PLASMALEMMA ATPase OF THE MAIZE SCUTELLUM

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
AMP-PNP	adenylyl imidodiphosphate
ATP	adenosine 5'-triphosphate
CDP	cytidine 5'-diphosphate
CMP	cytidine 5'-monophosphate
CTP	cytidine 5'-triphosphate
DCCD	N-N'-dicyclohexylcarbodiimide
DES	diethyl stilbesterol
DNP	2,4-dinitrophenol
EDCD	1-ethyl-3 (3-dimethyl-amino propyl)-carbodiimide
G-6-P	glucose-6-phosphate
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
IAA	indole-3-acetic acid
M-6-P	mannose-6-phosphate
MES	2 (N-morpholino) ethane sulfonic acid
MOPS	morpholinopropane sulfonic acid
NEM	n-ethyl maleimide
PCMB	p-chloromercuribenzoic acid
PCMBS	p-chloromercuriphenyl sulfonic acid
PEP	phosphoenol pyruvate
PNPP	p-nitrophenyl phosphate

P-Pi	pyrophosphate
TMP	thymidine 5'-monophosphate
TPT	triphenyltin chloride
TTP	thymidine 5'-triphosphate
UDP	uridine 5'-diphosphate
UDPG	uridine 5'-diphosphoglucose
UTP	uridine 5'-triphosphate

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An ATPase in the plasmalemma of the maize scutellum cell was characterized. This enzyme had a pH optimum of 6.5, was stimulated by $Mn^{2+} > Mg^{2+} \geq Ca^{2+} > Co^{2+}$, and was further stimulated by Na^{+} and K^{+}. The K_m for ATP was 0.65 mM. Nucleoside 5'-triphosphate was the preferred substrate.

This enzyme was capable of recognizing the substrate from the outside of the cell, and the products of the reaction remained in the bathing solution. It was stimulated by DNP at pH's below 5.0.

The inhibitors DCCD (10 μM), PCMB (0.5 μM) and NEM (10 μM) inhibited the enzyme from 0-20%, and PCMBS (5 μM) and EDCD (10 μM) gave 32% and 33% inhibition respectively.

Fresh and frozen HCl-treated slices gave nearly identical values for K_m, pH optima, stoichiometry, cation stimulation, kinetics, inhibition, and substrate specificity.

Cytological localization showed clear ATPase activity in the plasmalemma.

INTRODUCTION

There are well-characterized ATPases (E.C. 3.6.1.5) in bacteria [1], mitochondria [2], chloroplasts [2], and animal cells [3,4,5]. Higher plant plasmalemma ATPases have also been reported [6,7,8,9], but they have not been as well characterized.

Plant plasmalemma ATPases have been implicated in the transport of various mineral ions, amino acids and sugars into the cell [10,11,12,13,14]. Studies with higher plant plasmalemma ATPases have been done primarily with partially purified membrane fractions [6,7,8,9], and do not necessarily give much information about how the enzymes behave *in situ*. Since ATPases are thought to be vectorial enzymes, their biological role cannot be clearly understood from studies with cell fractions or isolated membrane vesicles. Attempts to isolate intact right-side-out vesicles by osmotic lysis of spheroplasts have resulted in vesicles which are not comparable to intact cell plasmalemma in the orientation of certain plasmalemma-associated enzymes [15,16].

This investigation was undertaken to determine the existence and location of an ATPase on the surface of the intact maize scutellum cell, and to determine some of the characteristics of the enzyme.

LITERATURE REVIEW

The Role of ATPases in Membrane Transport

The properties and functional characteristics of ATPases from animal cells and various subcellular organelles such as mitochondria and chloroplasts are well known [2]. There is also a large amount of information about bacterial-membrane ATPases [1].

Mitchell [17] proposed the "chemiosmotic hypothesis" as a model in which ATP production is linked to oxidative phosphorylation in the mitochondria. The model proposes an enzyme (ATPase) situated in the inner mitochondrial membrane with two functional portions. The catalytic portion is shown as protruding from the membrane into the matrix and is proposed to be responsible for ATP production. The other portion of the enzyme is embedded in the membrane and is proposed to function as a proton pump. Such a pump would be driven by the energy from oxidative phosphorylation and thus create the potential gradient across the inner mitochondrial membrane. This gradient has been implicated in the transport of various mineral ions, amino acids and sugars [10,11,12,13, 14].

Giaquinta [12] suggested that the uptake of sucrose from the apoplast into the phloem sieve tube element was coupled to an active influx of protons, and the efflux of K^+. The

phloem content had a relatively high K^+ concentration and a low H^+ concentration (pH 8-8.5), and a high sucrose concentration. If the apoplast pH was between 5 and 5.5, a substantial electrochemical potential could exist across the phloem plasmalemma, which could then be coupled to sugar translocation.

Proton uptake in yeast cells was studied at pH 4.5 to 5.5 [14]. There was good evidence that certain amino acids (glycine, leucine, lysine, and phenylalanine) were co-transported with the H^+ into the cells.

West and Mitchell [18] found that the rate of lactose inflow into E. coli cells which were kept in an anaerobic, nonmetabolizing state was correlated with the rate of simultaneous effective inflow of H^+. They postulated that the lactose and H^+ pass together via a β-galactoside symporter, and that the pH difference that is consequently built up across the membrane causes an effective outward leakage of H^+. The two transport processes, therefore, seemed to be strictly coupled with a 1:1 stoichiometry initially, but then there was a progressive drop in the observed ratio of effective net H^+ translocation to lactose translocation with time. Since the cells were obligate aerobes, the conditions of their growth prevented the production of the needed ATP. There had to be a source of ATP within the cell to pump out the H^+ as they entered in order to keep up the initial stoichiometry.

