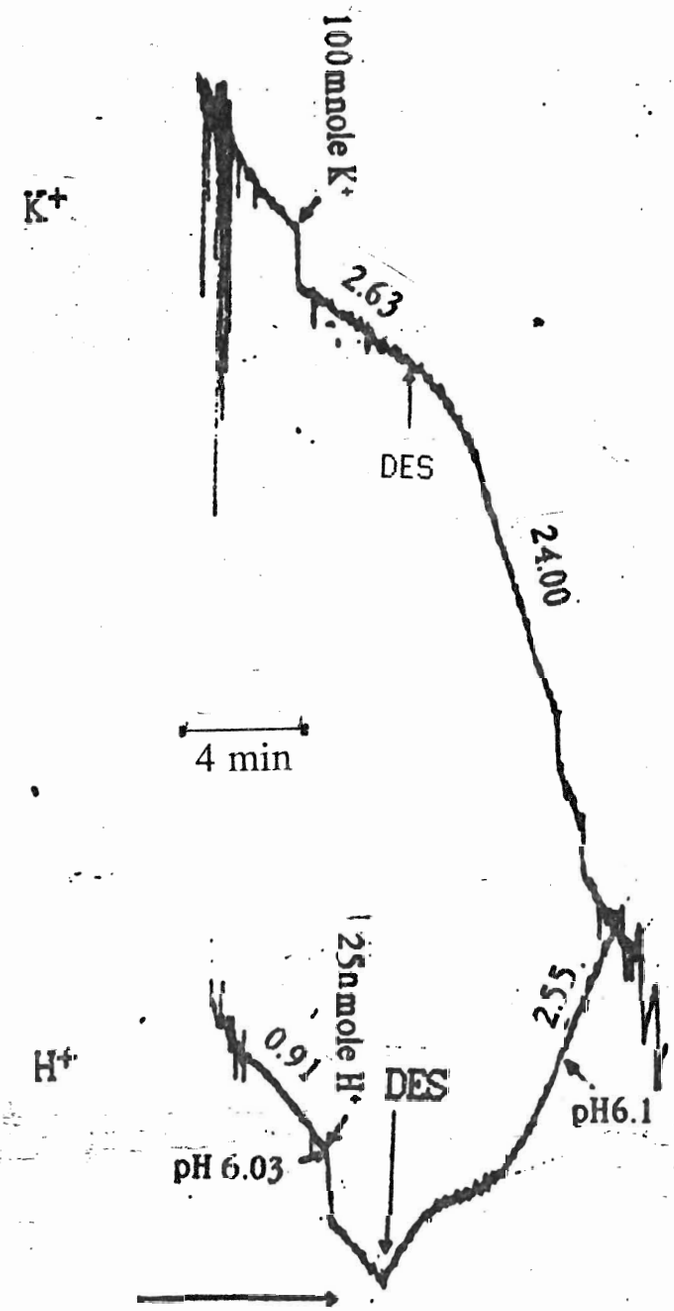

100 mmole K+
K+
2.63
DES
24.00
4 min
125 mmole H+
0.91
2.55
H+
DES
pH6.1
pH 6.03

Table 22. Influence of DES on Flux of H^+ and K^+

Conditions:

——— Rates of H^+ influx as net medium alkalinization are expressed in nmole H^+/min 10^6 cells.

——— Rates of K^+ efflux are expressed in nmole K^+/min · 10^6 cells.

——— The reaction vessel contained 10 X 10^6 cells in 10 ml of 1 mM $CaSO_4$ and 0.05 mM K_2SO_4.

——— The cell suspension was stirred in the dark.

——— The concentration of DES in the reaction vessel was 100 µM.

——— At the end of the experiment, the contents of the reaction vessel were titrated with 1 mM H_2SO_4 and 10 mM K_2SO_4.

——— Negative value indicates H^+ influx.

	Rate (nmole /10^6 cells·min)	
	Before adding DES	After adding DES
	H^+ flux	
Expt. 1	0.91	-2.55
Expt. 2	0.50	-3.33
Expt. 3	0.16	-0.33
Mean	0.53	-2.07
SD	0.37	1.56
	K^+ efflux	
Expt. 1	2.63	24.00
Expt. 2	1.92	24.00
Expt. 3	3.00	10.00
Mean	2.52	19.33
SD	0.55	8.08

Table 23. Influence of Erythrocin B, Oligomycin and Fusicoccin on the Fluxes of H^+ and K^+

Conditions:

- Each value is the mean of 3 trials (with S.D. in brackets)
- Rates of H^+ influx as net medium alkalinization are expressed in nmole H^+/min · 10^6 cells.
- Rates of K^+ efflux are expressed in nmole K^+/min · 10^6 cells.
- The reaction vessel contained 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ and 0.05 mM K_2SO_4.
- The cell suspension was stirred in the dark.
- The concentrations of oligomycin, erythrocin B or fusicoccin in the reaction vessel were 10 µg/ml, 100 µM or 100 µM, respectively.
- At the end of the experiment, the contents of the reaction vessel were titrated with 1mM H_2SO_4 and 10 mM K_2SO_4.

	Rate (nmole/min · 10^6 Cells)	
	H^+ Flux	K^+ Flux
Before addition of oli.	-o.77	4.44
	(0.62)	(0.64)
After addition of oli.	-2.35	4.05
	(1.06)	(0.80)
Before addition of EB	-2.27	1.26
	(0.29)	(0.22)
After addition of EB	-3.78	5.03
	(0.68)	(1.06)
Before addition of FC	-1.08	3.35
	(0.28)	(0.34)
After addition of FC	1.48	2.35
	(0.56)	(0.40)

C. Does H^+ Efflux compensate for the Electrogenic H^+/L-Glu Symport?

An electrogenic proton symport system will depolarize the membrane potential and acidify cytoplasm. Changes in both the membrane potential and pH of the cytoplasm may stimulate the plasma membrane ATPase to pump H^+ out. Thus the electrogenic ATP dependent H^+ efflux may regulate the membrane potential and internal pH driving the electrogenic proton symport. The experiments described in this section were designed to determine the relationship between the ATP dependent H^+ efflux and H^+/L-Glu symport.

1. Influence of K^+ and Na^+ on H^+ Efflux

The transport of K^+ and Na^+ into cells is thought to depolarize the membrane potential. If H^+ efflux acted as a compensating process for this electrogenic transport, it would increase in response to K^+ or Na^+ influx. The purpose of this experiment was to investigate this response under the conditions of both light and dark. The investigation was made using the pH vs time method (Method 2.4).

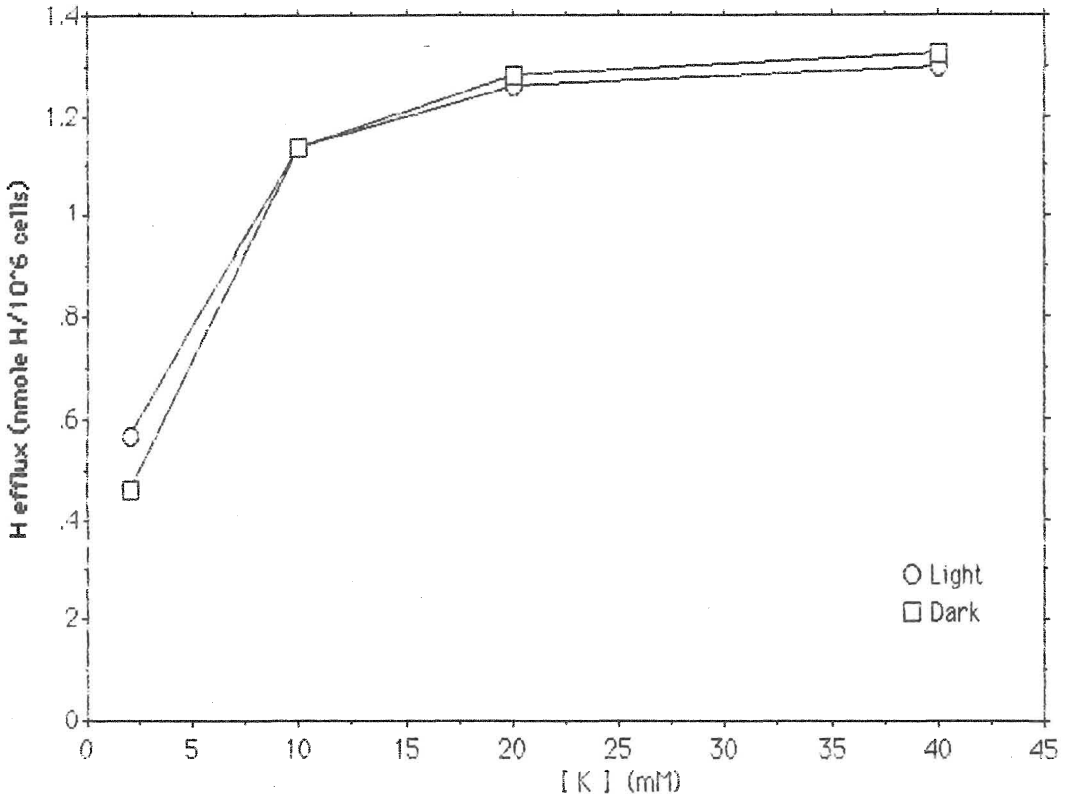
Fig. 10 and 11 show that H^+ efflux is increased with increases in the concentrations of K^+ as K_2SO_4. or Na^+ as Na_2SO_4. No significant difference were obtained between the dark and the light.

In the presence of 2, 10, 20 and 40 mM K^+, the mean rates of H^+ efflux were 0.46, 1.16, 1.36 and 1.35 nmole H^+/min · 10^6 cells in the dark and 0.40, 0.89, 1.14 and 1.12 nmole H^+/min · 10^6 cells in the light(Table 24).

In the presence of 2, 10, 20 and 40 mM Na^+, the mean rates of H^+ efflux were 0.50, 0.83, 1.04 and 1.18 nmole H^+/min · 10^6 cells in the dark and 0.37, 0.85, 1.00 and 1.07 nmole H^+/min · 10^6 cells in the light(Table 25).

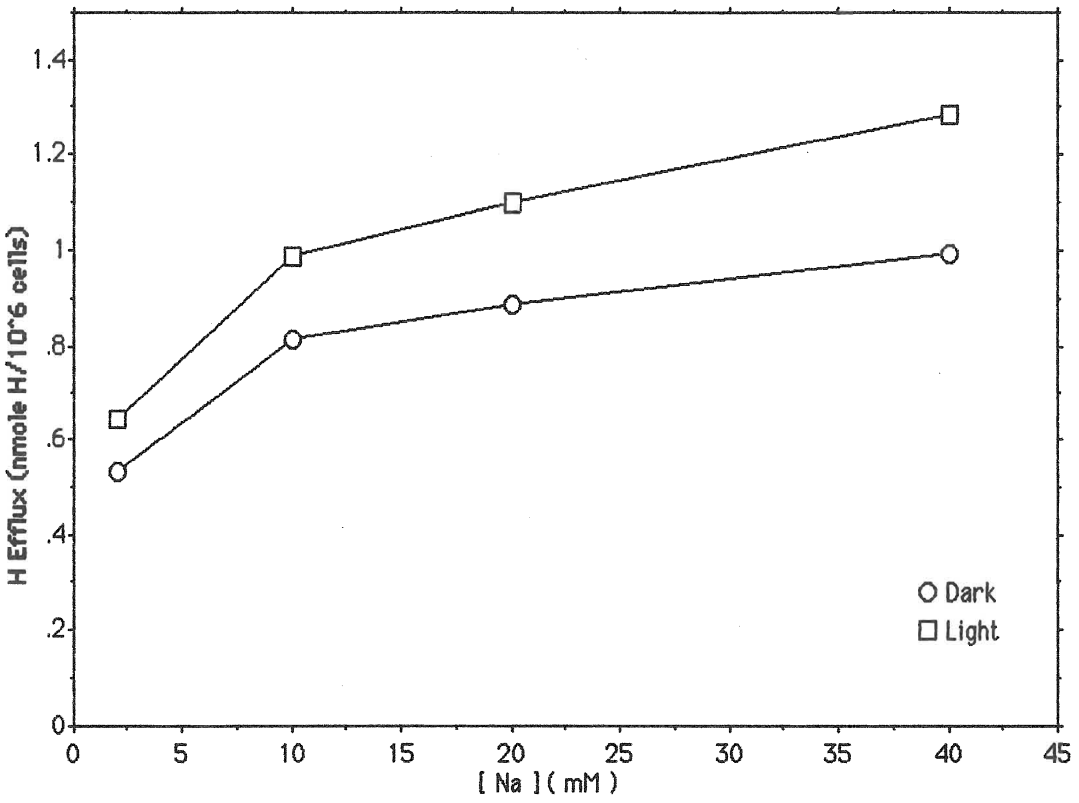
The results show that H^+ efflux increases in response to the presence of K^+ or Na^+ addition to the cell suspension medium. This result is consistent with H^+ efflux being activated in the presence of a depolarizing process such as K^+ influx or H^+/L-Glu symport.

