

ATPase proton translocation across isolated tonoplast vesicles of wheat
by Glenn Michael Magyar

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
Montana State University
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Abstract:
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The presumptive TP enriched fraction is also enriched in a 70 kD polypeptide which strongly cross-reacts with antiserum developed against an amino terminus peptide of the 70 kD subunit of the carrot (Daucus carota) TP ATPase.

AlF4^- (fluoroaluminate), which strongly inhibits the orthovanadate sensitive PM ATPase activity, has relatively little effect on the nitrate sensitive TP ATPase activity.

This work shows for the first time, the identification and characterization of a nitrate sensitive, proton translocating ATPase activity in the vacuolar membrane of wheat.

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Glenn Michael Magyar

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency and is ready for submission to the College of Graduate Studies.

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Date

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Approved for the Major Department

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ABSTRACT

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INTRODUCTION

Plant Cell Vacuoles

One of the major differences between plant and animal cells is the existence of a prominent vacuole found in most plant cells. This organelle is bounded by a lipid bilayer membrane known as the tonoplast. In some cells the vacuole is so large that nearly 90% of the total cell volume lies within it whereas the cytoplasm constitutes only about 4% (10). This organelle is so large, that it accounts for the majority of eukaryotic biomass on the earth (20). Because of the sugars and other nutrients that accumulate within the vacuole it has been argued that this is one of the most important parts of the entire plant. If this concentration of cell sap did not occur, most fruits and vegetables would have no appealing taste at all (30).

Vacuolar Functions

Perhaps the most important function of the plant cell vacuole is the maintanance of cell turgidity. A high concentration of solutes is commonly found within the vacuole, which results in a subsequent influx of water. This causes the vacuole membrane to be pressed against the

cytoplasm which in turn is pressed against the cell wall. This pressure enables non-woody plants to stand erect and is also responsible for cell enlargement in growing plants (12, 15, 22, 28).

There is strong evidence to support a lytic function of the vacuole thus making it similar to the lysosomes that are found in animal cells (14, 15, 19, 22). The various types of hydrolytic enzymes that have been found within the vacuole include proteinases, acid phosphatase, phosphodiesterase, RNase, DNase, alpha and beta-amylases, beta-glucosidase and beta-galactosidase. These enzymes are all specific to different cells and physiological functions, e.g. leaf abscission, plant senescence and seed germination (15, 19).

Another function of the vacuole is storage. Many different types of biomolecules can be found within the vacuole, including: sucrose; organic ions such as malate, citrate and oxaloacetate; inorganic ions such as K^+, Na^+ and Cl^- and also various amino acids and proteins. The high concentration of these organic acids contributes to the low pH of the vacuole, which is typically around 5.5 (31). Many of these metabolites are stored within the vacuole until they are needed by the plant (15,19).

The vacuole is involved in at least one other function of importance to the plant cell. It has been proposed (19) that the vacuole serves the same extracytoplasmic purpose in

the plant cell as the intercellular spaces do in animal cells. In other words, they are able to act as a compartment that mediates the exchange of components to and from the cytoplasm. The concentrations of these various components are maintained by transport systems at both the plasmalemma and the vacuole membrane.

Vacuolar Biochemistry

The vacuole membrane is also called the tonoplast, a name that implies a very elastic nature. This appears to be true as tonoplasts have been shown to be able to increase in surface area by 1.5 times upon uptake of water into the cell and also to withstand the intense shearing forces of cytoplasmic streaming (30).

The tonoplast is an asymmetrical lipid bilayer that surrounds the plant vacuole. It has been shown to be composed of a high percentage of saturated fatty acids, a property which is thought to be important in maintaining the fluidity of this membrane (20,30).

The proteins that are associated with the vacuole membrane consist of both integral and peripheral proteins on both faces. It has been shown with freeze-fracture faces from electron microscopy that the cytoplasmic side of the membrane appears to contain more globular proteins than the exoplasmic or interior side. However these proteins do not

appear to be anchored into place by microfilaments as they tend to line up into a row when the membrane is ruffled (30). Polyacrylamide gel electrophoresis, has shown that none of the major polypeptides associated with the tonoplast have a molecular weight of greater than 100,000 (20).

Carbohydrates have also been identified on the interior or exoplasmic side of the tonoplast (20), but not on the cytoplasmic side. Transmission electron micrographs show that the inner portion of the membrane appears thicker than the outer portion. The carbohydrates associated with the inside face could account for this observation.

Electrogenic Pumps and Proton Gradients

By the 1960's, it was known that differences in electrical potential existed between the vacuole, the cytoplasm and the surrounding medium. Since the differences were greater than what could be attained by simple diffusion, an electrogenic "pump" was postulated to be the cause for the gradients that were observed. Higinbotham (10) stated in 1970 that in the plasma membrane,

> "....an efflux pump for H^+ is a likely possibility; this is consistent with the hypothesis of Mitchell (21).....the source of energy could be the potential energy represented by sharp H^+ gradients or to reducing energy, e.g., NADH, as well as to ATP."

A cellular electrogenic pump is defined as a transport system that directly contributes to the electrical potential across a membrane (25). In 1974, several papers were published from a symposium that dealt with the subject of ion transport in plants (11, 24, 27, 32). Two conclusions drawn from these investigations were that the pH of the cytoplasm remains relatively constant in a cell and that the proper physiological pH must be maintained by the active transport of H^+'s across the membrane by a suitable proton pump (31).

Proton gradients formed by pumps are found in several subcellular locations in plants. Both the mitochondria and the chloroplast produce adenosine triphosphate (ATP) from the flow of H^+'s down a concentration gradient. These gradients are established from the action of an electron transport system. Whereas this type of proton electrochemical gradient is used to synthesize ATP via the action of complex ATP synthetases, there are at least two types of enzymes in plants that hydrolyze ATP to form an electrochemical proton gradient (18, 34, 35). These H^+ translocating adenosine triphosphatases (ATPases) are found in both the plasma membrane (PM) and the tonoplast (TP) of higher plants and are responsible for the establishment of an electrochemical proton gradient across these membranes.

Recently, another type of proton translocating enzyme has been shown to exist in the tonoplast. This pump

utilizes pyrophosphate (PP_i) as an energy source and also contributes to the pH gradient that is maintained across the tonoplast (29). It has also been reported that this pump is capable of establishing and maintaining a pH gradient in isolated vacuoles when PP_i is used as the sole energy source for proton pumping (8).

Once this proton gradient is established it subsequently drives a number of important processes such as the transport of nutrients (1) the maintanence of cytoplamic pH (34) and the transport of ions (7).

Tonoplast ATPase

While it has been pointed out that there are discrepancies in the literature regarding the TP ATPase (17), there is enough complimentary information available to review the general characterstics of this enzyme and to compare them to the general characteristics of the PM ATPase.

The primary function of both the TP and the PM ATPase is to establish and maintain a proton gradient within the plant cell by active transport of hydrogen ions. The potential energy of this proton gradient is then used to transport other solutes across the membrane. (6, 7, 12, 14, 18, 34, 35, 37).

These enzymes are oriented so that the ATP binding site faces the cytoplasm (23, 34), this allows for the hydrolysis of ATP which in turn provides the energy needed to power the electrogenic proton transport (18). As the gradient becomes steeper, the rate of ATP hydrolysis slows down and finally stops when the transmembrane difference in pH is about 1.5 - 2.0 units and the membrane potential is about 30 mV. Proton ionophores, (molecules that can dissipate a proton gradient), form holes in the membrane and cause the hydrolysis of ATP to begin again. Therefore it may be inferred that the ATPase is affected by the electrochemical proton gradient (34). Figure 1 shows a schematic diagram of the transport proteins located in both the tonoplast and the plasmalemma.

The ATPase of the tonoplast is distinguished from other known ATPases because of several unique properties. These properties were not very clearly defined before 1980 because of the difficulty encountered in the isolation of viable, intact, non-leaky vacuole membranes from plant cells.