Harold et al. [19] measured the internal pH of Streptococcus faecalis, undergoing glycolysis. They found that these cells maintained an internal pH which was more alkaline than that of the medium by 0.5 to 1.0 pH units. This pH difference disappeared upon exhaustion of glucose. The pH gradient could also be abolished by DCCD and chlorhexidine. It was inhibited by tetrachlorosalicylanilide, carbonyl-cyanide-m-chlorophenylhydrazone, nigericin, and monensin, which are all proton conductors. The antibiotics, valinomycin and monactin, which facilitated the K^+ diffusion did not affect the pH gradient as long as the external K^+ concentration was kept high. At low K^+ concentrations, these antibiotics lowered the internal pH. They concluded that pH gradients are formed by an energy-dependent extrusion of protons from the cell, thus creating a membrane potential. K^+ accumulation then resulted with the movement of K^+ down an electrochemical gradient.

Beffagna et al. [20] reported work on a compound (fusicoccin or FC) which is a diterpene glucoside, and is active in stimulating the H^+/K^+ exchange in plants, and also increases in vitro K^+-Mg^{2+} stimulation of the ATPase activity from plasmalemma-enriched preparation of maize coleoptile and spinach leaves. Their results were consistent with the hypothesis that FC stimulated the proton extrusion and cation uptake by being able to activate, at the cell membrane, a H^+/K^+ exchange system which depends on ATP as an energy source. The evidence suggests that the plasmalemma-bound, cation-dependent ATPase mediated the utilization of metabolic energy for K^+ transport.

Walker and Smith [21] measured the intracellular pH in Chara. The pH difference across the plasma membrane arose from an ATPase which extruded 2 protons for each ATP hydrolyzed. The electric potential difference was determined by the ATP/ADP concentrations with the external pH lower than the internal. This electric potential difference across the plasmalemma of Chara was thought to be to a large extent a result of H^+ extrusion.

When Streptococcus lactis was depleted of metabolic energy, a loss of transport function occurred [22]. Active transport of sugar could be induced in these nonmetabolizing cells when a proton motive force was artificially generated either by a pH gradient or by inducing membrane potential with a valinomycin-induced K^+ diffusion.

ATPases in Whole Cells or Tissues

Electron microscopy of ATPase localization in sieve cells of Pinus showed prominent ATPase activity in the plasma membrane at neutral pH with ATP or UTP as substrates [23]. No such localization was observed with β-glycerol phosphate as the substrate. The substrates were introduced from the outside of the cell, yet the cells showed localization in the plasmalemma.

Bentwood and Cronshaw [13] reported similar results in phloem of Nicotiana. The plasma membrane and endoplasmic reticulum were free of reaction product when β-glycerol phosphate was used as the substrate. Acid phosphatase activity was very high on the vacuolar membrane with this substrate.

Acid phosphatase activity showed no Mg^{2+} or Ca^{2+} stimulation.

Gilder and Cronshaw [24] reported ATPase localization in the phloem of Cucurbita in the petiole and minor veins. The enzyme activity was localized in the sieve elements, companion cells, and parenchyma cells. The activity was found at the cells' surfaces and associated with the dispersed P-proteins of mature sieve tube elements. They concluded that the activity of the ATPase on the plasmalemma was directly related to the ion assimilation, and those in the P-proteins to callose deposition. They found activity in the mitochondria and dictyosomes and regions of the phloem cell walls. They also looked at the distribution of ATPase activity in differentiating and mature phloem cells of tobacco and its relationship to phloem transport [25]. In a later paper [26], they ran substrate specificity and inhibition studies with fresh tobacco leaf homogenates. They found no inhibition by NEM, but 96% inhibition with PCMB. Substrate specificity, when expressed as a percent of ATP activity, gave 132% with GTP, 90% with ITP, 82% with CTP, 83% with ADP, 77% with UTP and 1% with AMP. They suggested a possible role of the ATPase as the energy source for phloem transport.

Hall [27] reported the cytochemical localization of ATPase activity in corn root cells by using the lead phosphate precipitation procedure. The activity was associated with the plasma membrane, nucleus, mitochondria, golgi, vacuole, and endoplasmic reticulum. Most cells examined showed high ATPase activity at the root surface, plasma membrane, and

plasmodesmata. The ATPase activity seemed especially to be associated with vesicles close to the surface of the cell, which suggested that ion transport may involve a process of pinocytosis, using ATP as the energy source. He suggested in another paper [28] that the ion transport mechanism may be by way of invagination and uptake by the formation of small vesicles. Surface β-glycerol phosphatase activity had a different localization pattern than did the ATPase. The activity of the former was localized predominantly in the cell walls. Biochemical studies [27] confirmed this finding.

In experiments with isolated barley roots, oligomycin at 5 µg/ml did not show any effect on the rate of hydrolysis of ATP in the presence of Mg^{2+} and K^{+}, indicating that specific ATPase-driven transport on the outer surface of the roots must be absent, and that hydrolysis of ATP on this surface is accomplished by nonspecific phosphatases [29]. They were able to show that the use of ATP as a qualitative substrate for determining the presence of ATPase in plant cells is inappropriate since the phosphatase, which is widely distributed in these cells, can usually readily hydrolyze the same spectrum of phosphoorgano substrates in which ATP is found.

Hanson and Kylin [10] reported a Na^{+}-K^{+}-activated ATPase in sugar beet roots. Kylin and Gee [11] related the Na^{+}-K^{+} ATPase activity to salt tolerance in Avicennia. They found that the ATPase activity with salt stimulation which occurred with peaks at three different Na^{+}-K^{+} ratios was greatest at pH 6.75. Salt stimulation of ATPase was found in acid as

well as slightly alkaline regions. In general, plant ATPases seem to have a pH optimum of about 7.0, and a strong requirement for divalent cations [30]. There are indications that ATPase may have many isoenzymes [30].

Hodges et al. [31] reported the absorption of inorganic ions by plant root cells to be an energy-requiring process, dependent on aerobic respiration with ATP as the energy source. Ion uptake was inhibited by DNP, arsenate, and oligomycin. There was a high correlation between KCl- and RbCl-activated ATPase and K^+ and Rb absorption by root tissues. The kinetics of monovalent ion transport and monovalent ion-stimulated ATPase activity were similar.