Fig. 10. Effect of Varying K^+ Concentrations and H^+ Efflux



These are curves showing that K^+ stimulates H^+ efflux. To a reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ was added 2, 10, 20 or 40 mM K^+. The cell suspension was stirred and aerated in the dark or light. At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH. Each value is the mean of 3 trials.

Fig. 11. Effect of Varying Na^+ Concentrations on H^+ Efflux



These are curves showing that Na^+ stimulates H^+ efflux. To a reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ was added 2, 10, 20 or 40 mM Na^+. The cell suspension was stirred and aerated in the dark or light. At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH. Each value is the mean of 3 trials.

Table 24. The Influence of K^+ on the Efflux of H^+

		[K^+] (mM)			
		2	10	20	40
		Rate (nmole $H^+/10^6$ cells·min)			
Dark					
18 05 88	Expt. 1	0.36	0.56	0.66	0.58
20 05 88	Expt. 2	0.60	1.10	1.07	0.88
27 05 88	Expt. 3	0.44	1.82	2.40	2.60
	Mean	0.46	1.16	1.36	1.35
	SD	0.13	0.64	0.93	1.09
Light					
18 05 88	Expt. 1	0.40	0.51	0.71	0.79
20 05 88	Expt. 2	0.50	0.84	1.28	1.26
30 05 88	Expt. 3	0.30	1.31	1.42	1.12
	Mean	0.40	0.89	1.14	1.06
	SD	0.10	0.41	0.38	0.24

——— To a reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ was added 2, 10, 20 or 40 mM K^+.

——— The cell suspension was stirred and aerated in the dark or light.

——— At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH.

Table 25. Influence of Na^+ on Efflux of H^+

	[Na^+] (mM)			
	2	10	20	40
	Rate (nmole $H^+/10^6$ cells·min)			
Dark				
19 05 88 Expt. 1	0.48	0.98	1.17	1.52
24 05 88 Expt. 2	0.42	0.64	0.63	0.81
27 05 88 Expt. 3	——	0.88	1.28	1.21
Mean	~~0.50~~ 0.45	0.83	1.04	1.18
SD	0.26	0.17	0.33	0.35
Light				
19 05 88 Expt. 1	0.57	0.88	1.23	1.32
24 05 88 Expt. 2	0.17	0.68	0.60	0.85
30 05 88 Expt. 3	0.39	0.99	1.17	1.04
Mean	0.37	0.85	1.00	1.07
SD	0.16	0.16	0.35	0.24

—— To a reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ was added 2, 10, 20 or 40 mM Na^+.

—— The cell suspension was stirred and aerated in the dark or light.

—— At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH.

2. The Effect of DES, Oligomycin, DCCD and Fusicoccin on K^+ Stimulated H^+ Efflux

The purpose of this experiment was to see if K^+ stimulated H^+ efflux was driven by the plasma membrane ATPase. Such H^+ efflux would be inhibited by the inhibitors of the ATPase or ATP synthesis such as DES, DCCD and oligomycin or stimulated by fusicoccin. The experiment was performed using the pH vs time method (Method 2.4). All factors affecting the activity of the plasma membrane ATPase were added 6 min after 20 mM K^+ was introduced to the cell suspension.

Fig. 12 and 13 show that the medium acidification induced by K^+ was completely stopped after addition of DES or DCCD and then changed to alkalinization. The presence of oligomycin (light or dark) or antimycin (dark) inhibited K^+ stimulated medium acidification by 100% but did not cause medium alkalinization. In the light, antimycin inhibited K^+ stimulated medium acidification slightly. The mean rates of H^+ efflux are shown in Table 26 (+ DES), Table 27 (+ DCCD), Table 28 (+ oligomycin) and Table 29 (+ antimycin). These results show that K^+ stimulated H^+efflux was completely inhibited by DES, DCCD or oligomycin both in the light and in the dark, and by antimycin in the dark. They are consistent with K^+ induced H^+ efflux being mediated by the plasma membrane proton ATPase.

K^+ stimulated medium acidification became stronger upon addition of fusicoccin both in the dark and in the light. The mean rates of K^+ stimulated efflux in the presence of fusicoccin are shown in Table 30. Fusicoccin stimulated K^+ induced H^+ efflux by 160% in the dark and 190% in the light.

The results described above indicated that K^+ stimulated H^+ efflux was ATP dependent.

Fig. 12. The Effect of DES, Oligomycin and DCCD on K^+ Stimulated H^+ Efflux in the Dark

These are pH vs time tracings showing the influence of DES, oligomycin and DCCD on K^+ stimulated H^+ efflux in the dark. To a reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ was added 20 mM K^+. The cell suspension was stirred and aerated in the dark. 100 µM DES, 10 µg/ml oligomycin or200 µM DCCD was added to the reaction vessel 6 min after the addition of K^+. At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH.

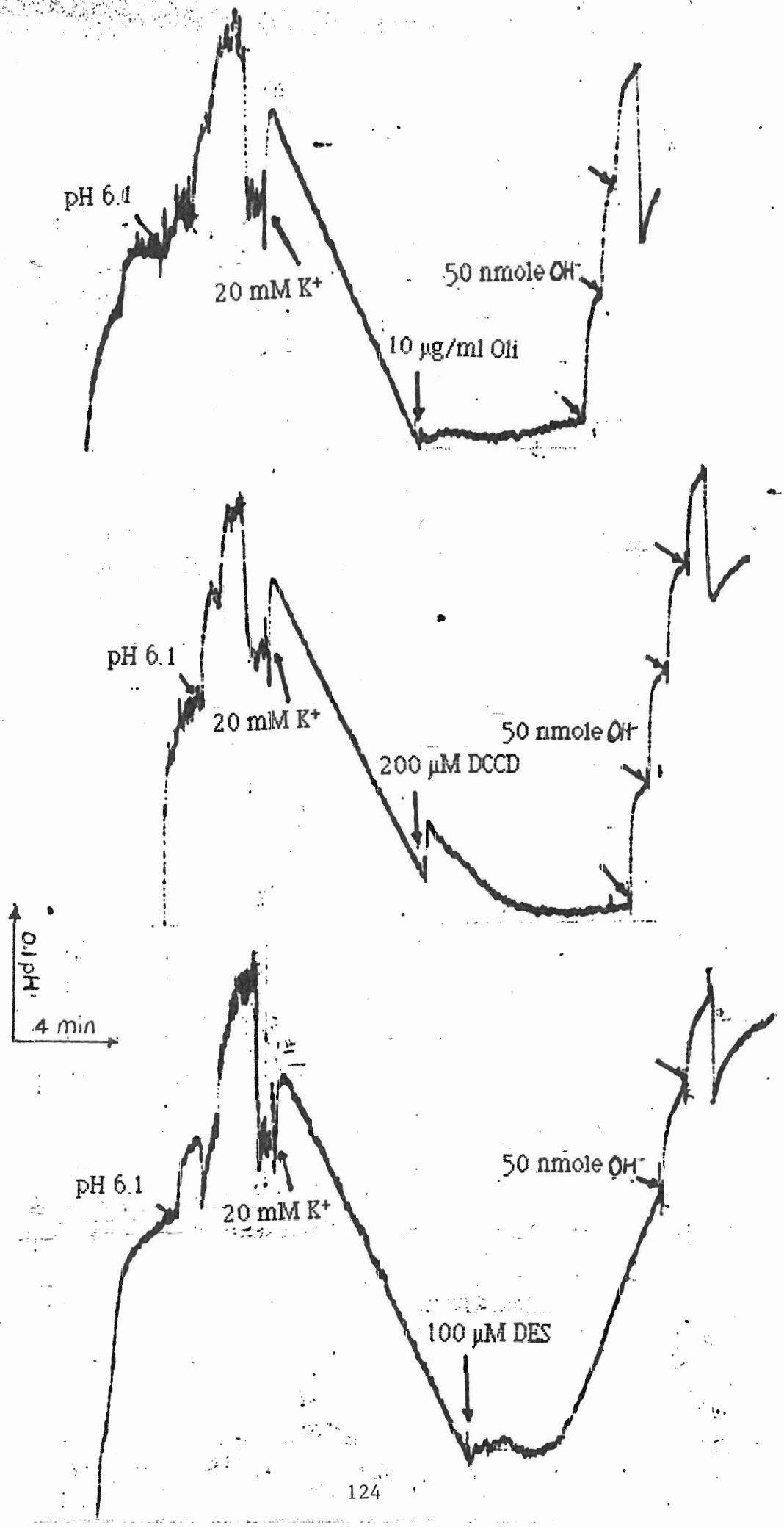

pH 6.1
20 mM K+
50 nmole OH-
10 µg/ml Oli
pH 6.1
20 mM K+
50 nmole OH-
200 µM DCCD
0.1 pH
4 min
pH 6.1
20 mM K+
50 nmole OH-
100 µM DES

Fig. 13. The Effect of DES, Oligomycin and DCCD on K^+ Stimulated H^+ Efflux in the Light

These are pH vs time tracings showing the influence of DES, oligomycin and DCCD on K^+ stimulated H^+ efflux in the light. To a reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ was added 20 mM K^+. The cell suspension was stirred and aerated in the dark. 100 µM DES, 10 µg/ml oligomycin or 200 µM DCCD was added to the reaction vessel 6 min after the addition of K^+. At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH.

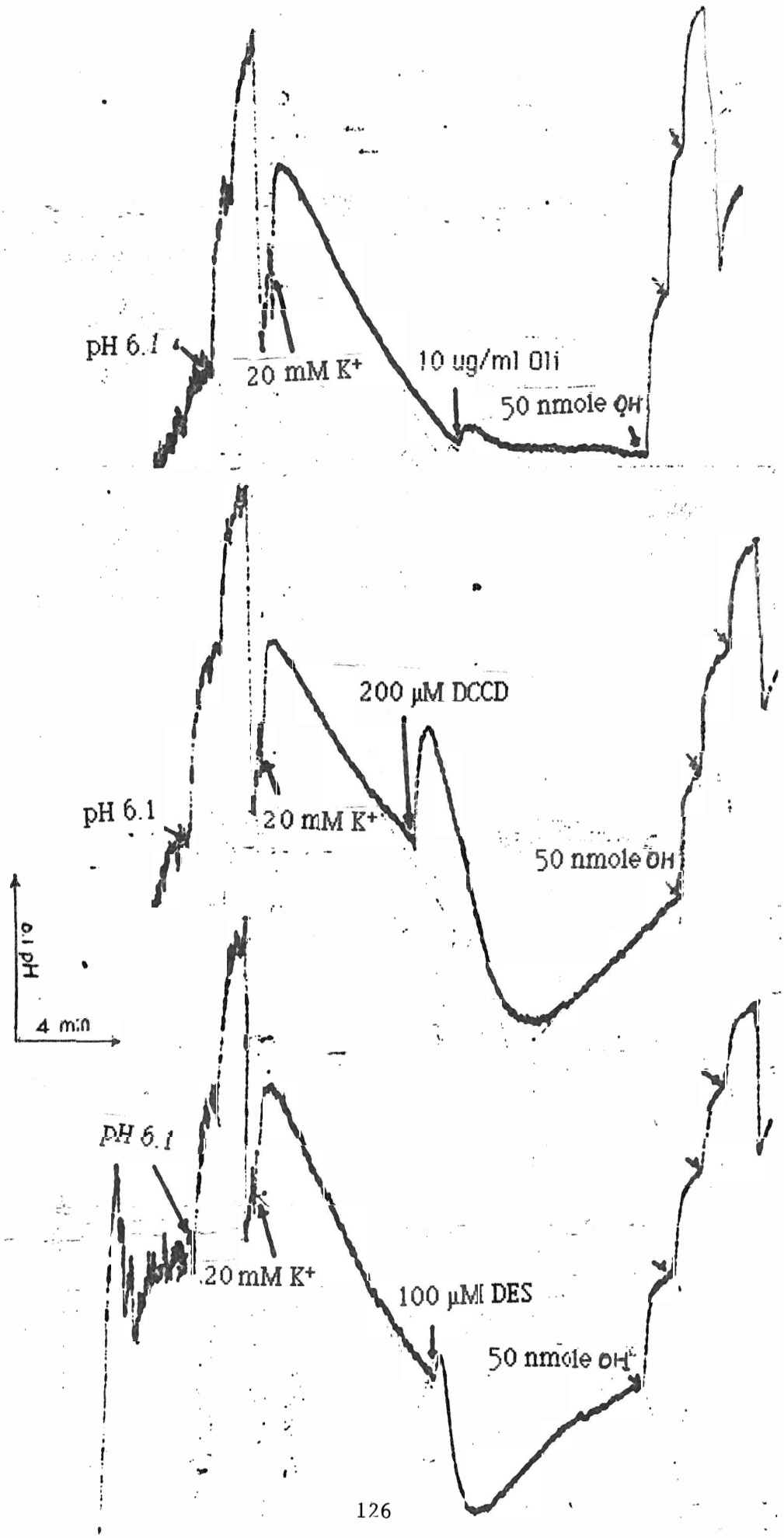

pH 6.1
20 mM K+
10 ug/ml Oli
50 nmole OH
200 µM DCCD
pH 6.1
20 mM K+
50 nmole OH
0.1 pH
4 min
pH 6.1
20 mM K+
100 µM DES
50 nmole OH

Table 26. Inhibition of K^+ Stimulated H^+ Efflux by DES

	Rate of H^+ Efflux	
	K^+	K^+ + DES
	nmole $H^+/10^6$ cells·min	
Dark		
09 06 88 Expt. 1	0.511	-2.778
10 06 88 Expt. 2	0.952	-3.000
13 06 88 Expt. 3	1.310	-2.679
Mean	0.924	-2.819
Light		
09 06 88 Expt. 1	0.365	-0.667
10 06 88 Expt. 2	0.667	-0.781
13 06 88 Expt. 3	0.845	-1.136
Mean	0.626	-0.861

——— The reaction vessel contained 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$.