Currently, the tonoplast and other endomembranes are separated from large organelles in homogenized plant tissue by differential centrifugation into a so-called microsomal fraction. The membrane vesicles found in this fraction of plant cells include tonoplast, endoplasmic reticulum, golgi, plasma membrane and small fragments of both the mitochondrial and chloroplast membranes (18, 34, 35). Since the

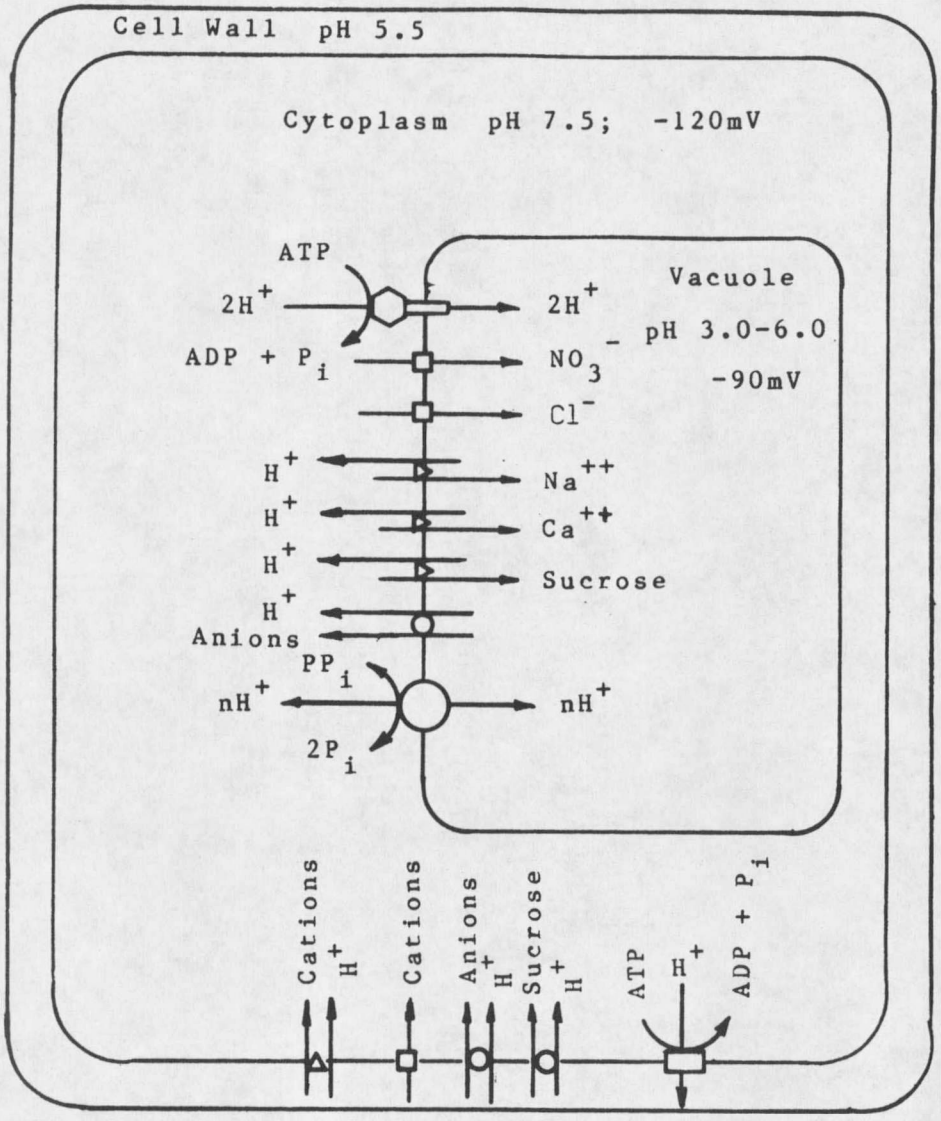


Figure 1. Schematic diagram of a typical plant cell and the transport proteins that have been shown to be associated with the tonoplast and plasma membranes (adapted from Sze, 1984 and Rea and Sanders, 1987).

density of the tonoplast membrane (1.0 - 1.13 g/cm^3) has been shown to be less than that of the PM (1.13 - 1.17 g/cm^3), the separation of these two major components of the microsomal fraction has been possible by utilizing the technique of density gradient centrifugation (4, 5, 35).

The TP ATPase has been shown to be sensitive to negatively charged ions. A most potent inhibitor of this enzyme is the anion nitrate (NO_3^-) which strongly inhibits the TP ATPase. Because of this factor, nitrate inhibition of ATPase activity has been used to identify the tonoplast vesicles of the microsomal fraction (35). Other anions appear to stimulate the activity of the TP ATPase, apparently by dissipating the electrochemical gradient. The ranking of anions in order of stimulation is as follows: $Cl^- > Br^- > I^- > HCO_3^- > SO_4^-$ (34).

Other inhibitors that have been shown to be effective against the TP ATPase include N,N'-dicyclohexylcarbodiimide, (DCCD); 7-chloro-4-nitro-benzo-2-oxa-1,3-diazole, (NBD-Cl); and 4,4'-diisothiocyano-2,2'stilbene disulfonic acid, (DIDS) (16, 26, 34, 35). It has been suggested that DIDS directly inhibits the enzyme by interacting with the anion sensitive sites.

The PM ATPase is strongly inhibited by orthovanadate, a molecule which has no effect on the TP ATPase. This allows for the identification of plasma membrane vesicles in the microsomal fraction. The effect of orthovanadate suggests

that there is a covalent phosphoenzyme intermediate formed in the mechanism of hydrolysis for the PM ATPase since orthovanadate competes with the phosphate for binding. However, because the TP ATPase is not affected by orthovanadate, the mechanism of action for hydrolysis of ATP would seem to be different than that for the PM ATPase (34, 35).

The subunit structure of the TP ATPase has only recently been determined. None of the polypeptides that are found to be associated with the tonoplast have a molecular weight that is greater than 100 kD. It has been shown that there are three major polypeptides that typically make up the TP ATPase in higher plants (2, 16). A 70-72 kD polypeptide which has been implicated as the catalytic subunit. A 60-62 kD polypeptide which appears to function as a regulatory subunit, and a 14-18 kD subunit that binds DCCD and is considered to be a proton channel through the vacuole membrane (16, 26). Together these subunits comprise a holoenzyme of about 400-500 kD. This is quite large when compared to the PM ATPase that is made up of only a single subunit of about 100 kD (23).

Other notable characteristics of the TP ATPase include: insensitivity to azide (which completely inhibits the mitochondrial ATPase), a pH optimum of around 8, substrate specificities of ATP > PPi > GTP > NTP, a K_m for ATP of

about 0.1 - 0.2 mM, and a dependance upon Mg^{++} for activity (14, 26, 34).

There has recently been a report that describes the isolation of a cDNA clone of the gene that encodes the 69 kD subunit of the TP ATPase in carrot (38). From this clone the primary amino acid sequence of the subunit was determined to be 623 residues in length with a molecular weight of 68,835. Interestingly, the amino acid sequence was 70% homologous to the exons of a 69 kD genomic clone from the fungi Neurospora. When compared to a 275 amino acid core sequence of the B subunit of mitochondrial ATPase from several non-plant sources, there was a sequence homology of 34.3%. These results lead to a proposal that the TP ATPases of higher plants may be closely related to the F_0F_1 type ATPases found in both chloroplasts and mitochondria (38).

The activity of the TP ATPase has been characterized in many different species of plants including barley, beets, corn and oats. While the PM ATPase has been characterized in wheat, to date there have been no reports concerning the activity of the TP ATPase in this important crop plant. The goal of this project is to investigate the possible occurrence of a nitrate sensitive, H^+-translocating ATPase activity in wheat.

EXPERIMENTAL PROCEDURES

Plant Material and Membrane Isolation

Wheat (Triticum aestivum L. cv. Winalta) or Oat (Avena sativa L. cv. Cayuse) seeds were sown in moist vermiculite and grown in the dark at 22^0 to 24^0 for 6 days. The roots (10 to 15 cm in length) were excised and rinsed in cold water.