Fisher and Hodges [32] and Kylin and Kahr [33] have reported Ca^{2+}- and Mg^{2+}-activated ATPase from oat and wheat roots. Much of the work is done with plant root tissues, since there is a great interest in the role of the ATPase activity in ion uptake [34]. Sexton and Sutcliffe [35] looked at ATPase activity in various tissues of the pea root. They found a peak activity in the region of the apical meristem with a maximum at 1.5 mm behind the apex. The activity fell rapidly from this point and leveled off until about 24 mm from the tip, when it again increased in the zone of lateral root formation. On a per cell basis, activity reached maximum at about 10 to 12 mm from the apex. The ATPase activity in the cortex appeared to be associated with the plasmalemma or cell wall. Such work implicated the ATPase with the role of ion uptake in the root. Ratner and Jacoby [36] studied

the nonspecific salt effect on Mg^{2+}-dependent ATPase from grass roots. They found KCl, NaCl, ethanolamine chloride, and choline chloride were all stimulators of the ATPase activity in corn root microsomal fraction. They concluded that the cation activation of ATPase from grass roots was rather nonspecific and not necessarily related to the capability of the roots to absorb cations.

ATPases from Plant Cellular Fractions

A common technique in work dealing with plant enzymes is to quick-freeze the tissue, perchloric-acid extract the protein, and neutralize with KOH. Such a procedure yielded, from several plant tissues, an acid-resistant ATP-hydrolyzing enzyme with a pH optimum at about 5 and no activity below 3 or above 9 [37]. This enzyme did not hydrolyze sugar phosphates, nucleoside monophosphates, PEP, or UDPG.

Another approach in the characterization of plant ATPases has been to homogenize the tissue, separate the various fractions with differential centrifugation, and test each fraction for enzyme activity [38]. Leonard et al. [39] have identified at least five membrane-associated ATPases, as well as a soluble ATPase which was most likely a nonspecific acid phosphatase. They found a plasmalemma fraction with an ATPase which was more effective at pH 6.0 than at 9.0, and was KCl-sensitive. The mitochondrial fraction ATPase had greater activity at pH 9.0.

Jolliot et al. [40] looked at the three ATPases associated with membrane fractions from potato tubers: the plasma

membrane, the mitochondrial membrane and the microsomal membrane. The microsomal ATPase seemed to be insensitive to the presence of Na^+ and K^+. Other workers [41,42,43,44], however, found that the microsomal fraction ATPase was indeed stimulated by Na^+ and K^+. Jolliot et al. [40] found that the plasma-membrane ATPase activity was stimulated by Mg^{2+} at pH 6.75, whereas the mitochondrial ATPase activity was stimulated by Mg^{2+} at pH 9.5 or above. The mitochondrial ATPase was also found to be oligomycin-sensitive, whereas the plasma-membrane enzyme was not. The stimulation of the mitochondrial ATPase was about 10 times more effective when stimulated with monovalent cations than that of the plasma-membrane ATPase.

Kasamo and Yamaki [45] found that IAA promoted the activity of Mg^{2+} ATPase in mung bean hypocotyl cell homogenates enriched for plasmalemma. The IAA seemed to bind to the ATPase and supply the additional protons to the cell wall. They confirmed that Mg^{2+} ATPase was associated with the plasmalemma, and that IAA binds to the ATPase and promotes its activity in vitro.

Tikhaya et al. [9] isolated a membrane fraction enriched with plasmalemma fragments by differential centrifugation in a sucrose density gradient. The fraction contained ATPase activity which was increased by Mg^{2+} but not by Na^+-K^+ or by Na^+ and K^+ alone. Addition of both Na^+ and K^+ in the presence of Mg^{2+} resulted in a considerable activation, with a maximum at a ratio of Na^+/K^+ = 2.0 to 2.5. Ouabain (0.1 mM) completely inhibited the Na^+-K^+-stimulated activity and

decreased the activity to that with Mg^{2+} alone. Ouabain had no effect on the enzyme in the presence of Mg^{2+} alone, Mg^{2+} and K^+, or Mg^{2+} and Na^+. The pH optimum of ouabain-inhibited ATPase was 8.0. They see a role of the plant ATPase in the transport of ions analogous to that in animal systems.

Lai and Thompson [6] were able to obtain purified plasma membrane from bean plants. Large levels of ATPase activity were found in the membrane preparation. The purified plasma membrane had from 3 to 6 times the ATPase activity of the crude homogenate on a specific activity basis. The levels of contamination were determined by glucose-6-phosphate succinate dehydrogenase and 5'-nucleosidase activities. All were low or not detectable.

Leonard and Hotchkiss [7] reported the properties of an isolated plasma-membrane ATPase from corn root. Maximum activity was observed at pH 7.0 with Mg^{2+} alone and 6.5 with Mg^{2+} and K^+. When no ion was present, pH had no effect over a range of 6.0 to 9.0. Other cations, such as Ca^{2+} and La^{3+}, were potent inhibitors, but only in the presence of Mg^{2+}. Oligomycin did not inhibit the ATPase activity but DCCD was a good inhibitor. Several cations, K^+, Rb^+, Na^+, and Co^{2+}, were all good stimulators. The best substrate proved to be ATP, followed by GDP and UDP. Nurminen et al. [46] reported various enzymes located on the cell wall and plasma membrane of baker's yeast. The wall was digested enzymatically and the released enzymes were assayed. During the enzymatic digestion, saccharases and acid phosphatases were released to

the medium. The Mg^{2+}-dependent ATPase was not. It appeared, therefore, that the Mg^{2+}-dependent ATPase was bound to the plasma membrane.

Hendrix and Kennedy [47] reported an ATPase activity in plasma-membrane-enriched fraction of soybean root and callus tissue. The enzyme from both sources was activated by divalent cations, Mg^{2+}, > Mn^{2+}, > Zn^{2+}, > Ca^{2+}, > Sr^{2+}, and further stimulated by monovalent salts, K^+, > Rb^+, > Cl^-, > Na^+, > Li^+, > NH_4^-, > Cs^+, > Tris. No synergistic effects between Na^+ and K^+ were seen. The pH optimum for ATP hydrolysis was 6.5 and the substrate preference was ATP >> ADP > GTP > CTP > UTP.