——— The cell suspension was stirred and aerated in the dark or light.

——— The concentration of K^+ in the reaction vessel was 20 mM.

——— 100 µM DES was added to the reaction vessel 6 min after addition of K^+.

——— At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH.

——— Each value is the mean of 3 trials. Negative value indicates H^+ influx.

Table 27. Inhibition of K^+ Stimulated H^+ Efflux by DCCD

	Rate of H^+ Efflux	
	K^+	K^+ + DCCD
	nmole $H^+/10^6$ cells·min	
Dark		
09 06 88 Expt. 1	0.681	0.000
10 06 88 Expt. 2	1.451	0.000
13 06 88 Expt. 3	1.411	0.000
Mean	1.181	0.000
Light		
09 06 88 Expt. 1	0.617	-0.358
10 06 88 Expt. 2	0.966	-0.947
13 06 88 Expt. 3	0.698	-0.781
Mean	0.760	-0.695

——— The reaction vessel contained 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$.

——— The cell suspension was stirred and aerated in the dark or light.

——— The concentration of K^+ in the reaction vessel was 20 mM.

——— 200 µM DCCD was added to the reaction vessel 6 min after addition of K^+.

——— At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH.

——— Each value is the mean of 3 trials. Negative value indicates H^+ influx.

Table 28. Inhibition of K^+ Stimulated H^+ Efflux by Oligomycin

	Rate of H^+ Efflux	
	K^+	K^++OLigomycin
	nmole $H^+/10^6$ cells·min	
Dark		
09 06 88 Expt. 1	1.010	0.000
10 06 88 Expt. 2	1.286	0.000
13 06 88 Expt. 3	1.648	-0.084
Mean	1.314	-0.028
Light		
09 06 88 Expt. 1	0.219	0.022
10 06 88 Expt. 2	0.446	0.000
13 06 88 Expt. 3	0.842	0.053
Mean	0.502	0.025

——— The reaction vessel contained 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$.

——— The cell suspension was stirred and aerated in the dark or light.

——— The concentration of K^+ in the reaction vessel was 20 mM.

——— 10 µg/ml oligomycin was added to the reaction vessel 6 min after addition of K^+.

——— At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH.

——— Each value is the mean of 3 trials. Negative value indicates H^+ influx.

Table 29. Inhibition of K^+ Stimulated H^+ Efflux by Antimycin

	Rate of H^+ Efflux	
	K^+	K^++Antimycin
	nmole $H^+/10^6$ cells·min	
Dark		
06 06 88 Expt. 1	1.45	0.00
07 06 88 Expt. 2	1.30	0.09
Mean	1.38	0.05
Light		
06 06 88 Expt. 1	1.00	0.68
07 06 88 Expt. 2	0.50	0.38
Mean	0.75	0.54

—— The reaction vessel contained 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$.

—— The cell suspension was stirred and aerated in the dark or light.

—— The concentration of K^+ in the reaction vessel was 20 mM.

—— 100 µM antimycin was added to the reaction vessel 6 min after addition of K^+.

—— At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH.

Table 30. Stimulation of K^+ Dependent H^+ Efflux by Fusicoccin

	Rate of H^+ Efflux	
	K^+	K^+ + FC
	nmole $H^+/10^6$ cells·min	
Dark		
20 07 88 Expt. 1	0.69	3.13
26 07 88 Expt. 2	1.30	2.08
Mean	1.00	2.60
% Stimulation		160
Light		
20 07 88 Expt. 1	0.05	3.13
21 07 88 Expt. 2	0.04	0.28
26 07 88 Expt. 3	1.22	2.13
Mean	0.41	1.19
% Stimulation		190

——— The reaction vessel contained 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$.

——— The cell suspension was stirred and aerated in the dark or light.

——— The concentration of K^+ in the reaction vessel was 20 mM.

——— 10 µM fusicoccin was added to the reaction vessel 6 min after addition of K^+.

——— At the end of the experiment, the contents of the vessel were backtitrated with 1mM NaOH.

3. Influence of Weak Acids on ATP Dependent H^+ Efflux

Weak acids diffuse though the plasma membrane into the cells as protonated neutral molecules. Because of a higher internal pH a fraction would dissociate to produce free H^+ after uptake. As a result, the cytoplasm would be acidified. If the plasma membrane ATPase is regulated by this acidification, in a mechanism of internal pH regulation, an increase in ATP dependent H^+ efflux would be observed. The purpose of this experiment was to test the influence of internal acidification on ATP dependent H^+ efflux. The study was performed using the pH vs time method (Method 2.4). To a reaction vessel containing 10 X 10^6 cells in 10 ml of 1mM $CaSO_4$ was added 1.5 mM butyric acid or acetic acid, both as sodium salts. The buffering capacity of the medium was measured at pH6.0 by titrating with 1 mM H_2SO_4 or I mM NaOH before and after the addition of the weak acids. Innitially, an alkalinization process was observed. Subsequently, medium acidification induced by the weak acids was observed .After10 minutes, oligomycin was introduced to the cell suspension medium to determine whether weak acid induced H^+ efflux was ATP dependent.

Fig. 14 showes the relationship between the presence of butyric acid (pH 6.0) and H^+ flux. Before addition of butyric acid, H^+ efflux was 0.26 nmole H^+/min · 10^6 cells. After butyric acid was introduced to the medium, the rate inereased to 2.08 nmole H^+/min · 10^6 cells. This increase followed a rapid alkalinization resulting from protonated neutral butyric acid entering the cells. The butyric acid induced H^+ efflux was completely inhibited by 10 µg/ml oligomycin, indicating that this H^+ efflux process was ATP dependent. The experiment was repeated three times. The mean rates of of H^+ efflux are shown in Table 31 . The percent stimulations of H^+ efflux were 449% by butyric acid and 319% by acetic acid.

These results showed that ATP dependent H^+efflux was stimulated by

cytoplasmic acidification induced by the addition of weak acids to the cell suspension medium. Thus, if L-Glu transport into the cell also causes acidification, the actual rate of L-Glu dependent alkalinization may be reduced by a stimulation of ATP dependent H^+ efflux.

Fig. 14. Stimulation of ATP-dependent H^+ Efflux by Butyric Acid and Acetic Acid

These are pH vs time tracing showing the stimulation of ATP-dependent H^+ efflux by butyric acid (BA) and acetic acid (AA). To a reaction vessel containing 10 X 10^6 cells in 10 mM of 1mM $CaSO_4$ was added 1.5 mM BA or AA (adjusted to pH 6.0). The cell suspension was stirred and aerated in the light. Before and after addition of BA or AA and at the end of the experiment, the contents of the reaction vessel were titrated with 1 mM H_2SO_4 or 1 mM NaOH to measure the buffering of the medium and calculate the rate of H^+ flux at pH 6.0. 10 µg/ml oligomycin was added to the cell suspension approximately 10 minutes after the addition of BA or AA.

200 nmole H+
100nmole H+
2.08
pH5.95
OLI
BA
20 nmole H+
0.26
pH 6.0
005 pH
2 min

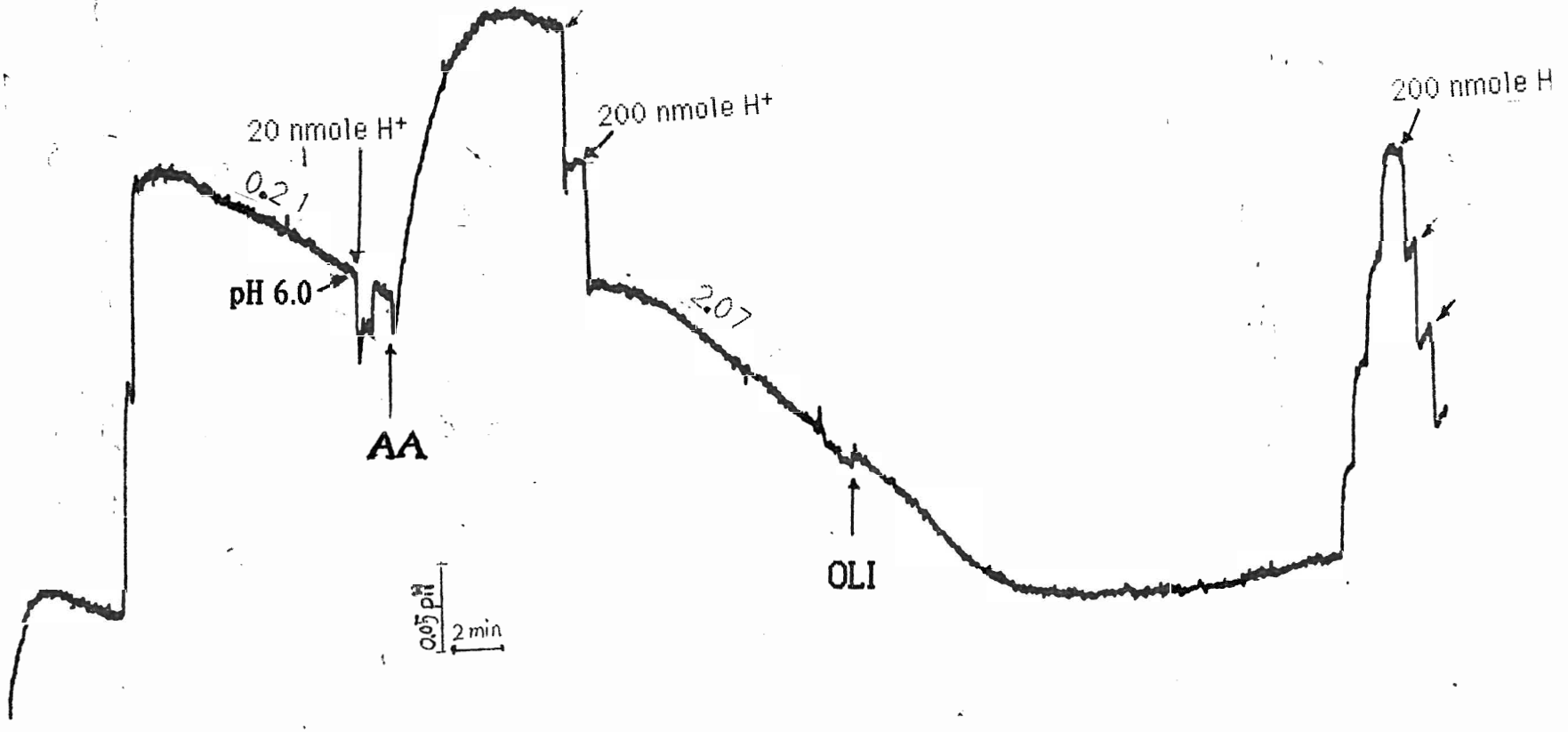

20 nmole H+
0.2 /
pH 6.0
AA
200 nmole H+
2.07
OLI
200 nmole H
0.05 pH
2 min

Table 31. Stimulation of ATP-dependent H^+ Efflux by Weak Acids

Conditions:

——— The reaction vessel contained 10 X 10^6 cells in 10 mM of 1mM $CaSO_4$.

——— The cell suspension was stirred and aerated in the light.

——— The concentration of BA in the reaction vessel was 1.5 mM.

——— The concentration of AA in the reaction vessel was 1.5 mM.

——— Before and after the addition of BA and at the end of th experiment, the contents of the reaction vessel were titraed with 1 mM H_2SO or 1 mM NaOH to measure the buffering of the medium and calculate the rate of H^+ flux.

——— 10 µg/ml oligomycin was added to the cell suspension 10 min after the addition of BA or AA.