Membranes were isolated essentially as described by DuPont et al. (5), and all procedures were carried out at 0^o to 5^o C. The excised roots were ground in a cold mortar and pestle, and washed sea sand was added to the mixture to aid in homogenization. The grinding buffer consisted of 0.25 M sucrose, 4 mM dithiothreitol (DTT), 50 mM Tris-HCl (pH 7.8), 8 mM ethylenediaminetetraacetic acid (EDTA), and phenylmethylsulfonylfluoride (PMSF) (7.2 mg/ml, added just prior to use). The roots were re-ground four times in fresh grinding buffer. Typically, 400-500 ml of grinding buffer were used for 50-60 g fresh weight of roots. The homogenate was strained through 4 layers of cheesecloth and centrifuged for 10 min. at 10,000g. The 10,000g supernatant was then centrifuged for 30 min. at 80,000g. The pellet was resuspended in 6 ml of resuspension buffer consisting of 0.25 M sucrose, 2.0 mM DTT, 5.0 mM

piperzine-N,N'-bis[2-ethanesulfonic acid (PIPES)-KOH (pH 7.2). The resupended pellet was layered over discontinuous sucrose gradients, consisting of 12 ml of 40% (w/w) sucrose and 8 ml each of 34%, 30%, and 22% (w/w) sucrose in 1 mM DTT, 1mM EDTA and 1mM Tris-HCl (pH 7.2). The sucrose gradients were centrifuged at 80,000g for 3 hours in a Beckman SW 28 rotor. The membrane fractions at the interfaces of the step gradient were collected with a Pasteur pipette bent at the tip and diluted at least 5 times with a dilution buffer consisting of 150 mM KCl, 2mM DTT and 25 mM Tris-HCl (pH 8.0). Each diluted fraction was repelleted at 80,000g, and the pellets were resuspended in 1 ml of the resuspension buffer and stored at -70^{0} C.

Protein Determination and Enzyme Assays

Protein was determined as described by Lowry et al. (13) using bovine serum albumin as a protein standard.

Adenosine triphosphatase (ATPase) activity was measured in a 0.5 ml reaction volume containing from 15 to 30 ug protein, and the release of inorganic phosphate was determined by the method of Stout and Cleland (33). The standard assay contained either 30 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) - Tris (pH 7.5) or 30 mM 2[N-morpholino] ethanesulfonic acid (MES) - Tris (pH 6.5), 3.5 mM $MgSO_4$, 0.6 mM NaN_3, 0.6 mM $NaMoO_4$,

3 mMATP-tris, and 0.025% Triton X-100. Potassium chloride (KCl), potassium nitrate (KNO_3), fluoride (KF), aluminum ($AlCl_3$), and orthovanadate (Na_3VO_4) were added as indicated in the individual assays.

Spectrofluorometric Assays

Proton translocation across sealed membrane vesicles was measured as a quenching of the two permeant fluorescent dyes, acridine orange and quinacrine. The optimum parameters for fluorescence excitation (acridine orange = 426 nm; quinicrine = 448 nm) and emission wavelengths (acridine orange = 529 nm; quinicrine = 500 nm) were determined for both of these dyes and used in all subsequent experiments. The total volume in a quartz cuvette was 3 ml including 40 mM (1,3-bis[tris(hydroxy-methyl)-methyl-amine]-propane (BTP)- HEPES (pH 7.55), 0.15 M KCl, 0.2 mM (ethylene-glycolbis)-B-(aminoethyl ester)-N,N,N,N-tetra-acetic acid (EGTA), 0.005 mM acridine orange or quinacrine, 5 mM $MgSO_4$, 0.5 mM sodium azide and approximately 250 ug of membrane protein. Evolution of fluorescence response with time was monitored using a SPEX Fluorolog2 spectro-fluorometer (model f211, with single photon counting and real time data acquistion). The bandpass was set for resolutions of 2.25 nm and 4.5 nm for the excitation and emission monochromators, respectively. Depolarizing wedges

in both monochromators eliminated correction due to polarization dependencies. The assay mixture in the absence of ATP was monitored for 120 seconds to establish a baseline. At 120 seconds, ATP-Tris (pH 7.5) was added to the cuvette to a final concentration of 3 mM, and the fluorescence was read until 400 seconds had elapsed. Six ug of carbonyl cyamide m-chlorophenyl hydrazone (CCCP) were then added from a stock solution of 2 mg/ml in 100% ethanol to dissipate the proton gradient. Various other additions are noted in the figure legend.

Gel Electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was essentially performed using the procedure of Chua (3), utilizing gradient gels (1.5 mm x 13 cm x 15 cm) of 5 to 15% acrylamide. Electrophoresis was carried out overnight at 8 mA constant current with approximately 25 to 50 ug of protein applied to individual wells. In some cases, gels were stained with 0.2% Coomassie Brilliant Blue in 50% (v/v) methanol and 10% (v/v) acetic acid and destained in several changes of 10% (v/v) methanol and 7% (v/v) acetic acid.

Electrotransfer (Western Blots) and Immunostaining

Immediately after SDS-PAGE, the gel was soaked in a solution of 10% glycerol in 50 mM Tris-HCl (pH 7.5) for 30 to 60 minutes. Proteins from the gels were transferred to nitrocellulose using a Bio-Rad Transblot Cell. The transfer buffer consisted of 10 mM sodium bicarbonate, 3 mM sodium carbonate, 20% methanol, and 0.01% SDS. Transfer was performed for 4 hours at 0.25 A, followed by 0.50 A for 1 hour. Nitrocellulose blots were air-dried, cut into 5mm wide strips, and then either stained with India Ink (9) or immunostained essentially as described by Towbin, et al. (36). The electrophoretic blots were first soaked for 1 hour at room temperature (about 22^0 C) in Tween 20-Tris buffered saline/bovine serum albumin (TTBS/BSA) which consisted of 20 mM Tris (pH 7.5), 0.5 M NaCl, 0.05% Tween 20 and 1% (w/v) BSA. They were then washed once in TTBS/BSA and incubated for 2 hours in antiserum or normal rabbit serum diluted in TTBS/BSA as a control. Rabbit antiserum against the amino-terminus peptide of the 70 kD subunit of the vacuolar ATPase from carrot (Daucus carota) was a gift from Professor Lincoln Taiz of the University of California at Santa Cruz. The blots were then washed four times in TTBS and incubated for 1 hour in the secondary (indicator) antibody, goat anti-rabbit immunoglobulin, conjugated to

alkaline phosphatase and diluted 1/2000 in TTBS/BSA. The blots were then washed twice in TTBS and twice in Tris buffered saline (TBS). The color development solution consisted of a 1 ml solution containing 30 mg of p-Nitro Blue Tetrazolium chloride in 70% (v/v) dimethylformamide (DMF) plus a 1 ml solution of 15 mg 5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt) in 100% DMF both dissolved in 100 ml of carbonate buffer, (0.1 M $NaHCO_3$-NaOH, pH 9.8, and 1 mM $MgCl_2$). After 2 to 10 minutes, the reaction was terminated by transferring the blots to double-distilled water. Following several washes in double-distilled water, the blots were allowed to air dry.

RESULTS

Membrane Isolation

Membrane fractions from wheat roots were obtained by differential and sucrose density gradient centrifugation. The crude membrane preparation was separated into four fractions. The 30/34% sucrose interface was discarded because it contained very little membrane material. SDS-PAGE was then used to compare the protein patterns of the three remaining membrane fractions (Figure 2). The protein profiles of the 0/22% sucrose interface (A) and the 22/30% sucrose interface (B) appear to be qualitatively very similar, however there are noticeable differences between them. There is a very faint band seen at about 100 kD in A but not in B. The band at about 75 kD is darker in A than B. There is a group of bands at about 38 kD that is prominent in A but not seen in B. Two large dark staining bands at 30 and 32 kD appear to be in much greater concentration in A than in B. The band at 27 kD is much darker in A than in B and finally the two bands at 12 and 14 kD are visible in A but not seen in B.

The band at 85 kD in fraction B is darker than the one seen in fraction A. This is also seen for the bands at 72, 60, 50, 45 and 35 kD.