Hodges and Leonard [8] reported ATPase activity in isolated plant root plasmalemma, which was Mg^{2+}-dependent, and was further stimulated by monovalent cations. The enzyme did not require the simultaneous presence of Na^+ and K^+ for maximum activity and was not inhibited by ouabain. They felt that the monovalent ion stimulations of the ATPase were associated with the plasma-membrane fraction. Since there were many membrane-associated ATPases, the separation and purification procedures were difficult. The authors used discontinuous density gradients to separate the various membrane fractions. They verified the plasma-membrane fraction by staining with phosphotungstic acid stain and found that 75% of the membrane in this fraction was plasma membrane.

Scarborough [48] reported the presence of a plasma-membrane ATPase in Neurospora. He used thiocyanate, which

penetrates biomembranes and becomes asymmetrically distributed across the membrane in the presence of a membrane potential difference. The uptake of Mg^{2+}-ATPase-dependent ^{14}C-SCN^- was used as a measure of ATP-hydrolysis-dependent generation of an electrical potential across isolated plasma-membrane vesicles. He concluded that the ATPase of this plasmalemma co-ordinated the movement of ions in one direction, thus producing an electro-potential gradient across the membrane.

Adenosine 5'-triphosphatase containing plasma membrane of oat roots formed vesicles in isolation [49]. It was Mg^{2+}-activated, but high concentrations of Mg^{2+} and ATP were inhibitory. The K_m was between 0.64 and 1.24 mM, due to the variable amounts of Mg^{2+} ATP complex and free ATP present. The true substrate for the ATPase was believed to be Mg^{2+} ATP.

Primary roots of Zea were ground and the plasma-membrane vesicles obtained with sucrose density gradient [50]. They found a K^+-stimulated ATPase, with a pH optimum of 6.5. The mitochondrial ATPase fraction had its maximum activity at pH 9.0.

Leonard and Hodges [51] reported an ATPase from the plasma membrane of oat roots. This enzyme was activated by Mg^{2+}, > Mn^{2+}, > Zn^{2+}, > Fe^{2+}, > Ca^{2+}, and further activated by K^+. The pH optimum with Mg^{2+} activation was 7.5 and 6.5 for Mg^{2+} and K^+. The K_m for Mg^{2+} activation was 0.84 mM, and for K^+ activation it was 0.72 mM at pH 6.0.

Maize root homogenates were found to contain two distinct KCl-stimulated ATPases [45,51]. One was associated

with the plasma-membrane fraction and the other with a fraction of the smooth intracellular membrane which could not be positively identified.

In preparation of plasma-membrane vesicles from Avena roots, Sze and Hodges [52] found that some were closed, some partially closed and others completely unsealed. The sealed ones were presumably inside out as well as right side out. These isolated membrane vesicles were found to be similar in their response to the plasma membrane in situ as far as passive influx and efflux of inorganic ions were concerned.

Washing excised corn root resulted in an increase in the Mg^{2+}-K^{+} ATPase [41]. This was correlated with ion accumulation. The increase in activity was limited to the microsomal fraction. There seemed to be a close relation between the increase in the ATPase activity and phosphate absorption. Washing seems to augment or activate the membrane transport mechanism by way of protein synthesis.

The ATPase from plasma membrane of marine diatoms required Mg^{2+} for basal activity and was further stimulated by Na^{+} and K^{+} [53]. Divalent ions Mn^{2+} and Co^{2+} were able to be partially substituted for Mg^{2+}, but Ca^{2+} inhibited the enzyme. The preferred substrate was ATP. The apparent K_m was 0.8 mM. The enzyme was insensitive to ouabain, but PCMB and NEM were inhibitory.

Adenosine 5'-triphosphatase in plasma membrane has also been implicated in the opening and closing of the guard cells [54]. Epidermal cell extracts of Commelina benghalensis had

two isoenzymes of ATPase. The first had a pH optimum of 5.5 and was activated by Ca^{2+}. The second, with a pH optimum of 7.5, was activated by K^+. The authors associated the stomatal movement with the activity of these enzymes, primarily by regulating the K^+ influx and efflux. The first form of the enzyme was associated with closure and the second with the opening of the stomata.

Cassagne et al. [55] reported a Na^+-K^+-stimulated Mg^{2+} ATPase from the plasma membrane fraction of leek epidermal cells. The rapid ion movement in the guard cells was associated with ATPase activity. The question of a Na^+-K^+ ATPase occurring in plant membranes is not entirely settled, but there seems to be support for its existence [6,56,57].

The presence of an ATPase in the plasma membrane does not seem to be universal. Heinrich [58] looked at various enzymes including ATPase in the nectaries of the Aloe plant. He found ATPase activity in the ER, but not in the plasmalemma. These cells are thought to be involved in the secretion of sugars. He felt, therefore, that the sugar-secreting activity was limited to the involvement of the ER in these cells.

Bacterial Membrane ATPases

Alder and Rosen [15] reported that lysed membrane vesicles from E. coli spheroplasts were right side out, and various enzyme markers indicated this to be so. However, the plasmalemma ATPase which is normally associated with only the inner side of the membrane was found on both sides of the

vesicle in about 50-50 distribution. They postulated that certain membrane proteins may be translocated from the inner surface to the outer surface, creating a mosaic.

Rosen and McClees [59] reported that Ca^{2+} accumulation in lysed E. coli vesicles was stimulated by ATP. These vesicles were believed, therefore, to be inside out. The accumulation of Ca^{2+} by these vesicles reflected a system which in vivo may be responsible for the active extrusion of Ca^{2+} from the cells.