	Rate of H^+ Efflux (nmole H^+ / 10^6 cells · min)		%Stimulation
	Before adding weak acid	After adding weak acid	
Butyric Acid (1.5 mM):			
Expt. 1	0.15	0.95	
Expt. 2	0.26	2.08	
Expt. 3	0.38	1.25	
Mean	0.26	1.43	449%
SD	0.14	0.59	
Acetic acid (1.5 mM):			
Expt. 1	0.24	1.47	
Expt. 2	0.21	2.07	
Expt. 3	0.80	1.56	
Mean	0.41	1.70	319%
SD	0.32	0.32	

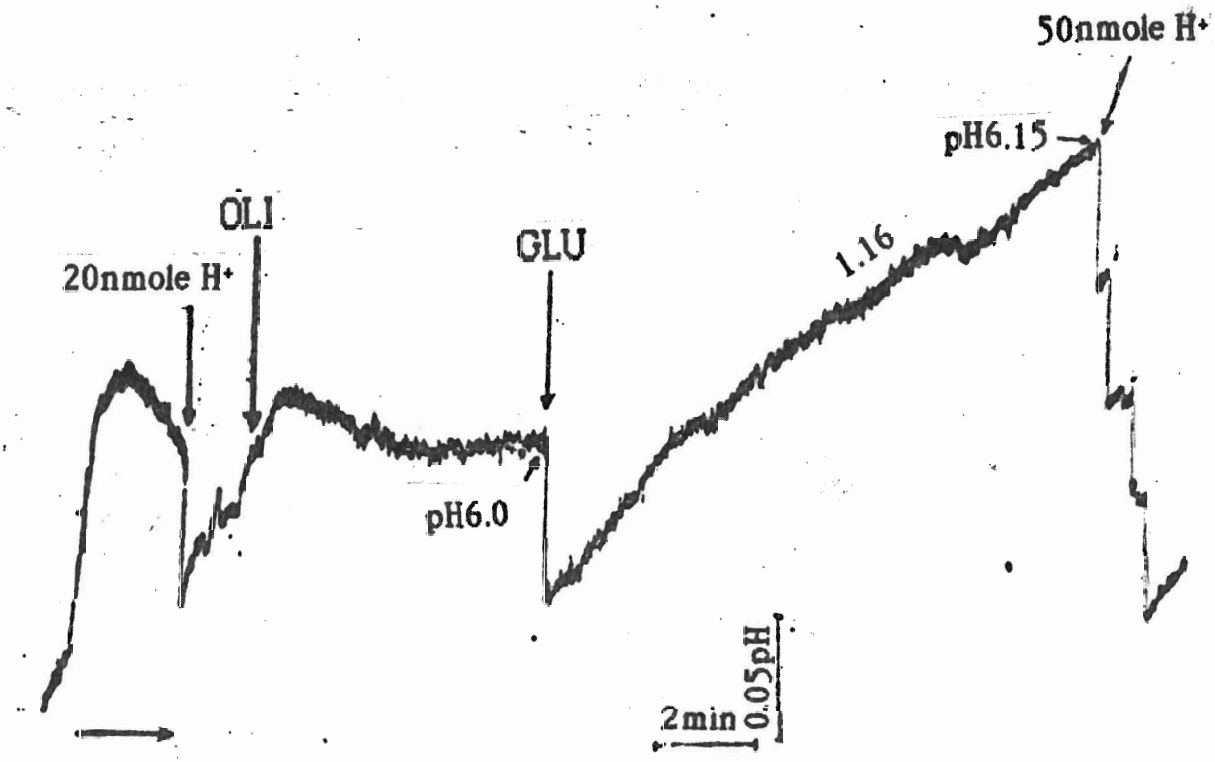
4. The Effect of ^{14}C-U-L-Glu Uptake on ATP Dependent H^+ Efflux

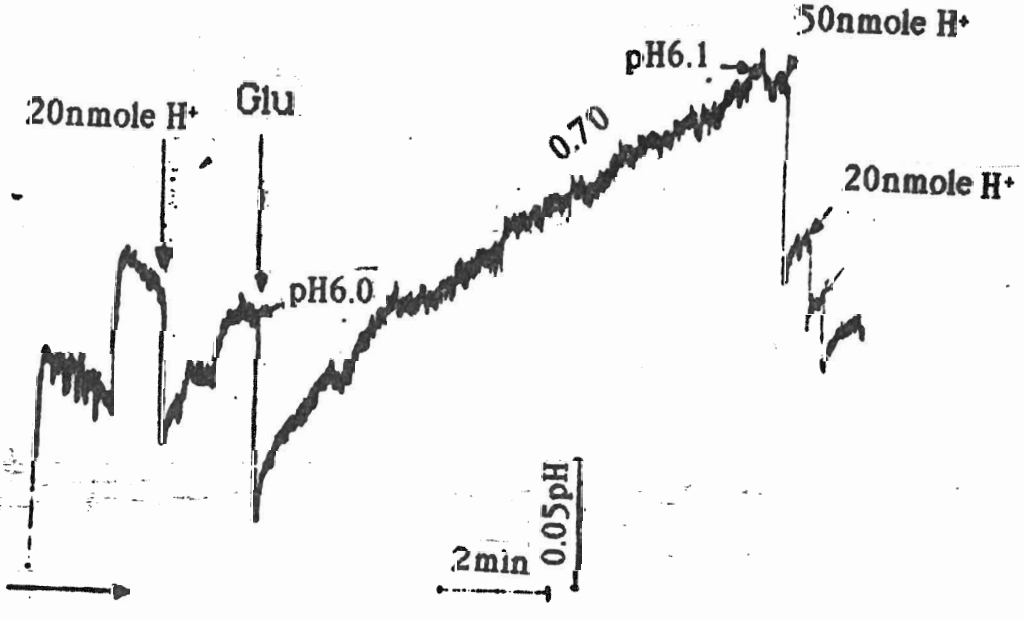
If H^+/L-Glu transport is an electrogenic process, every anion of L-Glu would be coupled to the entry of two or more H^+ into the cells. As a result, the membrane potential would be depolarized and the cytoplasm would be acidified. Both processes might in turn stimulate ATP dependent H^+ efflux. The purpose of this experiment was to investigate the relationship between L-Glu uptake and ATP dependent H^+ efflux and to determine whether ATP dependent H^+ efflux compensated for H^+/L-Glu symport. The data were obtained from the experiments concerned with the stoichiometry of H^+/L-Glu symport described in section Results A. 11. Here, the relationship between L-Glu uptake and ATP dependent H^+ efflux is examined.

In the presence of oligomycin which inhibited ATP dependent H^+efflux, the rate of the medium alkalinization induced by ^{14}C-U-L-Glu was 1.16 nmole H^+/min · 10^6 cells. In contrast, the rate decreased to 0.70 nmole H^+/min · 10^6 cells in the absence of oligomycin(Fig. 15). In Table 19, the data show the symport of 2 H^+ for every anion of L-Glu entering the cells in the presence of oligomycin, but the symport was 1 H^+/L-Glu in the absence of oligomycin. It indicates that one H^+ is pumped out for every L-Glu taken up. Thus L-Glu stimulated H^+ efflux may help regulate both internal pH and the membrane potential.

Fig. 15 The Effect of ^{14}C-U-L-Glu Uptake on ATP Dependent H^+ Efflux

These are pH vs time traces showing the effect ^{14}C-U-L-Glu uptake on the ATP dependent H^+ influx. To a reaction vessel containing 15 X 10^6 cells in 3 ml of 1mM $CaSO_4$ was added 1 mM ^{14}C-U-L-Glu with 3311 dpm/nmole. The cell suspension was stirred in ambient light. When usded, oligomycin at 10 µg/ml was added 5 min before addition of ^{14}C-U-L-Glu. At indicated times, 100 µl of the cell suspension containing 0.5 X 10^6 cells was removed to measure ^{14}C-U-L-Glu uptake or ^{14}C-GABA release. Before and after the addition of ^{14}C-U-L-Glu, the contents of the reaction vessel were titrated with 1mM H_2SO_4 or 1 mM NaOH to measure the buffering capacity of the medium and to calculate the rate of net medium alkalinization.


50nmole H+
pH6.15
OLI
20nmole H+
GLU
1.16
pH6.0
0.05pH
2min


50nmole H+
pH6.1
20nmole H+
Glu
0.70
20nmole H+
pH6.0
0.05pH
2min

Discussions

A. Electrogenic H^+/L-Glu Symport

A H^+ symport process may result in depolarization of the plasma membrane. Depolarization is regarded as evidence in favour of an electrogenic symport process. Transient depolarization measured with a microelectrode indicated that H^+/amino acid symport into oat coleoptiles was electrogenic (Kinraide & Etherton, 1980). However, this technique is hard to apply to many other systems involving small cells with thick cell walls (see Section B of Literature Review). For small cells, permeable lipophilic cations are often used as a membrane potential probe. Lipophilic cations have been used to estimate the membrane potential in algae (Komor & Tanner, 1976), fungi (Boxman et al, 1982, 1984, Eilam et al, 1985, Bakker et al, 1986 & Budd, 1989) and plants (Murphy et al, 1975 & Rubinstein, 1978). The results showed that the membrane potential was inside negative and that accumulation of lipophilic cations was inhibited by K^+ and uncouplers. However, Ritchie (1984) has cautioned against the use of lipophilic cations for estimating the plasma membrane potentials due to their accumulation in organelles. Astle & Rubery (1984) observed that $MTPP^+$ association with living and dead suspension-cultured runner bean cells increased with increasing external pH, indicating that $MTPP^+$ binding occurred. They concluded that $MTPP^+$ was not a suitable probe of membrane potential in their system. The use of lipophilic cations to estimate the membrane potential is circumspect because the probe does not distribute freely either side of the membrane.

Although the equilibrium distribution of lipophilic cations to estimate the membrane potential has problems, the fact that the accumulation of lipophilic cations is inhibited by K^+ or uncouplers indicates that the rate of uptake of

lipophilic cations may be governed by the membrane potential. Thus the rate of uptake of lipophilic cations may be used to monitor changes in the membrane potential. This method was used to indicate depolarizations associated with glucose uptake. It was concluded that the sugar transport process was electrogenic (Komor & Tanner, 1976). The rate of $MTPP^+$ uptake was also used to monitor changes in the membrane potential during K^+ efflux induced by ultraviolet radiation (Huerta & Murphy, 1988).

For studies on the electrogenic nature of H^+/L-Glu symport into *Asparagus sprengeri* mesophyll cells, it is difficult to measure changes in the membrane potential using a microelectrode due to the small size of the cells. Similarly, the ^3H-$MTPP^+$ equilibrium method can not be used due to binding. However, ^3H-$MTPP^+$ transport across the membrane of these cells appears to be driven by the membrane potential since ^3H-$MTPP^+$ uptake was inhibited by K^+ or CCCP (Fig. 3 & Table 10). This demonstrates that the rate of ^3H-$MTPP^+$ uptake can be used to study the electrogenic nature of H^+/L-Glu symport process in *Asparagus* cells. When 5 mM L-Glu was applied to the cell suspension medium, the rate of ^3H-MTPP uptake was inhibited (Fig. 3 & Table 10). This indicates that the uptake of L-Glu caused depolarization of the membrane and that H^+/L-Glu symport is electrogenic.

An electrogenic H^+ symport process would be expected to show a dependence on the membrane potential. The greater the depolarization, the more inhibition of substrate uptake is expected. The results showed that increases in the concentrations of permeable $MTPP^+$ resulted in decreases in the rate of ^{14}C-U-L-Glu uptake (Table 15). This result is in accord with an electrogenic H^+/L-Glu symport process. However, the addition of K^+ to the cell suspension medium, which could also depolarize the membrane potential (Fig. 3 & Table 10), did not inhibit the uptake of ^{14}C-U-L-Glu (Table 9). This may result from an increase in the H^+

chemical gradient (ΔpH) resulting from K^+ stimulated H^+ efflux (Fig. 8, 10 & Table 24). Thus, a reduction in the membrane potential ($\Delta\Psi$) induced by K^+ may be associated with an increase in ΔpH. As a result, the driving force ($pmf = \Delta\Psi - 0.06\ \Delta pH$) for H^+/L-Glu symport would not be changed.

If a H^+ symport process is electrogenic, the specifity of substrate dependent depolarization and alkalinization should be consistent with the specifity of the uptake. The satisfactory agreement between the depolarization induced by the L-isomers of amino acids and the uptake of L-isomers was reported (Felle, 1981). The H^+/L-Glu symport system in *Asparagus sprengeri* mesophyll cells was suggested to be stereospecific for the L-isomer because L-Glu induced medium alkalinization but D-Glu did not (McCutcheon & Bown, 1987). In my studies, the rate of ^3H-MTPP uptake was inhibited by L-Glu but not by D-Glu (Fig. 5 & Table 12). Thus, the sterospecific inhibition of $MTPP^+$ uptake indicates that inhibition is due to the electrogenic and sterospecific uptake of L-Glu.