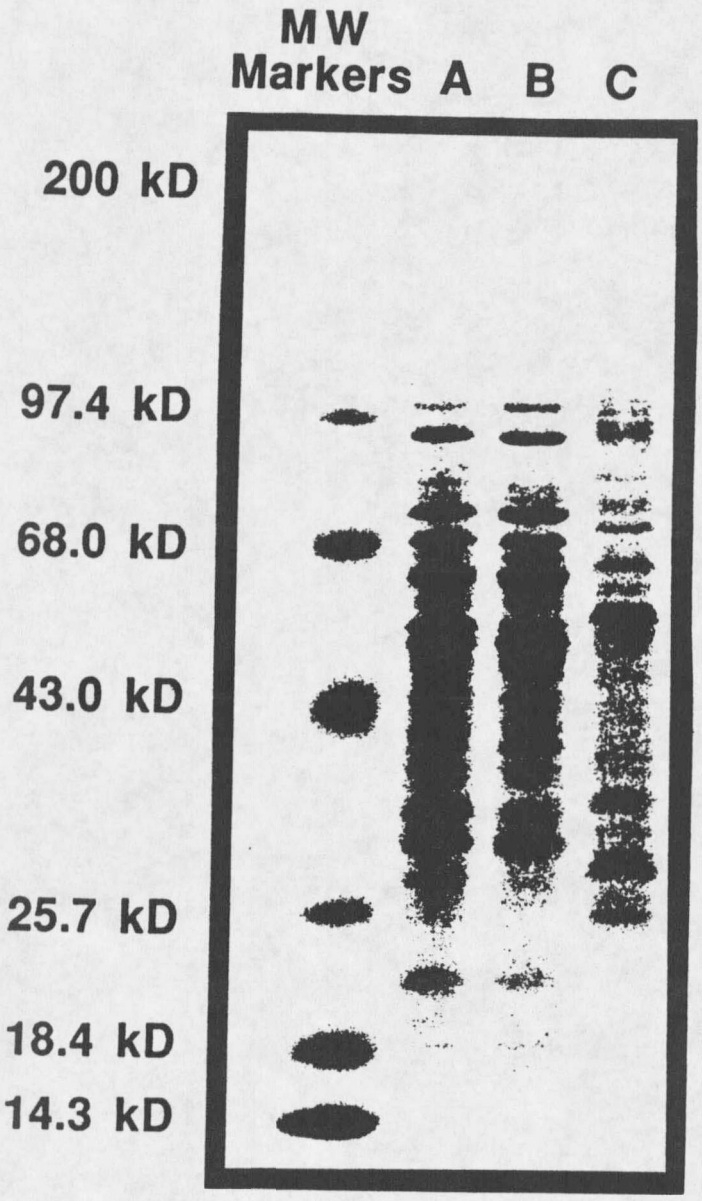


Figure 2. SDS-PAGE of the separated membrane fractions from wheat roots. Lane A is the 0/22% interface, Lane B is the 22/30% interface and Lane C is the 34/40% interface.

The profile of the 34/40% sucrose interface (C) is seen to be very different from either A or B. These results are comparable to those shown by DuPont et al. in barley roots (5). Based on the data that they had reported, fraction B was expected to be enriched in tonoplast derived vesicles and fraction C to be enriched in plasma membrane vesicles. To test this the three membrane fractions were assayed for nitrate sensitive and vanadate sensitive ATPase activity.

Enzymatic Characterization of Membrane Fractions

Nitrate inhibition of chloride stimulated ATPase activity, (in the presence of azide, at pH 7.5), is indicitive of the activity of the TP ATPase (29). Table I shows the results of ATPase assays on the three membrane fractions. These data support the idea that fraction B from wheat roots is relatively enriched in tonoplast vesicles. Fraction C contains the highest orthovanadate sensitive ATPase activity. In the presence of molybdate (to inhibit non-specific phospha- tases), this type of inhibition has been shown to be a marker of the PM ATPase.

Once the TP enriched fraction had been identified, a nitrate concentration curve experiment was run in order to determine the effects of increasing amounts of nitrate upon the activity of the ATPase. Figure 3 shows an inhibition of ATPase activity with increasing amounts of nitrate. An

Fraction	Protein conc*	NO_3^- sensitive ATPase activity**	Van sensitive ATPase activity
A. 0-22% interface	1.3 mg/ml	9.7	5.0
B. 22-30% interface	2.5mg/ml	17.0	21.9
C. 34-40% interface	1.3mg/ml	6.9	33.0

* average of 4 membrane isolations

** Specific Activity= µmoles of P_i liberated/hour•mg protein

Table 1. Membrane fractions and their associated protein concentrations and enzymatic activities. Protein concentrations were determined by using the method of Lowry et al. (13) and are an average of four different membrane isolations. ATPase activity was determined by using the method of Stout and Cleland (33). In all ATPase assays, the detergent Triton-X was included to ensure that all membrane bound proteins were solubilized.

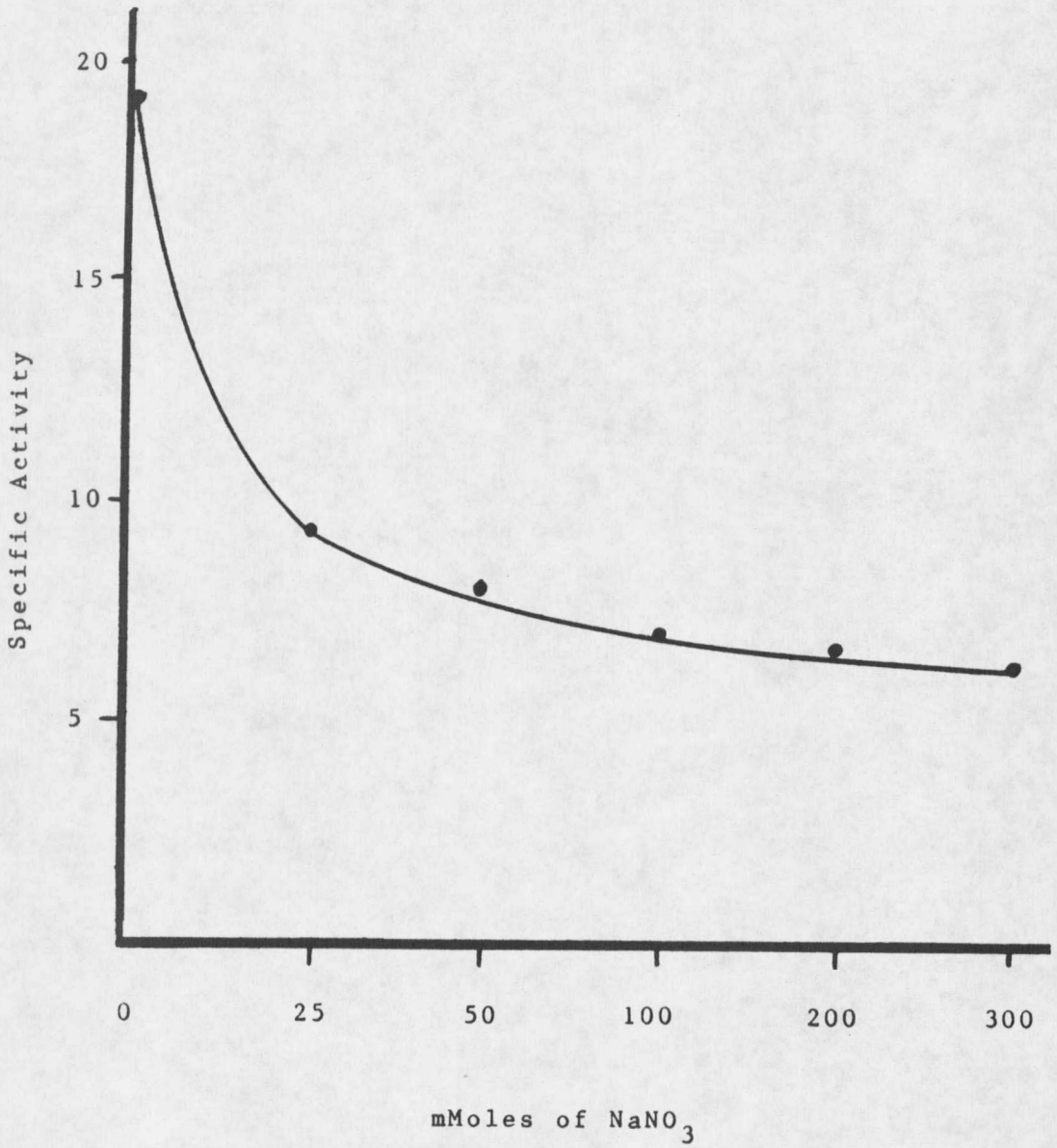


Figure 3. The effect of nitrate on ATPase activity of membranes from fraction B. Specific activity is in umoles P_i/hour/mg protein. Average of two different tonoplast enriched membrane fractions.

inhibition of about 50% was observed at 25 mM nitrate. This is consistant with previous reports of TP ATPase nitrate sensitivity.