Right-side-out membrane vesicles from E. coli were capable of ATP production [60] when the vesicles were loaded with ADP and Pi, and an artificial proton gradient across the membrane was produced with a more acid outside and a more basic inside. The synthesis of ATP required, in addition, Mg^{2+}. It was inhibited by DCCD and carbonyl cyanide. It was stimulated by valinomycin in the presence of KCl. Altendorf and Staehlin [16] obtained E. coli membranes via osmotic lysis. This technique has been used widely to obtain right-side-out vesicles. Such lysed vesicles, however, exhibited some 50% to 60% of the total ATPase activity found in the whole cell when the substrate was introduced from the outside.

In bacterial membranes, inverted and right-side-out vesicles showed Mg^{2+} ATPase activity and kept up a proton gradient [61]. In the absence of Mg^{2+} ATPase, little respiration-driven Ca^{2+} transport could be observed. The results suggested the presence of a Ca^{2+}/H^{+} antiport.

When the EDTA-lysozyme method of membrane vesicle preparation was used, Hare et al. [62] reported a 1:1 ratio of inverted and right-side-out vesicles.

Abrams and Smith [63] reviewed the general properties of bacterial membrane ATPase. There seemed to be good evidence that the ATPase, by operating in reverse, acted as a coupling enzyme in the oxidative phosphorylation of ADP to ATP. It seems to mediate an ATP-driven transport of various solutes against an electrochemical gradient under normal conditions. The K_m for ATP was found to be 2.5, 1.0, or 0.6 mM depending on the species, and the pH optima ranged from 6.0 to 9.5. The compound DCCD failed to inhibit solubilized enzyme, but inhibited strongly when reattached to the membrane. The carbodiimide sensitivity factor was thought to reside in the lipid bilayer.

Riebeling and Jungermann [64] found an ATPase in the membrane fraction from Clostridium pasteurianum. The enzyme was Mg^{2+}-dependent, but Co^{2+} and Mn^{2+} could replace Mg^{2+} in the reaction. Ca^{2+} could not replace Mg^{2+}. The effect of Mg^{2+} was slightly antagonized by Ca^{2+}. The monovalent cations Na^{+} and K^{+} had no stimulatory effect even in the presence of Mg^{2+}. The effect of ADP was inhibitory. The membrane-bound ATPase was inhibited 80% by DCCD, but oligomycin, ouabain, and sodium azide had no effect. The enzyme could be solubilized by 2 M LiCl in the absence of Mg^{2+}, and this resulted in the instability of the enzyme. The ATPase was nearly completely released from the membrane by one washing with 250 mM Tris HCl at pH 7.5. The solubilized enzyme was not stable. The pH optimum was between 7.0 and 8.5.

Hanson and Kennedy [65] found that ATPase from E. coli membrane, purified via gel electrophoresis, was cold-labile after release from its membrane. There was an 80% decline in specific activity after 6 days at 4°. The best activators of the enzyme were Mg^{2+} and Ca^{2+}. Purine nucleoside phosphates were more effective than pyrimidines as substrates. The Lineweaver-Burke plots of the enzyme activity were linear, suggesting a single enzyme.

The use of a French press or sonication on E. coli cells caused the membrane vesicles to be formed inside out. About 90% of the membrane vesicles formed with these techniques were determined to be inside out [66]. The assumption was made that there were certain enzyme markers which determined sidedness of the membrane. The ATPase was used as one such marker. The author assumed that the hydrolytic portion of the enzyme was directed internally, and that hydrolysis or synthesis could only occur if the substrate could reach the active site.

The ATPase from Paracoccus denitrificans [67] could be solubilized from its membrane with washing in low salt concentrations. This ATPase resembled the coupling ATPase of mitochondria, chloroplasts, and other bacteria. It was a protein with a MW of about 300,000 and was negatively charged. An inhibitor protein was tightly bound to the enzyme in vivo and could be destroyed by trypsin treatment. The ATP and ADP were tightly bound to the enzyme and the ratio of ATP to ADP was greater than 1.

Animal Cell ATPases

Shigekawa et al. [3] reported an ATPase in canine cardiac sarcoplasmic reticulum which seems to be involved in Ca^{2+} transport. This enzyme had a K_m of 0.18 mM and a pH optimum of 6.8. It seemed to require both Ca^{2+} and Mg^{2+}. They postulated that Ca^{2+} was necessary for the enzyme substrate intermediate and Mg^{2+} was required for the decomposition of the complex to release the enzyme and product. The concentration of Ca^{2+} necessary for 1/2 maximal activation was 4.7 μM. The same authors found [68] that this ATPase was further stimulated by K^+, > Na^+, > Rb^+, > NH_4^+, > Cs^+, > Li^+ > Tris.

Ca^{2+} ATPase has been removed from sarcoplasmic reticulum membrane, purified, and then incorporated into an artificial lipid membrane [4]. The enzyme so treated was then able to catalyze ATP-dependent cation transport.

Two cell membrane fractions have been isolated from two Erlich cell types. The two cell membrane fractions had nonidentical stimulatory responses to amino acids in their Mg^{2+}-dependent activity to cleave ATP, despite the presence of ouabain and the absence of Na^+ and K^+ [5]. The first fraction showed little Na^+-K^+ ATPase activity and the second fraction showed a Na^+-K^+ enhancement.

ATPases from animal tissues, primarily muscle and microsomal fractions, were Mg^{2+}-, Ca^{2+}- or Na^+-K^+-activated enzymes [69,70,71,72]. ATPase from sarcoplasmic reticulum [69] was Ca^{2+}-Mg^{2+}-activated and could account for the large portion of the structure of the membrane itself. The ATPase

aggregate particles were about 90 Å in diameter and had a MW of about 102,000. Bovine brain microsomal fraction contained a K^+-activated ATPase which was ouabain-sensitive [71]. The purified ATPase from membrane fraction of rectal glands of the dogfish shark was stable at 0^o for as many as 10 days, whereas chloroplast ATPase was cold-labile [72].

Grisham and Mildvan [73] reported on the properties of ATPase obtained from sheep kidney medulla. They found Mn^{2+} and Mg^{2+} had the same binding site on the enzyme. The ATPase required the presence of a divalent metal ion bound at the active site. Their findings confirm the extrusion of protons driven by ATP hydrolysis.