B. The Stoichiometry of the H^+/L-Glu Symport

In considering the H^+/L-Glu symport stoichiometry, it is assumed that the transported L-Glu is in the anion form. The pK value for the α-carboxlate group is 2.19, for the γ-caboxlate group is 4.25 and for the α-amino group is 9.67 (Armstrong, 1983). Based on these data, the anionic form of L-Glu is more than 98% of the total L-Glu present at pH 6.0. In my studies on the stoichiometry of H^+/L-Glu symport, the extracellular medium was 1 mM $CaSO_4$ adjusted to pH 6.0. This indicates that the anionic form of L-Glu is about 98% of the total L-Glu present in my study system. Thus the H^+/L-Glu symport stoichiometry is defined as the number, ' n ', of protons coupled to the entry of one anion of L-Glu with net charge

of -1 . If n is 1. the symport would be electroneutral. Otherwise, when n is 2 or more, the symport is expected to be electrogenic because net charge is transported. If the protonated neutral form of L-Glu entered, the affinity of the symport system would have to be very high. In addition the entry of protonated neutral L-Glu with one proton is operationally the same as the entry of the anion form with 2 protons

The stoichiometry of the H^+/L-Glu symport was calculated from rates of L-Glu dependent alkalinization, rates of ^{14}C-L-Glu uptake and rates of ^{14}C-GABA release (Fig. 7, Table 16). The values of the measured molar stoichiometry of H^+/L-Glu uptake were 2 : 1 in the presence of oligomycin and 1 : 1 in the absence of oligomycin (Table 19). In these calculations the rate of L-Glu entry was obtained from the sum of the radioactivity in the cell plus the radioactivity in GABA released to the medium.

The difference in the values probably involves the measurement of H^+ movement. Because H^+ rapidly equilibrates with a molar excess of water, unidirectional measurement of H^+ movement with isotopes of hydrogen is impossible and only net movement can be quantified. Although measurements of amino acid dependent net alkalinization are often considered to approximate H^+ influx coupled to amino acid transport, it is necessary to consider how accurately the values for net alkalinization of the medium reflect the real rate of H^+ influx coupled to amino acid entry. The rate of amino acid coupled H^+ influx may be underestimated due to outwardly directed H^+ pump activity stimulated by H^+/amino acid entry resulting in a reduction in cytoplasmic pH. In addition, measurements may be affected by the release of products of amino acid metabolism, which change the pH of the medium.

In *Asparagus* mesophyll cells, CO_2 and GABA are released from the cells on addition of L-Glu. Because GABA is a neutral molecule, it should not change the pH of the medium. CO_2 release may acidify the medium resulting in an

underestimation of H^+ influx. Most of the CO_2 released from labelled L-Glu is refixed when the cells are illuminated (Chung, 1989). Her data showed that the uptake of ^{14}C-U-L-Glu label in the dark was 73% of uptake found in the light and the uptake of ^{14}C-1-L-Glu in the dark was 28% of uptake measured in the light. Higher uptake of label in the light than that in the dark, particularly with ^{14}C-1-L-Glu, suggested that the lost carbon-1 released was refixed by photosynthesis. The results indicate that the amount of CO_2 evolved from illuminated cells is small so that loss of label as CO_2 is not significant. Stirring of the 3 ml suspension will prevent CO_2 accumulation and possible associated pH change

L-Glu dependent H^+ influx may be underestimated due to increased H^+ pumping activity. The activity of the plasma membrane H^+ ATPase has been considered to be stimulated by both a depolarization process and the acidification of the cytoplasm (see reference in section D of Discussion). A depolarizing electrogenic H^+/L-Glu process will cause both depolarization and acidification. As a result, alkalinization due to L-Glu may be underestimated due to increases in the activity of the H^+ ATPase during H^+/L-Glu symport. Thus the stoichiometry of 1 : 1 obtained in the absence of oligomycin would be an underestimation . In the presence of oligomycin, the effect of ATP driven H^+ extrusion on the rate of alkalinization could be excluded due to the inhibition of the H^+ATPase activity. Therefore, the stoichiometry of 2 : 1 obtained in the presence of oligomycin more accurately indicates the quantitative relationship between H^+ and L-Glu coupled entry. This stoichiometry is consistent with a 2 : 1 stoichiometry for H^+/L-Glu symport in *Ricinus* cotyledons (Robinson and Beever, 1981) and in cultured sugarcane cells (Wyse and Komor, 1984). The latter showed that every 2 H^+/1 L-Glu entry would be compensated by the efflux of 1 K^+.

McCutcheon (1987) found for every anion of L-Glu entering, the coupled number of H^+ ranged from 3 to 8 as the external ^{14}C-L-Glu concentration was

increased from 0.5 to 10 mM. Thus in his studies, only ^{14}C-CO_2 evolved as the labelled product of the ^{14}C-L-Glu metabolism was taken into account. The stoichiometries obtained by McCutcheon were much higher than those found in literature (Wyse & Komor, 1984). This indicates that the stoichiometries may be overestimated due to loss of label. The lost label was later identified as GABA released to the medium (Chung, 1989). Because the molar excess of GABA measured in the medium is much greater than that of measured CO_2, the stoichiometries based on calculations of CO_2 evolved and radioactivity inside cells would be overestimated.

It is suggested here that 2 : 1 is the stoichiometry of H^+/L-Glu symport in *Asparagus* mesophyll cells. This stoichiometry indicates that H^+/L-Glu symport is a depolarizing electrogenic process. Thus, it is consistent with the data showing that H^+/L-Glu symport is electrogenic.

C. H^+/Glu Symport and K^+ Efflux

Electrophysiological studies demonstrated that when amino acids were added to the extracellular medium, the membrane potential of oat coleoptiles first depolarized, and then spontaneously repolarized (Kinraide, et al., 1980). The depolarizations were considered to reflect electrogenic H^+/amino acid symport, and the repolarizations were believed to depend on a depolarization-induced increase in K^+ efflux plus an increased H^+ efflux (Kinraide, 1984). Experimental evidence confirmed that passive K^+ efflux maintained charge balance during amino acid uptake in cultured sugarcane cells (Wyse and Komor, 1984). However, K^+ efflux during L-Glu dependent alkalinization was not significant in *Asparagus* mesophyll

cells. The results suggest that addition of L-Glu increased H^+ influx by approximately 2.73 nmol/10^6 cell·min. However, K^+ efflux increased by 0.41 nmol/10^6 cell·min (Fig. 8, Table 20). Assuming $2H^+$/1L-Glu, then the rate of entry of charge would be 1.36 nmol/10^6 cell·min which is over 3 times greater than the rate of K^+ efflux. Thus K^+ efflux may not be a universal feature of H^+/amino acid symport.

K^+ flux is thought to be mediated via K^+ channels located in the plasma membrane. There are at least two classes of K^+ channels characterized; one may be responsible for K^+ efflux and the other K^+ uptake (Schroeder, et al., 1987; Schroeder, 1988). Both classes of K^+ channels are strongly regulated by the membrane potential(Hedrich, 1989). Thus K^+ channels allowing K^+ efflux are activated by membrane depolarization (Hedrich, 1989) and play an important role in the restoration of the membrane potential (Ketchum, et al., 1989). This type of K^+ channel has been found in different kinds of higher plant cells such as motor cells of pulvini (Moran, et al., 1988), trap-lobe cells (Ijima, et al., 1987), aleurone layer cells (Bush, et al., 1988), cone suspension-culture cells (Ketchum, et al., 1989) and mesophyll cells of wheat (Moran, et al., 1984). This kind of of K^+ channel opened when a threshold level of depolarization was obtained (Homble, et al., 1987; Ketchum, et al., 1989). In various tissues the threshold voltage to open K^+ channels was about -40 mV (Hedrich, 1989), indicating that only a depolarization to values more positive than -40 mV could activate the K^+ channels. Although K^+ efflux via this type of K^+ channel may be a response to depolarization induced by electrogenic H^+/amino acid symport, it is possible that K^+ efflux may be dependent on how positive the membrane potential becomes. If depolarization occurs to values more positive than the threshold voltage, K^+ efflux may occur. If not, K^+ efflux may not be observed. Thus K^+ efflux during electrogenic H^+ symport may be detected in some studies but not in other studies. Although K^+ channels

probably exist in the plasma membrane of *Asparagus* mesophyll cells and play a role in restoration of the membrane potential after a depolarization event, significant K^+ efflux may not be observed because H^+/L-Glu symport into these cells does not reduce the membrane potential below the threshold.

It is also possible that no significant K^+ efflux occurred during H^+/L-Glu symport into *Asparagus* mesophyll cells due to the blockage of the K^+ channels. To date, most of the studies on factors blocking K^+ channels are concerned with extracellular Ca^{2+} and cytoplasmic Ca^{2+}. It was reported that K^+ channels were inhibited by external Ca^{2+} (Keifer & Lucas, 1982; Bisson, 1984; Homble, 1985; Sokolik & Yurin, 1986; Beilby, 1986). Little is known concerning the inhibition of K^+ efflux via effects on cytoplasmic Ca^{2+} levels or by Ca^{2+} effects on the external side of the K^+ channel. An increase in the cytoplasmic Ca^{2+} level using Ca^{2+} channel effectors lead to an increased rate of closure of K^+ channels. The same effect was obtained by increasing the external concentration of Ca^{2+} (Tester & MacRobbie, 1990). It was suggested that gating of K^+ channels was controlled by cytoplasmic Ca^{2+}, however, the effect of external Ca^{2+} might be on the external face of the K^+ channels. In my studies, Ca^{2+} was present as the calciun salt of L-Glu and the cell suspension medium was 1mM $CaSO_4$. This indicates that K^+ channels may be inhibited by the external Ca^{2+} so that no significant K^+ efflux during H^+/L-Glu symport was observed. Subsequent work in the laboratory indicates that K^+ efflux is not stimulated by L-Glu when Ca^{2+} is absent (Personal communication from Dr. A. Bown.)

K^+ efflux was detected as a response to addition of Na^+ (Table 21) or DES (Fig. 8, Table 22). Thus positive evidence for K^+ efflux from *Asparagus* mesophyll cells was obtained. However, it is not clear whether Na^+ and DES induced a depolarization in these cells and whether K^+ efflux was caused by the depolarizing mechanism. Although Na^+ entry may depolarize the membrane, K^+ efflux may be

mediated via a Na^+/K^+ antiport rather than by the depolarizing mechanism. The mechanism of the effect of DES on K^+ efflux may be complicated. DES is known as an inhibitor of ATPase. The presence of DES may cause a depolarization due to the inhibition of ATPase activity which contributes to the membrane potential. The result showed that the rate of K^+ efflux increased almost 8 times immediately after addition of DES. However, this dramatic increase may not be involved in the restoration of the membrane potential after addition of DES. The presence of DES was found to increase the accumulation ratio of TPP^+ which indicates hyperpolarization of the membrane (Budd, 1989). It is also possible that DES disrupts the membrane and stimulates an apparent K^+ efflux by increasing membrane permeability through a non-physiological toxic mechanism.

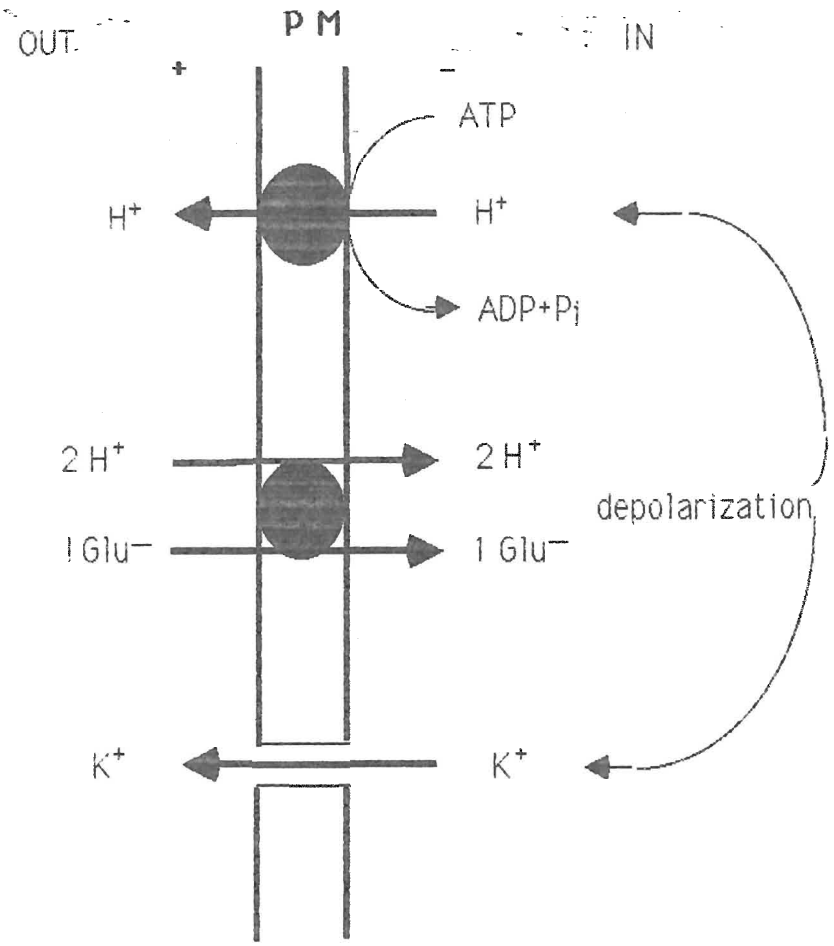
D. H^+/L-Glu Symport and Active H^+ Efflux

The simultaneous measurement of L-Glu dependent H^+ influx and K^+ flux showed no significant K^+ efflux in response to H^+ influx. This led to an initial suggestion that H^+/L-Glu symport is a neutral process. However, it is possible that H^+ efflux due to ATPase activity may play a role in charge compensation during depolarization. Experiments (Komor, et al., 1981; Kinraide & Etherton, 1981) also indicate that H^+/sugar symport and H^+/amino acid symport are driven by the membrane potential ($\Delta\Psi$) and H^+ gradient (ΔH), generated by the plasma membrane H^+ ATPase. If this were true, the transport of these solutes would collapse these gradients and continuing uptake would require continuing H^+ efflux via ATPase to maintain the gradients. Based on these results, a model is proposed to explain charge compensation during a depolarizing H^+/amino acid symport

(Model A). In this model, the coupling between a H^+/amino acid symport and passive K^+ efflux or between a H^+/amino acid symport and active H^+ efflux is indirect via the membrane potential. It is assumed that the H^+/amino acid symport depolarizes the membrane to a value above the threshold to open K^+ channels and or remove inhibition of the H^+ ATPase impaired by a high membrane potential. Passive K^+ efflux or active H^+ efflux or both together compensate the depolarizing symport process.