Spectrofluorometry

It was clear from the results discussed above that a membrane fraction from wheat roots contains nitrate sensitive ATPase activity. In order to determine if this ATPase activity was responsible for the pumping of protons across the vesicle membranes, spectrofluorometric studies were conducted.

Figure 4 shows the results of the spectrofluorometric assays for fraction B. The quenching of fluoresence of the dye is indicative of proton gradient formation across sealed membrane vesicles. This activity in the vesicles of fraction B is seen to be sensitive to nitrate but not to orthovanadate. This data further supports the idea that TP ATPase proton pumps are present in wheat.

Antiserum Cross-Reactivity

Western blots of the proteins separated by SDS-PAGE from the membrane fractions of wheat roots were used for antibody cross-reactivity studies. Antiserum was developed in rabbits against an amino-terminus peptide of the 70 kD

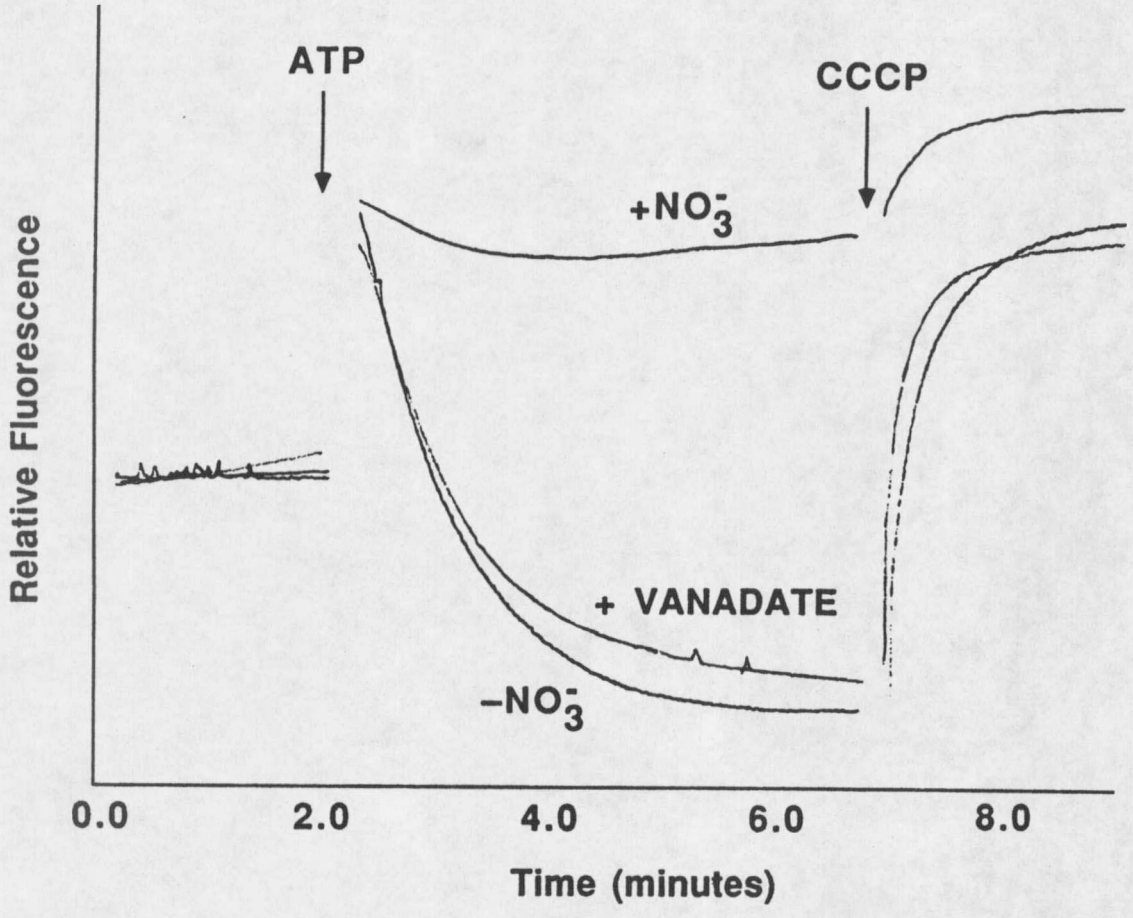


Figure 4. Fluorescence quenching/proton pumping studies. $-NO_3$ is the control. + NO_3 is the addition of 50 mM KNO_3 (TP^3 ATPase inhibitor); + Vanadate is the addition of 50 mM orthovanadate (PM ATPase inhibitor).

subunit of the TP ATPase in carrot root and was kindly provided by Professor Lincoln Taiz of the University of California at Santa Cruz. Figure 5 shows the cross-reactivity of this antiserum to a 70 kD polypeptide found in fraction B. This shows the apparent structural similarity between the amino-terminus of the 70 kD subunit of the carrot TP ATPase and a 70 kD polypeptide found in wheat root membranes. This antiserum also cross reacted to 70 kD polypeptides in both fraction A and fraction C, however the amount of binding in fraction B (the presumed tonoplast-enriched fraction) was much greater than these two (data not shown).

To further substantiate these results found in wheat, a microsomal fraction from oat roots was isolated and separated by sucrose density gradient centrifugation. Figure 6 shows the results of ATPase assays and the effects of nitrate. These results correlate with the data shown for the membranes of the microsomal fraction of wheat roots (Table 1), in that the nitrate sensitive ATPase activity is enriched in fraction B. The Western blots of these oat root membranes also correlate well with the immunoblots for wheat roots membranes and show nearly the same levels of antibody cross-reactivity to the carrot ATPase antibody (data not shown).