Sarcoplasmic reticulum had two distinct ATPases [74]. The first was a Ca^{2+} ATPase, and the second a Na^+-K^+ ATPase. The Ca^{2+} ATPase was an integral protein with a MW of approximately 102,000 and did not seem to have a subunit structure. It comprised nearly 65% by weight of the total protein in the sarcoplasmic reticulum. This enzyme hydrolyzed ATP in the presence of Ca^{2+} and Mg^{2+}. During the reaction, the enzyme was transiently phosphorylated by the terminal phosphate group of ATP and was Ca^{2+}-dependent. Dephosphorylation was Mg^{2+}-dependent.

This enzyme was thought to be surrounded by about 75 p-lipid molecules. Its shape was unknown, but if it were spherical it would be about 60Å in diameter. It was thought to be asymmetrically distributed with a greater portion in the cytoplasmic leaflet. Calcium-ion ATPase may also span the entire

thickness of the membrane, as does the Na^+-K^+ ATPase [74]. This enzyme was involved in the transport of Ca^{2+} from the sarcoplasmic reticulum to the cytoplasm during muscle contraction. Under certain conditions, Ca^{2+}-Mg^{2+} ATPase can synthesize ATP from ADP and Pi.

Sodium-potassium-ion ATPase [74] required Na^+, K^+ and Mg^{2+}. The requirement for the Na^+ was absolute, but other monovalent cations could substitute for the K^+. Its MW was about 250,000. It was thought to be composed of 2 or more subunits and was ouabain-sensitive. If the enzyme were spherical, it would be about 85 Å in diameter and capable of spanning the membrane, but functional asymmetry was implied. The ATPase from microsomal fraction of canine renal medulla seemed to have two subunits, a large polypeptide and a smaller polypeptide [74]. Antibodies were produced against the purified large chain of Na^+-K^+ ATPase and the antigenic site was determined to be located on the inside surface of the membrane. It was also found that this large chain was phosphorylated by MgATP from the inside.

Cardiac glycosides are known to bind to the ATPase only at the outside surface of the cell. It was shown to bind to a portion of the large chain of the Na^+-K^+ ATPase. Consequently, the large chain must have had a surface exposed to the outside of the cell and one exposed to the inside, and therefore spanned the membrane. If it were spherical, it would be about 75 Å in diameter, and more than large enough to span the membrane with adequate surface on each side.

Since the cardiac glycoside binding site was accessible from the outside, and the phosphorylation site from the inside, and both sites are on the large polypeptide unit of the enzyme, the polypeptide was thought to have sequences exposed to both sides of the membrane [75].

Although the ATPases normally function to hydrolyze the ATP to Pi and ADP, the reaction can at times be reversed under certain conditions. Lew and Glynn [76] reported the reversal of the ion pump which resulting in the synthesis rather than hydrolysis of ATP in red blood cell ghosts. These membranes were preincubated with Pi to load them up and then incubated in a high-Na^+ and K^+-free medium. This led to an increase in the ATP concentration which could be wholly or partially prevented with ouabain. They felt that there was strong evidence of a net synthesis of ATP as being linked with the reversal of the pump.

Chloroplast Membrane ATPase

Ca^{2+} ATPase from lettuce chloroplast [77] seemed to be composed of two parts: the portion embedded in the membrane (CF_o) and the portion protruding from the membrane (CF_1). The CF_1 was also known as the coupling factor and was directly involved in the terminal steps of photophosphorylation, and thus normally functioned in the synthesis of ATP. The enzyme would also catalyze the hydrolysis of ATP under artificial conditions. The CF_1 was shown to require heat activation and Ca^{2+}. Other metal ions were inhibitory. The K_m values

for Ca^{2+}, Mg^{2+} and Mn^{2+} stimulation were 2.7 mM, 2.0 mM and 4.0 mM respectively.

The chloroplast ATPase CF_1 seemed to be Mg^{2+}-Ca^{2+}-dependent and required light [78].

Membrane-bound ATPase in chloroplasts of Euglena showed a Ca^{2+}- and Mg^{2+}-dependent activity and could not be further activated by other ions [79]. This enzyme was highly speific for purine nucleosides and was inhibited by DCCD, phlorizine, ADP and Pi.

There were at least two distinct ATPases associated with the chloroplast from spinach. One was membrane-bound, light-activated, and sulfhydryl-dependent Mg^{2+} ATPase [80]. The other was a trypsin-activated Ca^{2+} ATPase. A chelating agent (4,7 diphenyl-1,10-phenanthroline) was able to inhibit the Mg^{2+} ATPase, but not the Ca^{2+} ATPase.

Certain properties of ATPase from spinach chloroplasts were reported [81]. It had a high specificity for ATP over other nucleoside triphosphates and Ca^{2+} was the best activator. The K_m at pH 6 was 0.11 mM. The enzyme was stimulated by maleate, and the K_m with 60 mM of maleate was 1.1 mM.

There seemed to be a necessity for the presence of lipids (p-lipids in particular) [82] for the proper functioning of the ATPase. Sulfolipids seemed to confer stability to the ATPase activity of the chloroplast CF_1, and the p-lipids were important in the binding of CF_1 to the membrane fragments, which resulted in highly increased specific activity of the enzyme.

ATPase from bacteria, mitochondria and chloroplasts seemed to serve two major functions [83] and share many similarities in the properties of the enzymes from the different sources. The first was to function as a proton translocator across the membrane. The second was to catalyze the ATP hydrolysis and synthesis and the regulation of these catalyses.

Malyan and Makarov [84] looked at the mechanism of ATP hydrolysis by soluble ATPases of chloroplasts (CF_1) in the presence of Mg^{2+} ions. A linear increase in reaction rate of ATPase with ATP concentration up to 1 mM was observed at constant Mg^{2+} concentrations. At high concentrations of $MgCl_2$ the dependence was more complex. At $MgCl_2$ concentrations of less than 0.1 mM, the reaction approached 2nd-order kinetics with respect to Mg^{2+}. An increase in $MgCl_2$ concentration resulted in a decrease in reaction order. It was assumed that MgATP is the true substrate for ATP hydrolysis in which the enzyme binds with a free Mg^{2+} to obtain its active conformation.