The plasma membrane ATPase is a universal feature of higher plant cells and it functions in regulating the electrical properties of the membrane (Spanswick, 1981; Sze, 1985). Evidence for a H^+ ATPase located in the plasma membrane of *Asparagus* mesophyll cells has been obtained. The rate of H^+ efflux was dependent on the ATP level inside the cells (Bown & Nicholls, 1985). In this study, H^+ efflux was inhibited by erythrocin B and oligomycin, but was stimulated by fusiccocin (Table 23). The H^+ efflux was stimulated by K^+ or Na^+ (Fig. 10; Table 24, 25). This K^+ stimulated H^+ efflux was strongly inhibited by DES (Table 26), DCCD (Table 27) oligomycin (Table 28) and antimycin (Table 29) but was stimulated by fusiccocin (Table 30). This confirms that the K^+ stimulated H^+ efflux is ATP dependent and not just a cation exchange process in the cell wall. In addition, the uptake of the lipophilic cation $MTPP^+$ was inhibited by K^+, indicating that K^+ transport into the cells caused a depolarization. Taken together, these data indicate that active H^+ efflux is coupled to K^+ or Na^+ uptake through depolarization and that H^+ efflux play a role in the restoration of the membrane potential after a depolarizing event. Experiments with other tissues also showed that active H^+ efflux was coupled to the uptake of cations such as K^+ (Cheesman & Hanson, 1978; Gloss, 1982; Kochian & Lucas, 1982; Behl & Rachke, 1987) and Na^+ (Pitman et al.,

Model A.



1975,). Data obtained from cation transport into membrane vesicles (Sze, 1985) and protoplasts (Lin, 1985) indicates that the uptake of cations is driven by a H^+ electrochemical gradient. Thus, continuing cation uptake would require active H^+ efflux to maintain the gradient. Moreover, the coupling between active H^+ efflux and K^+ uptake was observed to be dissociated by lipophilic cation (Bellando et al., 1979). This was *in vivo* evidence that coupling is electrical. The observation of active H^+ efflux and K^+ uptake stoichiomitries close to one also supported the electrical coupling proposed and indicated that one is compensating for the other (Behl & Raschke, 1987). In an *in vitro* system, the H^+ ATPase was shown to pump protons in the absence of K^+ (Serrano, 1985). However, some other researchers argued that the coupling was a direct chemical coupling (Poole, 1974; Cheeseman & Hanson, 1979, 1980). Because the external K^+ concentrations applied were quite different, ranging from μM to mM K^+ levels, interpretation of these studies is complicated. Over this concentration range, K^+ uptake may be mediated via multiple K^+ transport systems (Kochian, 1989). At low external K^+ concentrations (μM levels) K^+ uptake is active. However at high external K^+ concentrations (mM levels), the dominating K^+ uptake process is passive (Kochian, 1982, 1985). In this case the coupling between H^+ efflux and K^+ uptake would be indirect. It was proposed that micromolar concentrations of K^+ resulted in uptake rates high enough to stimulate H^+ ATPase activity through removal of the inhibition produced by its electrogenic activity (Kurkjian & Guern, 1989). Thus, in the present study, increases in the rate of active H^+ efflux at external K^+ concentrations ranging from 2 mM to 40 mM are most probably due to a deporlarizing mechanism. Thus, electrogenic H^+ efflux could play a role in the restoration of the membrane potential after a depolarization event. Therefore, active H^+ efflux may well serve as a charge compensation mechanism during depolarizing electrogenic H^+/L-Glu symport.

In addition, a 2 : 1 H^+/L-Glu symport stoichiometry indicates that the symport would result in acidification of the cytoplasm. This was consistent with data which showed acidification of the cytoplasm during H^+/L-Glu symport into *Asparagus* mesophyll cells (McCutcheon, 1987). This acidification may stimulate ATP driven H^+efflux which is considered to play a major role in the regulation of the cytoplasmic pH (Sze, 1984; Marre & Ballarin-Denti,1985; Serrano, 1988; Kurkdijian & Guern, 1989). Data obtained under different experimental conditions showed active H^+ efflux caused medium acidification when tissus was treated with weak acids (Bates & Goldsmith, 1983; Marre et al., 1983; Brummer et al., 1984; and Frachisse et al. 1988). Thus cytoplasmic pH regulation occured via the H^+ATPase (see above reference). The cytoplasmic pH may act as a signal to activate the H^+ ATPase (Reid et al., 1985). In *Asparagus* mesophyll cells, H^+ efflux increased more than 3 times when 1.5 mM butiric acid or 1.5 mM acetic acid was added (Fig. 14, Table 31). This H^+ efflux was entirely inhibited by oligomycin. This indicates that an ATP dependent system for the regulation of cytoplasmic pH exists in *Asparagus* mesophyll cells. Thus the H^+/L-Glu symport into these cells and resulting acidification could stimulate H^+ efflux via this regulatory system.

Electrogenic H^+/L-Glu symport into *Asparagus* mesophyll cells would result in both depolarization and acidification of the cytoplasm. Both depolarization and acidification could in turn stimulate the plasma membrane H^+ ATPase to pump H^+ out. The 1 : 1 H^+/L-Glu symport stoichiometry obtained in the absence of oligomycin compared with 2 : 1 obtained in the presence of oligomycin (Table 19, Fig. 7), indicates that active H^+efflux stimulated by the H^+/L-Glu symport was observed. It suggests that one H^+ was pumped out by the H^+ ATPase for every 2 H^+/1 L-Glu symport into the cells. This active H^+ efflux compensates for depolarizing electrogenic H^+/L-Glu symport and and partly restores the pH gradient. Thus the driving force for H^+/L-Glu symport, the membrane potential

($\Delta\Psi$) and H^+ gradient (ΔpH), could be maintained and L-Glu uptake could continue.

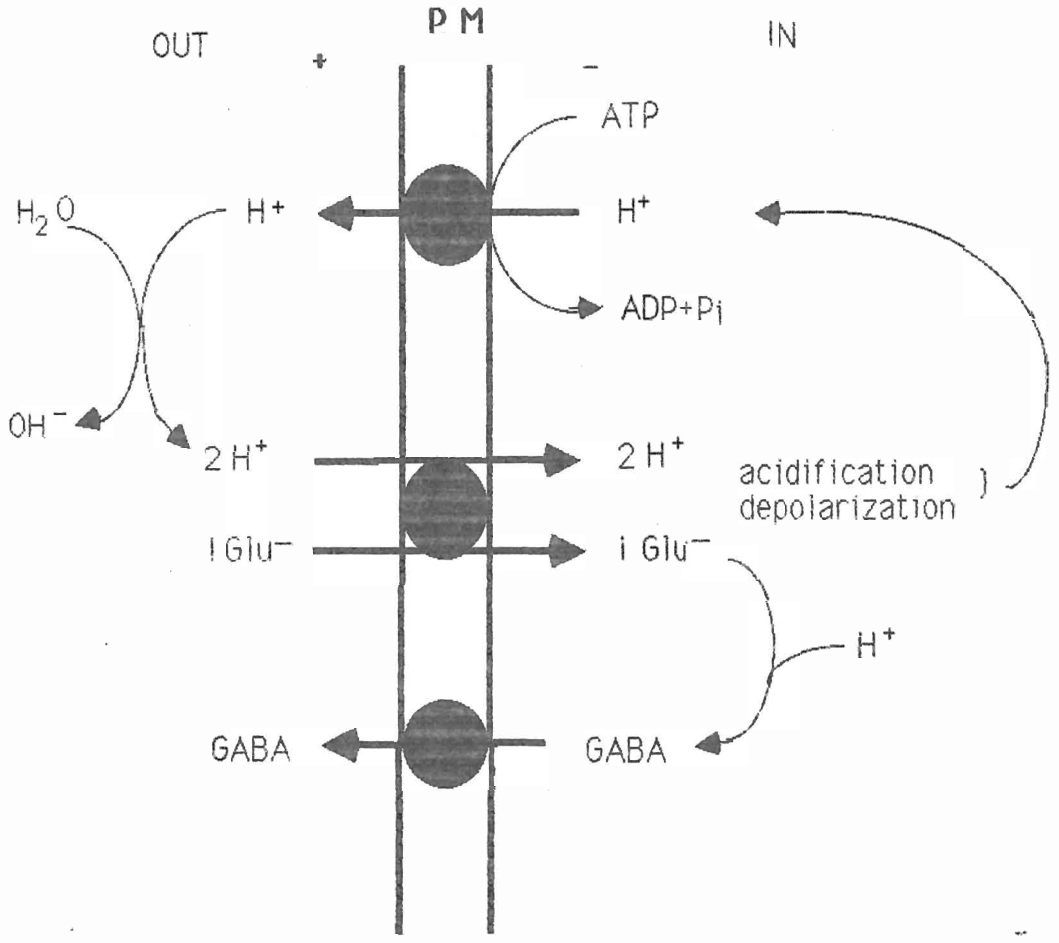
E. A Model for the Electrogenic H^+/L-Glu Symport

A model is drawn from the results obtained with *Asparagus* mesophyll cells. A H^+ ATPase is located in the plasma membrane of these cells. It drives H^+ out of the cells to generate a H^+ electrochemical gradient across the plasma membrane. This results in a negative inside membrane potential and an alkaline cytoplasm. L-Glu uptake is coupled to downhill H^+ influx with a stoichiometry of 2 H^+/1L-Glu. Thus the H^+/L-Glu symport is depolarizing.

The driving forces for this symport include the electrical gradient ($\Delta\Psi$), the H^+ chemical gradient (ΔpH) and the L-Glu gradient (ΔS). The electrogenic H^+/L-Glu symport would collapse these driving forces. Therefore, continuing L-Glu uptake would reguire maintenance of these driving forces. The activity of the plasma membrane H^+ ATPase is stimulated by both depolarization and acidification of the cytoplasm. As a result, one of the two H^+ coupled to the entry of 1 L-Glu is pumped out by the H^+ATPase. This active electrogenic H^+ efflux would compensate for the depolarizing process, and also partly maintain the H^+ gradient. As a result of L-Glu decarboxylation, the L-Glu gradient is also maitained. This model explains the continued L-Glu uptake by indicating how the driving forces for H^+/L-Glu symport are maintained.

Model B.

A Model for the Electrogenic H^+/L-Glu Symport



Conclusions

The present study was initiated to determine whether or not H^+/L-Glu symport into *Asparagus sprengeri* mesophyll cells is electrogenic. The role of passive K^+ efflux or active H^+ efflux in charge compensation for electrogenic symport was investigated. Data in this study is consistent with the following conclusions.

1. H^+/L-Glu symport into *Asparagus sprengeri* mesophyll cells is electrogenic. This symport inhibited lipophilic cation, ^{3}H-MTPP uptake, whereas high concentrations of MTPP inhibited ^{14}C-L-Glu uptake. The molar stoichiometry of H^+/L-Glu symport was 2 : 1. These data support the electrogenic nature of H^+/L-Glu symport.

2. Significant passive K^+ efflux was not observed during H^+/L-Glu symport into *Asparagus sprengeri* mesophyll cells. This indicates that passive K^+ efflux is not involved in charge compensation for the electrogenic H^+/L-Glu symport.

3. The data indicate that active H^+ efflux is involved in charge compensation for electrogenic H^+/L-Glu symport.

REFERENCES

Addision, R. and Scarborough, G. A. 1982. Comformational changes of the *Neurospora* plasma membrane H^+ ATPase during its catalytic cycle. *J. Biol. Chem.* 257: 10421.