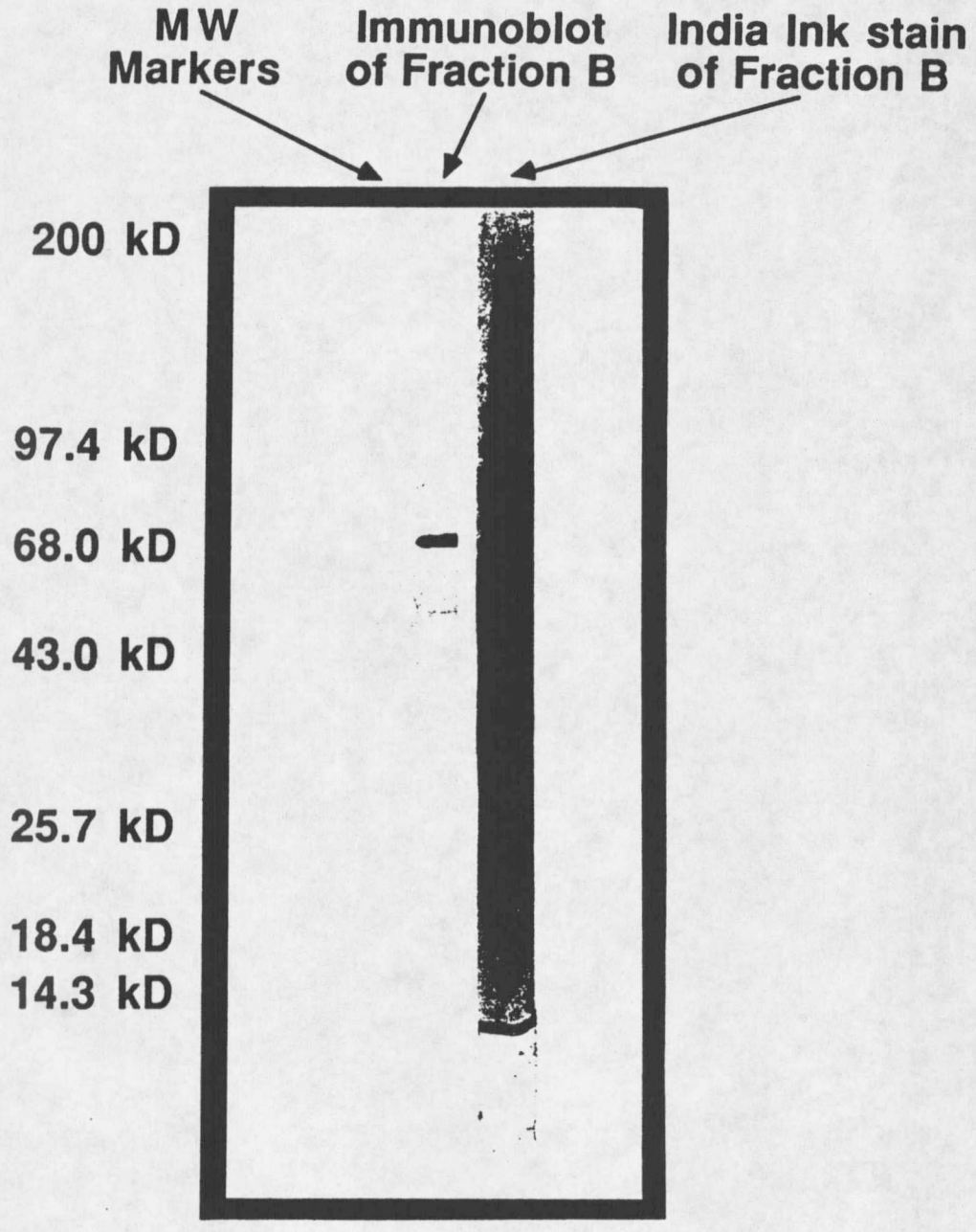


Figure 5. Antiserum cross-reactivity studies.

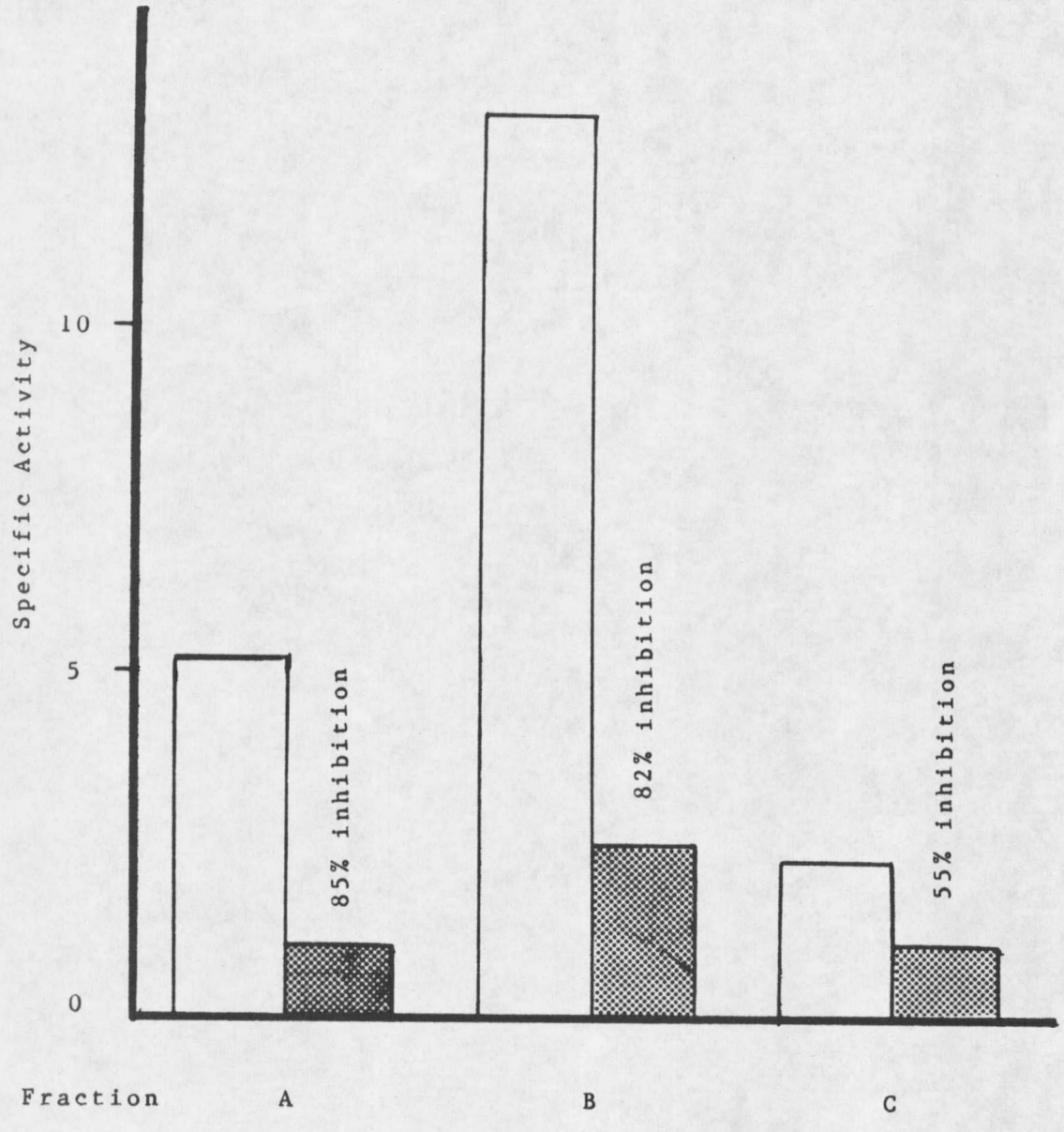


Figure 6. Effect of nitrate on ATPase activity of oat root membrane fractions. Open bars are controls, shaded bars contain 50 mM KNO_3^-. All reactions also include 50 mM orthovanadate, 50 mM KCl, 5 mM sodium azide and 1 mM ammonium molybdate. Specific activity is in umoles P_i/hour/mg protein.

Fluoroaluminate Studies

The PM ATPase of animal cells is classified in the same category of enzymes as the PM ATPase of plant cells, (those ATPases that have a phosphorylated intermediate). This enzyme in animal cells has been shown to be inhibited by fluoroaluminate (AlF_4^-). This molecule is believed to work in the same way as orthovanadate. To see if fluoroaluminate has any effect upon ATPase activity in wheat and oat roots, colorimetric enzyme assays were performed as shown in Figures 7 and 8. The fluoroaluminate was introduced into the reaction vessel by adding KF and $AlCl_3^-$ which combine to form AlF_4^- (fluoroaluminate). Figure 7 shows that KF and $AlCl_3^-$ alone have a minimal effect on enzyme activity, while fluoroaluminate and orthovanadate strongly inhibit the enzyme at comparable concentrations. The lack of inhibition by nitrate shows that there is very little TP ATPase activity in this fraction.

The data regarding the oat roots (Figure 8) shows essentially the same results as was seen for the wheat roots (Figure 7). The only difference is a greater inhibition upon the addition of KF alone. This may be attributable to possible aluminum contamination in the oat root membrane preparation.