Mitochondrial ATPase

Mitochondrial ATPases and ATPases from sub-mitochondrial particles of Jerusalem artichoke showed a maximum activity at pH 9.0 - 9.3. It had a higher specificity for ATP than other nucleoside triphosphates. Broken mitochondria also caused the rapid hydrolysis of ATP. The enzyme was inhibited by oligomycin [85].

Some general properties of mitochondrial ATPases were reported by Penefsky [81]. The isolated mitochondrial ATPase

was latent and was activated by the addition of DNP, which uncoupled the oxidative phosphorylation, or by physical alteration of the membrane. There was an absolute requirement for divalent cations, Mg^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+} or Ca^{2+}. The pH optimum was about 8.0, and they were not inhibited by sulfhydryl group reagents. They were highly sensitive to iodine, DCCD and tetranitromethane. The sensitivity to DCCD depended on the presence in the membrane of the oligomycin-sensitivity-conferring protein or FC_1 and a second protein FC_2. The enzyme was stimulated 15% to 20% by methanol and by bicarbonate.

The activity of mitochondrial ATPase seemed to vary somewhat from organism to organism. The ATPase activity from dicotyledonous plants studied [86] closely resembled the ATPase from animal and yeast mitochondria. They were inhibited by oligomycin and had elevated molecular weights when separated from the organelle. The mitochondria from monocotyledonous plants, on the other hand, had ATPases which behaved quite differently. Monocot mitochondrial ATPases were unaffected by oligomycin. Purified ATPases from monocots were more stable to cold and had a lower molecular weight than that obtained from dicots. Similar observations were made by Jung and Hanson [87].

There seems to be general agreement that mitochondrial ATPases from various sources have a requirement for divalent metal ions, and Mg^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+} and Co^{2+} were all effective [88]. In beef heart mitochondria, the pH optimum was reported to be 8.0 [88]. This ATPase was not inhibited by

sulfhydryl reagents, had a high sensitivity to iodine and tetranitromethane, and was virtually destroyed by DCCD. Jung and Laties [89] found a potato mitochondrial ATPase that showed a sharp optimum activity at pH 10.2.

Pullmen and Monroy [90] described a naturally occurring inhibitor of mitochondrial ATPase from beef heart. A similar phenomenon was reported by Jung and Laties [89] in potato mitochondria. They found the endogenous ATPase activity of intact potato mitochondria to be quite low and not stimulated by Mg^{2+}. Jung and Hanson [91,92] found that in cauliflower mitochondrial ATPase, respiratory priming (the addition of respiratory substrate in the presence of Mg^{2+} and Pi) and self priming (prolonged incubation with Mg^{2+} and ATP) caused stimulation. In potato mitochondrial ATPase, however, such treatments had no significant effect.

When membrane barriers were disrupted by sonication or with detergents, such as Triton X-100 treatment, a slightly Mg^{2+}-dependent ATPase-activity increase occurred in the cauliflower mitochondrial ATPase [87], but such treatment caused a ten- to fifteenfold increase in the activity of the potato mitochondrial ATPase. They found that trypsin treatment could induce ATPase activity in potato mitochondrial ATPase when 2 mM $MgCl_2$ were added. Cold lability seemed to be repressed in mitochondrial ATPases if the enzyme was integrated in the membrane [93]. Oligomycin inhibition was not observed in the isolated enzyme.

Boyer et al. [94] postulated a high-energy coupling system in which ATPase acts in the hydrolysis or synthesis capacity depending on the situation and the site. For example, the normal function in muscle contraction would be hydrolysis and in mitochondria, synthesis. The release of the ATP synthesized on the mitochondrial membrane requires an energy-dependent conformational change at the catalytic site.

Penefsky [95] reported that an ATP analogue, AMP-PNP, could not be hydrolyzed by mitochondrial ATPase. He found that AMP-PNP was a potent competitive inhibitor of soluble membrane-bound mitochondrial ATPase and could also inhibit other ATP-dependent reactions. However, AMP-PNP did not interfere with the synthesis of ATP from ADP and Pi. The conclusion from the data was that the hydrolytic site of the ATPase was not identical to the catalytic site for ATP synthesis.

Beef heart mitochondrial ATPase had tightly bound ATP and ADP in both membrane-bound and isolated forms [96]. At one site ADP was bound, from a second ATP was lost, and at a third ADP was phosphorylated. There were four equivalent sites in all and they passed through each of the four states in turn in a rotating-wheel manner.

Effect of DNP on ATPase

The inhibitor DNP increased the permeability of the mitochondrial and chloroplast membranes to H^+ [97,98] and had a great effect on sugar transport in the corn scutellum [99]. It increased sucrose transport out of the vacuole and inhibited the uptake across the plasmalemma [100,101]. At the

plasma membrane, DNP was reported to hydrolyze sucrose, which was thought to be a result of the compound's interference with the normal function of the disaccharide transport system [102]. The compound was thought to cause these changes as a direct result of its ability to increase the permeability of the membrane to H^+. Humphreys [103] showed rapid pH-dependent H^+ influx into slices induced by DNP. The H^+ influx was very slow at pH 5, but increased rapidly as the pH was lowered over the range of 5 to 3.5. The influx of H^+ was accompanied by a nearly equal K^+ efflux. He concluded that the H^+ influx was induced by DNP, but the H^+ carrier might be an ATPase.

Lambeth and Hardy [104] reported that DNP decreases the activity of purified rat liver mitochondrial ATPase. Mitchell [44] reported that DNP may be acting as an uncoupler of the ATPase by specifically equilibrating the electrochemical potential of H^+ across the coupling membrane. He postulated the existence of a proton pump which was separate from the redox and ATPase system, but activated by an energy-rich intermediate in equilibrium with the latter systems.