Armstrong, F. B. 1983. Biochemistry. *Oxford University Press InC.*

Assmann, S. M., Simon C. L. & Schroeder, J. I. 1985. Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba. Nature* 318: 285-87

Astle, M. C. & Rubery, P. H. 1984. Evaluation of triphenylphosphonium cation as probe of membrane potential in suspension-cultureed runner bean cells. *Plant Science Letters* 36: 43-49.

Bates, G. W. & Goldsmith, M. H. M. 1983. Rapid response of the plasma-membrane potential in oat coleoptiles to auxin and other weak acids. *Planta* 159: 231-37.

Beardall, J. & Raven, J. A. 1981. Transport of inorganic carbon and the " CO_2 concentrating mechanism" in *Chlorella emersonii* (Chorophyceae). *J. Phycol.* 17: 134-141.

Beffagna, N. & Romani, G. 1988. Effects of two plasmalemma ATPase inhibitors on H^+ extrusion and intracellular pH in *Elodea densa* leaves. *J. Exp. Botany* 39, 205: 1033- 1043.

Behl R, Rashke K (1987) Close coupling between extrusion of H^+ and uptake of K^+ by barley root. *Planta* 172: 531-538

Beilby, M. J. & Blatt, M. R. 1986. Simultaneous measurement of cytoplasmic K^+ concentration and plasma membrane electrical parameters in single membrane samples of *Chara corallina Plant Physiol.* 82: 417-422.

Bellando, M., Tratta, A., Bonetti, A., Colombo, R., Lado, R. & Marre, E. 1979. dissociation of H^+ extrusion from K^+ uptake by means of lipophilic cation. *Plant Cell Eviron* 2: 39-47.

Bennett, A. B., O'Neill, S. D. & Spanswick, R. M. 1984. H^+-ATPase activity from storage tissue of Beta vulgaris. 1. Identification and characterization of an anion-sensitive H^+-ATPase. *Plant Physiol* 74: 538-544.

Bidwai, A. P., Zhang, L., Bachmann, R. C., & Takemoto, J. Y. 1987. Mechanism of action of *Pseudomonas syringae* phytotoxin, syringomycin. *Plant Physiol* 83: 39-47

Bisson, M. A. 1984. Calcium effects on elects on electrogenic pump and passive permeability of the plasma membrane of *Chara corallina*. *J. Membr. Biol* 81: 59-67.

Bowman, B, J., Mainzer, S. E., Allen, K. E. & Slayman, C. W. 1978. Effect of inhibitors of the plasma membrane and mitochondrial ATPase of *Neuospora crassa*, *Biochim. Biophys. Acta* 512: 13.

Bowman, B. J., Blasco, F. & Slayman, C. W. 1981. Purification and characterization of the plasma membrane ATPase of *Neurospora crassa*. *J. Biol. Chem.* 256: 12343.

Bowman, B. J., Mainzer, S. E., Allen, K. E. & Slayman, C. W. Effect of inhibitors of the plasma membrane and mitochontrial ATPase of *Neurospora crassa*. *Biochim. Biophys. Acta* 512: 13.

Bown, A. L., Chung, I., Snedden, W. & Shelp, B. 1989. Specific glutamate cotransport into mesophyll cells and efflux of the major metabolite 4-aminobutyric acid. *Plant membrane transport. Elesevier Science publishers B. V.* 329-334.

Bown, A. W. & Nicholls, F. 1985. An investigation into the role of photosynthesis in regulating ATP levels and rates of H^+ efflux in isolated mesophyll cells. *Plant Physiol* 79: 928-934.

Bown, A. W. & Shelp, B. J. 1989. The metabolism and physiological roles of 4-aminobutyric acid. *Biochem.* 8: 21-25.

Briskin, D. P. & Poole, R. J. 1983. Characterization of a K^+-stimulated ATPase associated with the plasma mambrane of red beet. *Plant Physiol* 71: 350.

Briskin, D. P. and Leonard, R. T. 1979. Ion transport in isolated protoplasts from tobacco suspension cell. *Plant Physiol* 64: 959-962.

Brooker, R. J. & Slayman, C. W. 1983. Effect of Mg^{2+} on the plasma membrane H^+-ATPase of *Neurospora crassa*. I. Inhibition by N- ethylmaleimide and trypsic.

Brummer, B., Bertl, A., Potrykus, I., Felle, H., Parish, R. W. 1985. Evidence that fusicoccin and indole-3-acetic acid induce cytosolic acidification of *Zea mays* cells. *FEBS Lett* 189: 109-14.

Brummer, B., Felle, H. & Parish, R. W. 1984. Evidence that acid solutions induce plant cell elongation by acidifying the cytosol and stimulating the proton pump. *FEBS Lett* 174 (2): 223-227.

Budd, K. 1989. Role of the membrane potential in the transport of zinc by *Neocosmospora vasinfecta*. *Exp. Mycol* 13: 1-8.

Bush, D. R. & Langston-Unkefer, P. J. 1988. Amino acid transport into membrane vessicles isolated from *Zucchini Plant Physiol* 88: 487-490.

Bush, D. R. 1989. Proton-coupled sucrose transport in plasmalemma vesicles isolated from sugar beet (Beta vulgaris L. cv great Western) leaves. *Plant Physiol* 89: 1318-1323.

Cheeseman JM, Hanson JB (1980) Does active K^+ ion influx to roots occur? *Plant Sci Lett* 18: 81-84

Cheeseman, J. M. & Hanson, J. B. 1979. Energy-linked potassium influx as related to cell potential in corn roots. *Plant Physiol* 64: 842-845.

Chung, I. 1989. A thesis of M.Sc. L-glutamate uptake, decarboxylation to γ-aminobutyric acid and GABA effect in isolated *Asparagus sprengeri* mesophyll cells. *Brock University Libarary*

de Kerk-kiebert, Y. M., Kneppers, T. J. A., Matthijs H. C. P. & Verleur, J. D. 1983. Sugar uptake in soybean (*Glycine max*) cells in suspension culture. *Physiol Plant* 57: 217-221.

Desmaison, A. M. & Tixier, M. 1986. Amino acids content in germinating seeds and seedings from *Castanea sativa* L. *Plant Physiol.* 81: 692- 695.

Despeghel, J. P. & Delrot, S. 1983. Energetics of amino acid uptake by *Vicia faba* leaf tissues. *Plant Physiol.* 71: 1-6.

Dufour, J. P. & Goffeau, A. 1983. Molecular and kinetic properties of the purified plasma membrane ATPase of the yeast *Schizosaccharaomyces pombe. Eur. J. Biochem.* 105: 145.

Dufour, J. P., Goffeau, A. and Tsong, T. Y. 1982. Active proton uptake in lipid vesicles, reconstituted with the purified yeast plasma membrane ATPase. Fluoresence quenching of 9-amino-6-chloro-2-methoxyacridine. *J. Biol. Chem.* 257: 9365.

DuPont, F. M., Giorgi, D. L. & Spanswick, R. M. 1982. Characterization of a proton-translocating ATPase in microsomal vesicles from corn roots. *Plant Physiol.* 70: 1694-1699.

Felle, H. 1981. Stereospecificity and electrogenicity of amino acid transport in *Riccia fluitans. Planta* 152: 505-512.

Felle, H. 1982. Short-term pH regulation in plants. *Physiol. Plant.* 74: 583-591.

Felle, H., Portor, J. C., Slayman, C. L. & Kaback, H. R. 1980. Quantitative measurements of membrane potential in *Escherichia coli. Biochemistry* 19: 3585- 3590.

Fowden, L. 1958. *Biol. Rev.* 33: 393.

Frachisse, J. M., Johannes, E. & Felle, H. 1988. The use of weak acids as physiological tools: a study of the effects of fatty acids on intracellcular pH and electrical plasmalemma properties of *Riccia fluitans* rhizoid cells. *Biochim. Biophys. Acta* 938: 199-210.

Grrinius, L. L., Jasaitis, A. A., Kadziauskas, yu. p., Liberman, E. A., Skulachev, V. P., Topali, V. P., Tsofina, L. M., and Vladimirova, M. A. 1970. Conversion of

biomembrane-produced energy into electric form. *Biochim. Biophys. Acta* 216: 1-12.

Guinn. G. & Brinberoft, L. A. 1970. *Crop. Sci* 10: 175.

Hager, A., Moser, I. 1985. Acetic acid esters and permeable weak acids induce active proton extrusion and extension growth of coleoptile segments by lowering the cytoplasmic pH. *Planta* 163: 391-400

Hedrich, R. 1989. The physiology of ion channels and electrogenic pumps in higher plants. *Annu. Rev. Plant Physiol.* 40; 539-569.

Hodges, T. K., Leonard, R. T., Bracker, C. E., and Keenan, T. W. 1972. Purification of an ion-stimulated adenosine triphosphatase from plant roots: association with olasma membrane. *Proc. Nat. Acad. Sci. USA* 69: 3307-3311.

Hoek, J. B., Nicholls, D. G. & Williamson, J. R. 1980. Determination of the mitochondrial protonmotive force in isolated hepatocytes. *J. Bio. Chem.* 255: 1458-1464.

Hoffman, J. F. & Laris, P. C. 1974. Determination of membrane poxentials in human and *Amphiuma* red blood cells by means of a flurrescent probe. *J. Physiol* 239:: 519-552.

Homble, F. 1985. Effect of sodium, potassiun, magnesiun and teraethyammonium on the transient voltage response to galvonostatic step and of the temperature on the study membrane conductance of *Chara cornollino* a further evidence for the involvement of potassiun channels in the fast time variant conductance. *J. Exp. Bot.* 36: 1603-1611.

Hope, A. B. 1965. Ionic relations of cells of Chara australis. Effects of bicarbonate ions on electricaal properties. *Aust. J. Biol. Sci.* 18: 789-801.

Huerta, A J. & Murphy, T. M. 1988. The kinetics of efflux of K^+ from cultured rose cells treated with ultraviolet light.

Hutchings, V. M. 1978. Sucrose and proton cotransport in *Ricinus* cotyledons. I. H^+ inhlux associated with sucrose uptake. *Planta* 138: 237-241.

Inatomic, K. & Slaughter, J. C. 1975. *Biochem.* J. 147: 479.

Keifer, D. W., Lucas, W. J. 1982. Potassiun channel in *Chara cornollino* control and interaction with the electrogenic H^+ pump. *Plant Physiol.* 69: 781-788.

Kennedy, C. D & Stewart, R. A. 192. Glucose-induced H^+ efflux and transent currents in excised root: particularly those of *Zea mays. J. Exp. Bot.* 33: 1220-1238.

Ketchum, K. A., Shrier, A. and Poole, R. J. 1989. Votage-activated K^+channels in corn plasmalemma are Ca^+dependent. *Plant Physiol.* 89: 148.

Kinraide, T. B. & Etherton, B. 1980. Electrical evidence for different mechanisms of uptake for basic, neutral, and acidic amino acids in oat coleoptiles. *Plant Physiol.* 65: 1058-1089.

Kinraide, T. B., Newman, I. A. and Etherton, B. 1984. A quantitative stimulation mode for H^+-amino acids on membrane potential and extracellular. *Plant Physiol.* 76: 806-813.

Kishinami, I & Qjima, K. 1980. *Plant Cell. Physiol.* 21: 581.

Kitasato, H. 1968. The influence of H^+ on the membrane potential and ion fluxes of *Nitella. J. Gen. Physiol.* 52: 60.

Kochian LV, LucaS WJ (1982) Potassium transport in corn roots. I. Resolution of Kinetics into a saturable and linear component. *Plant Physiol.* 70: 1723-1731.

Kochian LV, Shaff JE, LucaS WJ (1989) High affinity K^+ uptake in maize roots. A lack of coupling with H^+ efflux. *Plant Physiol* 91: 1202-1211

Kochian, L. V., Xin-Zhi, J. & Lucas, W. J. 1985. Potassium transport in corn roots. IV. Characterization of the linear componert. *Plant. Physiol.* 79: 771-776.

Komor, E. 1977. Sucrose uptake by cotyledons of *Ricinus communis* L.: characteristics, mechanism, and regulation. *Planta* 137: 119-131.

Komor, E. and Tanner, W. 1976. The determination of the membrane potential of *Chlonella valgaris* *Eur. J. Biochem.* 70: 197-204.

Komor, E., Thom, M. & Maretzki, A. 1981. The mechanism of sugar uptake by sugarcane suspension cells. *Planta* 153: 181-192.

Kulkarni, I. & Sohonie, K. 1956. *Nature* 178: 925.

Kurkdjian, A. & guern, J. 1989. Introcellular pH: measurement and importance in cell activity. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 40: 271-303.

Ladror, U. S. & Zielinski, R. E. 1989. Protein kinase activities in tonoplast and plsmalemma membrane from corn roots. *Plant Physiol* 89: 151-58

Lane, T. R. & Stiller, M. 1970. Glutamic acid decarboxylase in *Chlorella* *Plant Physiol* 45: 1322-1327.