The possible inhibitory effects of fluoroaluminate on the presumed TP ATPase were also examined. Figure 9 shows a

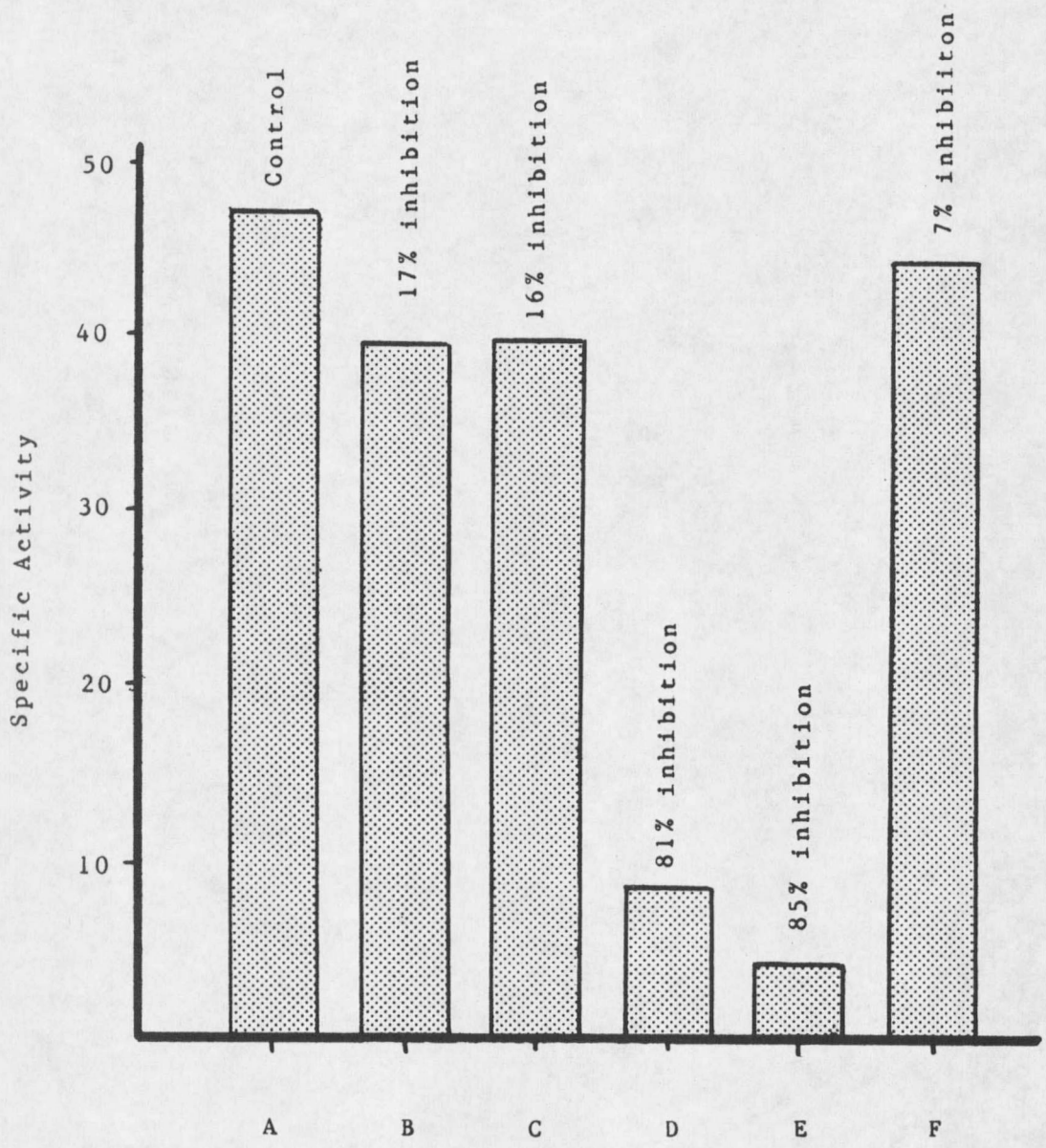


Figure 7. Effects of various inhibitors on ATPase activity of fraction C from wheat roots. All reactions include 50 mM KCl, 5 mM sodium azide and 1 mM ammonium molybdate. A. Control; B. 0.05 mM $AlCl_3$; C. 5.0 mM KF; D. 0.05 mM $AlCl_3$ + 5.0 mM KF to yield AlF_4; E. 50 mM orthovanadate; F. 50 mM KNO_3. Specific activity is in umoles P_i/hour/mg protein.

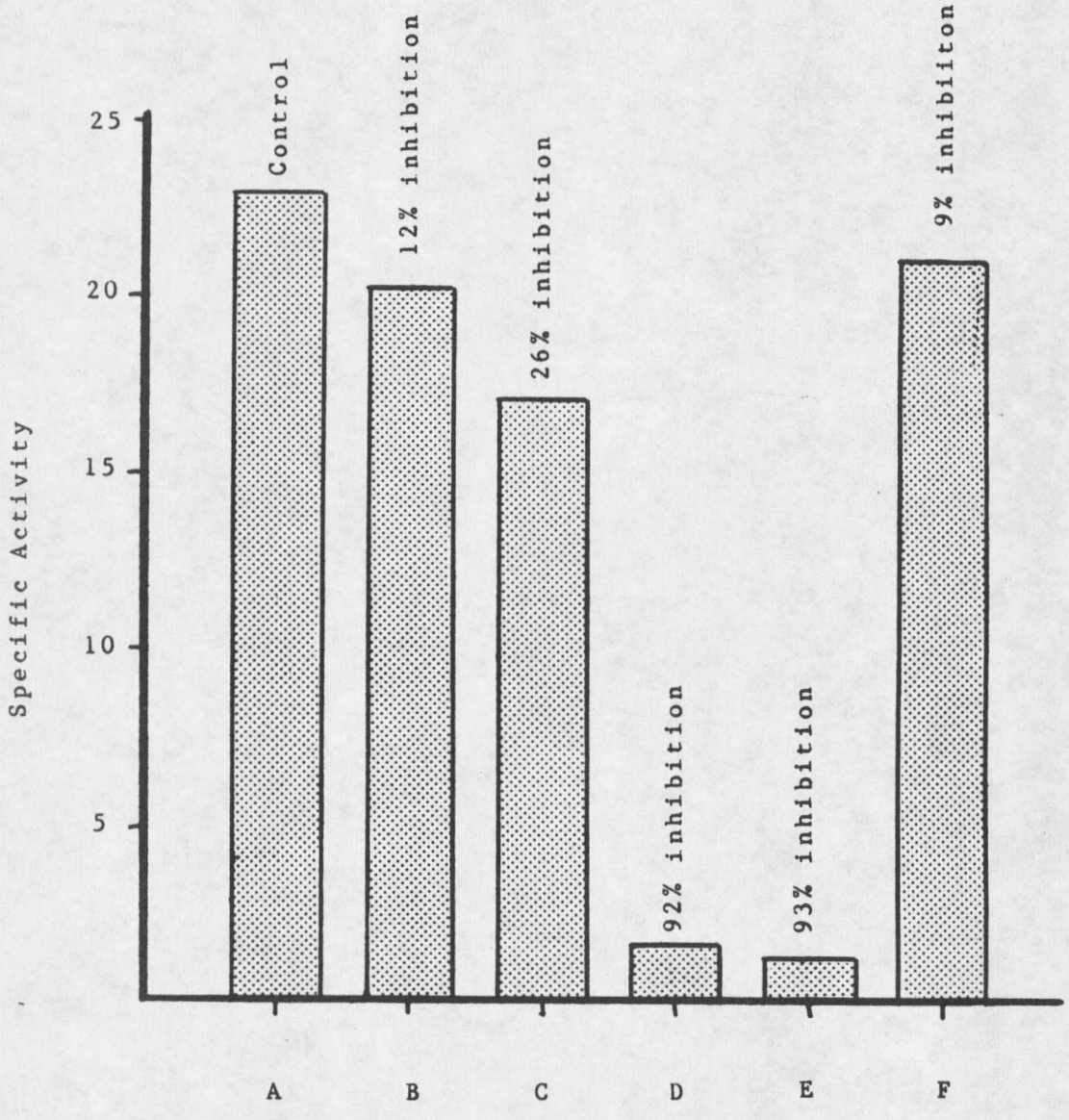


Figure 8. Effects of various inhibitors on ATPase activity of fraction C from oat roots. All reactions include 50 mM KCl, 5 mM sodium azide and 1 mM ammonium molybdate. A. Control; B. 0.05 mM $AlCl_3$; C. 5.0 mM KF; D. 0.05 mM $AlCl_3$ + 5.0 mM KF to yield AlF_4^-; E. 50 mM orthovanadate; F. 50 mM KNO_3. Specific activity is in umoles P_i/hour/mg protein.

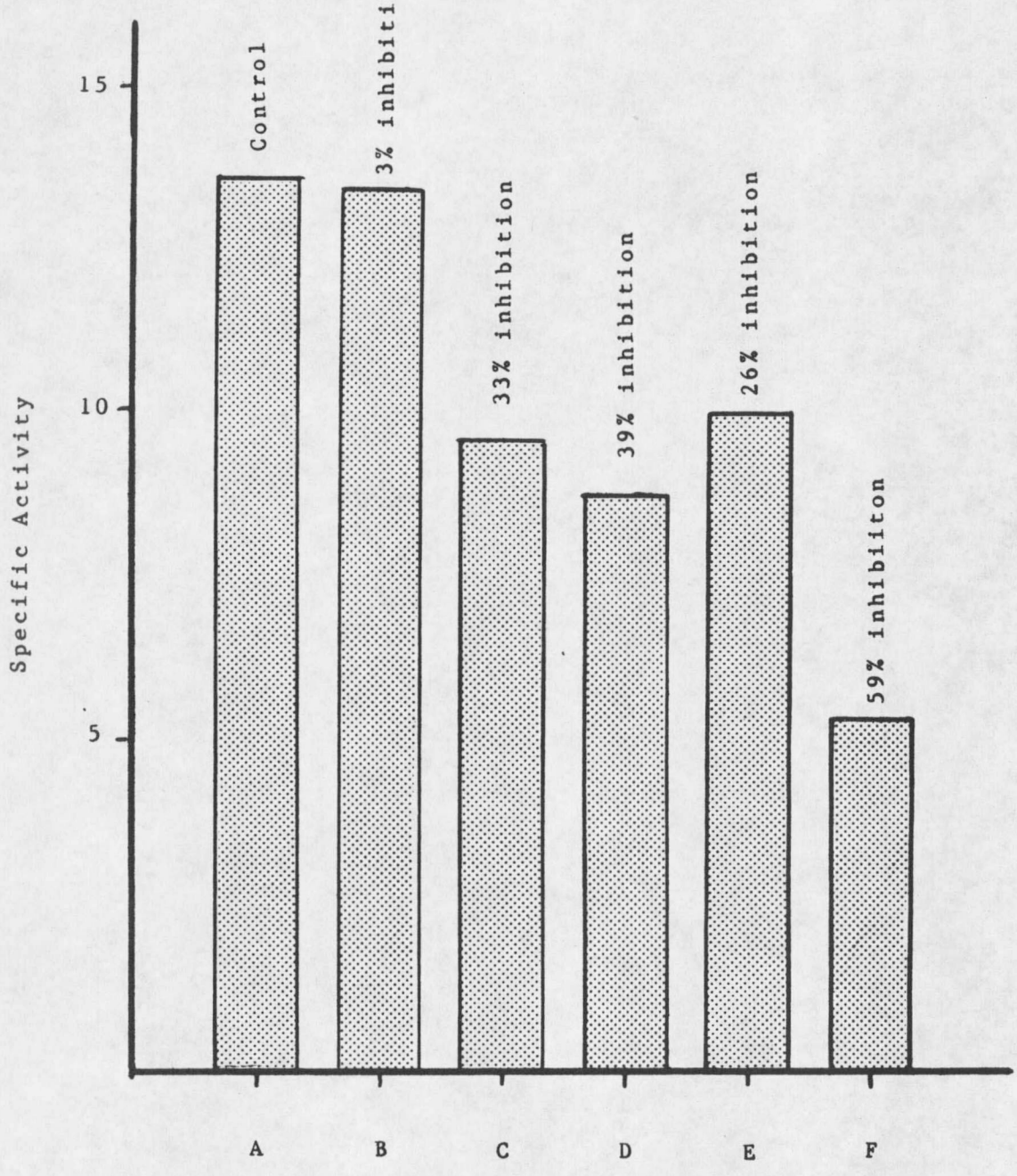


Figure 9. Effects of various inhibitors on ATPase activity of fraction B from wheat roots. All reactions include 50 mM KCl and 5 mM sodium azide. A. Control; B. 0.05 mM $AlCl_3$; C. 5.0 mM KF; D. 0.05 mM $AlCl_3$ + 5.0 mM KF to yield AlF_4; E. 50 mM orthovanadate; F. 50 mM orthovanadate + 1 mM ammonium molybdate + 50 mM KNO_3. Specific activity is in umoles P_i/hour/mg protein.

small inhibition by $AlCl_3^-$, about a 30% inhibition by KF, and a slightly higher amount of inhibition by AlF_4^-. This is comparable to the orthovanadate inhibition seen in the same sample. The effect of fluoroaluminate on the ATPase activity in wheat membrane fraction B may be chiefly due to contamination by the fluoroaluminate-sensitive PM ATPase.

The data in Figure 9 also shows that upon the addition of known inhibitors of identified ATPase activity there is still a residual ATPase activity detected. Upon heating of the membrane sample for 15 minutes at 75° C., this residual activity was able to be completely inhibited. This shows that the residual activity is attributable to an enzymatic activity of some type. Whether this activity is a yet undiscovered enzyme or simply the incomplete inhibition of the TP ATPase by nitrate is not known. However this same type of residual ATPase activity has been seen to occur with isolated tonoplast vesicles from barley (J. Garbarino, personal communication).

SUMMARY

The results presented here include findings that are new and unique while also confirming reports of similar work that has been performed in this field. This is the first report of a proton-translocating, nitrate-sensitive, ATPase activity in membrane vesicles isolated from wheat. This is also the first report of cross-reactivity of antiserum made against the vacuolar ATPase of a dicotyledon (carrot) with a similar polypeptide from a monocotyledon (wheat). From an evolutionary standpoint, these two plants are quite distinct from one another. However the cross-reactivity of the antiserum suggests that at least a portion of the structure of this enzyme, and perhaps the genetic structure as well, is highly conserved across a wide range of higher plants. While the use of fluoroaluminate as an inhibitor of PM-type ATPases has recently been shown in animal cells, this is the first report demonstrating this effect in higher plants. I would like to propose that this sensitivity to fluoroaluminate could be used along with orthovanadate as a way to distinguish PM ATPases from TP ATPases in future studies of plant membrane transport.

Along with these new findings the results described herein confirm other previous reports regarding membrane

transport in plants. The membrane isolation and fractionation techniques that I used appear to yield the same general results in wheat, barley, oats and corn. The proton-translocating ability of a nitrate-sensitive ATPase has been shown to exist in all of these cereals. There also appears to be some type of unidentified "ATPase" activity in all of these isolated membrane fractions that is insensitive to commonly used ATPase inhibitors.

As with any type of research, the results that have been reported only lead to more questions that warrant further investigation. Some of the problems that could be looked at in the future are listed below. The detection of a PP_iase (pyrophosphatase) mediated proton translocating activity in the tonoplast of wheat should be investigated as this enzyme has been shown to exist in oats and corn. The immunopurification of the wheat TP ATPase with antiserum could lead to the amino acid sequence of the 70 kD subunit of this enzyme. This data would in turn yield a cDNA sequence so that the genomic DNA of wheat could be probed in order to clone the gene for this polypeptide. Another very intriguing problem is the nature of the inhibitor-insensitive "ATPase" activity. So far it is known that this is due to an enzyme activity, and that it is not just an artifact of the assay system. This "ATPase" activity needs to be better identified if possible. Since there haven't been any other reports on the inhibiton of the PM ATPase in

a higher plant by fluoroaluminate, the nature of this sensitivity needs to be further characterized. The subcellular origins of the membrane fractions separated by discontinuous sucrose gradient centrifugation from wheat roots should be verified through the use of marker enzymes that have been associated with endomembranes in other higher plants. Finally, by isolating either vesicles or intact vacuoles from wheat roots and establishing an ATP driven proton gradient across the membrane, the proton-cotransport of other molecules such as sucrose or sodium ions across the tonoplast could be studied.

This study has provided much in the way of preliminary findings in regards to membrane transport in wheat root cells. Future investigations of transport systems across the wheat vacuole membrane may find this information a valuable foundation for their work.

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