Leonard, R. T. & Hotchkiss, C. W. Cation-stimulated adenosine triphosphatase activity and cation transport in corn roots.

Leonard, R.T. & Hotchkiss, C. W. 1976. Cation-stimulated adenosine triphosphatase activity and cation transport in corn roots. *Plant Physiol.* 58: 331-335.

Lew, R. R. 1989. Calcium activates an electrogentic proton pump in *Neurospora* plasma membrane. *Plant Physiol* 91: 213-16.

Lin, W. 1985. Energetics of sucrose transport in protoplasts isolated from developing soybean cotyledons. *Plant Physiol.* 78: 41-45.

Lin, W. 1984. Further characterization on the transport property of plasmalemma NADH oxidation system in isolated corn root protoplasts. *Plant Physiol.* 74: 219-222.

Linnett, P. E. & Beechey, R. B. 1979. Inhibitions of the ATPase synthesis system . *Methods Enzymol.* 55: 472.

Malpartida, F. & Serrano, R. 1980. Purification of the yeast plasma membrane ATPase solubilized with a novel zwetterionic detergent. *FEBS Lett.* 111: 69.

Malpartida, F. & Serrano, R. 1981. Proton translocation catalyzed by the purified yeast plasma membrane ATPase reconstituted in liposomes. *FEBS Lett.* 131: 351.

Marre, E. & Ballarin-Denti, A. 1985. The proton pumps at the plasmalemma and the tonoplast of higher plants. *J. Bioenerg. Biomembr.* 17: 1-21.

Marre, M. T. 1983. Transmembrane hyperpolarization and increase of K^+ uptake in maize roots treated with permeant weak acids. *Plant, Cell and Environment.* 6: 617-623.

McCutcheon, S. L. & Bown, A.W. 1987. Evidence for a specific glutamate/H^+ cotransport in isolated masophyll cells. *Plant Physiol.* 83: 691-697.

McCutcheon, S. L., Ciccarelli, B. W., Chung, I. Shelp, B. J. & Bown, A.W. L-glutamate-dependent medium alkalinization by *Asparagus* masophyll cells. *Plant Physiol.* 88: 1042-1047.

McCutcheon, S. L.1987. A thesis of M. Sc. Amino acid transport: the special case of a glutamate/H^+ cotransport system in *Asparagus sprengeri* masophyll cells. *Brock University Libarary*

Mercier, A.J. and Poole, R. J. 1980. Electrogenic pimp activity in red beet: it relation to ATP levels and to cation influx. *J. Membrane Biol.* 55: 165-174.

Mimura, T., Shimmen, T. & Tazawa, M. 1983. Dependence of the membrane potential on intracellular ATPase concentration in tonoplast-free cells of *Nitellopsis obtusa*. *Planta* 157: 97-104.

Mimura, T., Shimmen, T. and Tazawa, M. 1983. Dependence of the membrane potential on intracellular ATP concentration in tonoplast-free cells of *Nitellopsis obtusa*. *Planta* 157: 97-104.

Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature.* July 8 1961. 144.

Mitchell, W. J., Booth, I. R. & Hamilton, W. A. 1979. A quantitative analysis of proton-linked transport systems. Glutamate transport in *Staphylococcus aureus*. *Biochem. J.* 184: 441-449.

Mizusaki, S. Noyvehi, M. & Tamaki, E. 1964. Arch. *Biochem. Biophys.* 105: 599.

Murphy, T. M., wright, J. R. & Murphy, J. B. 1975. Inhibition of protein synthesis in cultured tobacco cells by ultraviolet radiation. *Photochem Photobiol.* 21: 219-225.

Newman IA, Kochian LV, Crusak MA, LucaS WJ (1987) Fluxes of H^+ and K^+ in roots. Characterzation and stoichiometries using ion-selective microelectrode. *Plant Physiol* 84: 1177-1184

Novacky, A., Ullrich-Eberius, C. I. & Luttge, U. 1980. pH and membrane potential changes during glucose uptake in *Lemna gibba* and their response to light. *Planta* 149: 321-326.

O'Neill, S .P. & Spanswick, R. M. 1984b. Characterization of native and reconstituted plasma membrane H^+-ATPase from the plasma membrane of *Beta vulgaris*. *J. Membrane Biol.* 79: 231- 243.

O'Neill, S .P. & Spanswick, R. M.1984a. Solubilization and reconstitution of a vanadate-sensitive H^+-ATPase from the plasma membrane of *Beta vulgaris*. *J. Membrane Biol.* 79: 231- 243.

Oka, K., Naitou, S., Yoshida, M., Ishikawa, H., Ohta, E., and Sakata, M.,1987. Membrane potential measurement of protoplast isolated from *Vigna mungo* Hypocotyl using fluorescent probe, diS-C_3-(5). *Plant Cell Physiol.* 28(5): 843-849.

Pantoja, O. and Willmer, C. M. 1986. Pressure effects on membrane potentials of mesophyll cell protoplasts and epidermal cell protoplasts of *Commelina communis* L. *J. Exp. Bot.* 37: 315-320.

Pedersen, L. P. & Carfolic, E. , 1987. Ion motive ATPase. I. ubiquity, properties and siginificance to cell function. *TIBS* 12: 146-150.

Poole, R. J. 1966. The influence of the intracellular potential on potassium uptake by beetroot tissue. *J. Gen.Physiol* 49: 551.

Poole, R. J. 1974. Ion transport and electrogenic pumps in storage tissue cells. *Can. J. Bot.* 52: 1023-1028.

Poole, R. J. 1978. Energy coupling for membrane transport. *Ann. Rev. Plant Physiol.* 29: 437-460.

Rasi-Caldogno, F., de michelis, M. I. & Pugbarello, M. C. 1981. Evidence for an electrogenic ATPase inmicrosomal vesicles from pea inlernoder. *Biochim. Biophys. Acta* 642: 37-45.

Reggiani, R., Cantic, C. A., Brambillka, I. & Bertani, A. 1988. Accumulation and interconversion of amino acids in rice roots under anoxia. *Plant Cell. Physiol.* 29: 981-987.

Reid, R. J. Field, L. D., Pitman, M. G. 1985. Effects of external pH, fusicoccin and butyrate on the cytoplasmic pH in barley root tips measureed by ^{31}P-nuclear magnetic resonance spectroscopy. *Planta* 166: 341-47.

Reinhold, L. & Kaplan, A. 1984. Membrane transport of sugar and amino acids. *Ann. Rev. Plant Physiol.* 35: 45-83.

Ritchie, R. J. 1984. A critical assessment of the lipophilic cation as membrane potential probes. *Prog. Biophys. Molec. Biol.* 43: 1-32.

Robinson, S. P. & Beevers, H. 1981. Evidence for amino : proton cotransport in *Ricinus* cotyledons. *Planta* 152: 527-533.

Rubenstein, B. 1978. Use of lipophilic cations to measure the membrane potential of oak leaf protoplasts. *Plant Physiol.* 62: 927-929.

Saddler, H. W. D. 1970. The membrane potential of *Acetabularia mediterranea*. *J. Gen. Physiol.* 55: 802.

Salimath, B. P. & Marme, D. 1983. Protein phosphorylation and its regulation by calcium and calmodulin in membrane fractions from zucchini hypocotyls. *Planta* 158: 560-68

Satya Narayan, N. & Nair, P.M. 1985. *Eur. J. Biochem.* 150: 53.

Satya Narayan, V. & Nair, P. M. 1986. The 4-aminobutyrate shunt in *Solanum tuberosum*. *Phytochem.* 25 (5): 997-1001.

Satya Nasayan, v. & Nair, P. M. 1986. Enhanced operation of 4-aminobutyrate shunt in γ-irradiated potato tubers. *Phytochem* 25 (8): 1801-1805.

Schaller, G. E. & Sussman, M. R. 1988. Phosphorylation of the pkasma-membrane H^+-ATPase of oat roots by a calcium-stimulated protein kinase. *Planta* 173: 509-18.

Schroeder, J. I. 1988. K^+ transport properties of U^+ channels in the plasma membrane of *Vicia faba* guard cells. *J. Gen. Physiol.* 92: 667-683.

Schroeder, J. I., Raschke, K. & Neher, E. 1987. Votage dependence of K^- channels in guard-cell protoplasts. *Proc. Nat. Acad. Sci. USA* 84: 4108-4112.

Selman, I. W. & Cooper, P. 1978. Changes in the free amino compourds in young tomato plants light and darkness with particular reference to γ-aminobutyric acid. *Ann. Bot.* 42: 627-636.

Serrano, R. 1984. Plasma membrane ATPase of funfi and plants as a novel type of proton pump. *Curr. Top. Cell Regul.* 23: 87-126.

Serrano, R. 1985. Plasma membrane ATPase of plants and fungi.CRC Press, Inc.

Serrano, R. 1987. H^+ ATPase from the plasma membranes of *Saccharomyces cenevisiae* (baker's yaest) and *Avena sativa* (oat) root. Purification and reconstitution. *Methods Enzymol.*

Serrano, R. 1988. Struture and function of proton translocating ATPase in plasma membrane of plants and fungi. *Biochim. Biophys. Acta* 947: 1-28.

Serrano, R., Cano, A. & Pestana, A. 1985. The plasma membrane ATPase of *Dictyostelium* discoideum. *Biochim. Biophys. Acta* 812: 553.

Shmitt, M. R., Hitz, W. D., Lin, W. & Giaquinta, R. T. 1984. Sugar transport into protoplasts isolated from developing soybean cotyledons. II. Sucrose transport kinetics, selective and modeling studies. *Plant Physiol* 75: 941-946.

Slayman, C. L., 1965. Electrial properties of *Neurospora crassa* respiration and the intracellular poterntial. *J. Gen. Physiol* 49: 93-116.

Smith, J. E. & Waygood, E. R. 1961. *Can. J. Biochem.* 150: 53.

Sokolik, A. I. & Yurin, V. M. 1986. potassium channels in plasmalemma of *Nitella* cells at rest. *J. Membr. Biol* 89: 9-12.

Spanswick, R. M. 1970. The effects of bicarbonate ions and external pH on the membrane potential and resistance of *Nitella translucens*. *J. Membr. Biol* 2: 59-73.

Spanswick, R. M. 1972. Evidence for an electrogenic ion pump in *Nitella translucens*. *Biochim. Biophys. Acta.* 288: 65-72.

Spanswick, R. M. 1974. Evidence for an electrogenic ion pump in *Nitella translucens*. II. Control of the light stimulated component of the membrane potential. *Biochim. Biophys. Acta.* 332: 387-398.

Spanswick, R. M. 1981. Electrogenic ion pump. *Ann. Rev. Plant Physiol* 32: 267-321.

Streeter, J. G. & Thompson, J. F. 1972. In vivo and in vitro studies on γ-aminobutyric acid metabism with the radish plant (*Raphanus satives* L.) *Plant Physiol* 49: 579-584.

Sze, H. & Churchill, K. A. 1981. Mg^{2+}/KCl-ATPase of the plasma membrane is an electrogenic pump. *Proc. Nat. Acad. Sci. USA* 78: 5578-5582.

Sze, H. 1980. Nigericin-stimulated ATPase activity in microsomal vesicles of tobacco callus. *Proc. Nat. Acad. Sci. USA* 77: 5904-5908.

Sze, H. 1984. H^+-translocating ATPase of the plasma membrane and toloplast of plant cells. *Plant Physiol* 61: 683-691.

Teser, M. & macRobbie, E. A. C. 1990. Cytoplasmic calcium affects the gating of potassium channels in the plasma membrane of Chara corallina: a whole-cell study using calcium-channel effectors. *Planta* 180: 569-581.

Thompson, J. F., Pollard, J. K. & Steward, F. C. 1953. *Palnt physiol* 28: 401.

Van Bel, A. J. E. & van der Schoot, C. 1980. Light-stimulated bi phasic amino-acid uptake by xylem praenchyma cells. *Plant Sci. Lett* 19: 101-107.

Vandewalle, I & Olsson, R. 1983. *Plant Sci Lett* 31: 269.

Vara, F. & Serrano, R. 1982. Partial purification and properties of the proton-translocating ATPase of plant plasma membranes. *J. Biol. Chem* 257: 12826.

Villalabo, A., Boutry, M. & Goffeau, A. 1981. Electrogenic proton translocation coupled to ATP hydrolysis by the plasma membrane Mg^{2+}-dependent ATPase of yeast in reconstituted proteoliposomes. *J. Biol. Chem* 256: 12081.

Wallace, W., Secor, J. & Schrader, L. E. 1984. Rapid accumulation of 4-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, ormechanical manipulation. *Plant Physiol* 75: 170.

Wayse, R. & Romon, E. 1984. Machanism of amino acid uptake by sugarcane suspension cells. *Plant Physiol* 76: 865-870.

Willsky, G. R. 1979. Characterization of the plasma membrane Mg^{2+}-ATPase from the yeast *Sacckaromyces cerevisiae*. *J. Biol. Chem* 354: 3326.