Jung and Hanson [92] reported an oligomycin-insensitive ATPase from cauliflower mitochondria which could be stimulated by DNP. Cauliflower mitochondrial membrane was apparently quite leaky, and therefore the organelle could not maintain an adequate amount of needed ions and cofactors in the matrix during senescence or starvation. Indirect comparisons of corn and rat-liver mitochondria did not seem quite as leaky.

It appeared that DNP caused an increase in the ATPase activity in the mitochondria isolated from Jerusalem artichokes [85]. This was perhaps due to the collapse of the oxidative phosphorylation, so that the ATPase had to work faster to maintain the potential across the membrane.

Inhibitors of ATPase

There are various inhibitors of ATPase reported in the literature. The state of the science is, however, far from clear. The same inhibitor which has a large inhibitory effect in one system may not affect another, even though both are characterized as ATPases.

Partially purified spinach ATPase was found to be 80% inhibited by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, and completely reversed by dithiothreitol. This enzyme was also inhibited by quercetin [5].

Abrams et al. [105] reported the effect of DCCD on ATPase derived from Streptococcus faecalis. They looked at wild-type and mutant forms of the bacteria in respect to sensitivity of the plasma-membrane ATPase to DCCD. They found that the sensitivity to DCCD required that the enzyme be attached to the membrane. The mutants were 100 times less sensitive to DCCD than the wild types, and the energized uptake of K^+ and cycloleucine was not affected in the mutant by DCCD.

The link between sensitivity to inhibitors and its association with the membrane is further supported by the work of Knowles, Guillory and Racker [106]. They found that sensitivity of the ATPase to oligomycin required the presence of

certain p-lipids. When dissociated from the membrane lipids, the enzyme was not inhibited by oligomycin or DCCD.

Simoni and Shandell [107] reported a mutant E. coli which was unable to couple energy from ATP hydrolysis to the active transport of proline. They found that the ATPase was poorly attached to the membrane, and the analysis of the membrane showed the absence of a single polypeptide with a MW of about 54,000 and the appearance of a new polypeptide with a MW of about 25,000. The oligomycin-sensitive protein in mitochondria was presumed to be in the stalk. Sensitivity to DCCD resided elsewhere. This inhibitor was effective only when the BF_1 was attached to the membrane. The ATPase of the mutant was insensitive to DCCD from 0 to 20 μM. In wild types, inhibition was maximal at about 5 μM. The conclusion drawn from these data was that either the strain was deficient in the DCCD-reactive protein, or the BF_1 was poorly attached to the membrane and thus was insensitive to DCCD.

Similar results were reported with another E. coli mutant with altered sensitivity to DCCD [108]. The wild type is highly sensitive to 50 μM of DCCD. In the mutant, as much as 200 μM of DCCD were needed to obtain inhibition. A highly water-soluble carbodiimide (EDCD), however, was equally effective on both the wild type and mutant in inhibiting the ATPase activity. This seemed to indicate that the DCCD preferentially entered the hydrophobic membrane phase of the mutant, thereby effectively decreasing the concentration of DCCD available for inhibition.

The coupling factor (F_1) proteins from beef heart, rat liver, and yeast mitochondria were similar and had a MW of about 360,000 [83]. The coupling factors from chloroplast (CF_1) were cold-labile and insensitive to DCCD when isolated from the membrane. Structurally, CF_1 is similar to the coupling factors from various mitochondria.

In mitochondria, DCCD was thought to react specifically with one polypeptide in the membrane portion of the complex [109,110].

Plasma-membrane ATPase from corn root [7] was inhibited by Ca^{2+} and La^{3+} in the presence of Mg^{2+}, but not in its absence. Oligomycin was not an effective inhibitor; however, DCCD at concentrations from 50 to 100 μM inhibited the enzyme by 50%. Complete inhibition occurred with 200 μM of DCCD.

An interesting observation in corn root mitochondrial ATPase was that DCCD was a good inhibitor of this enzyme when only low levels were present (less than 50 μM) [7]. Concentration higher than 50 μM did not have an inhibitory effect on the enzyme.

Lin et al. [111] isolated vacuoles from petals of Hippeastrum and Tulipa and found an ATPase on the tonoplast but not in the vacuolar contents. This ATPase was not inhibited by DCCD, but was inhibited by the water-soluble EDCD. The tonoplast membrane exhibited twice the activity at pH 6 than at 9 and was dependent on Mg^{2+} with stimulation by KCl. It was insensitive to oligomycin. They found no acid phosphatase activity with the tonoplast membrane when PNP was used as a

substrate. They felt that the ATPase on the tonoplast might be on the outer surface of the vacuolar membrane, and implicated it in the transport of ions in and out of the vacuole.

The Mg^{2+}-dependent ATPase from the envelope of spinach chloroplast was also insensitive to DCCD [112].

Sapir and Pederson [113] reported that ATPase from rat liver mitochondria was inhibited by 95% by 2.5 μM of oligomycin and 60% with 1-8 μM of DCCD. The isolated ATPase was less sensitive to oligomycin, venturicidin, and DCCD than was membrane-bound ATPase. They concluded that this may indicate the need for a conformational specificity. They also reported an oligomycin-insensitive ATPase in the same system. When oligomycin-sensitive ATPase was purified from rat liver mitochondria, detergent treatment decreased the enzyme's sensitivity to oligomycin.

Riebeling and Jungermann [64] reported an 80% inhibition of clostridial membrane-bound ATPase by DCCD, but oligomycin, ouabain and NaN_3 had no effect at concentrations ranging from 10^{-8} to 10^{-3} M.

Triphenyltin chloride (less than 1 μM) inhibited ATP formation and coupled electron transport in isolated spinach chloroplasts [114]. When the ATPase was purified, the Ca^{2+}-Mg^{2+} ATPase CF_1 protein was found to be insensitive to TPT. This compound is thought to inhibit the phosphorylation exchange and membrane-bound ATPase in chloroplasts by specifically blocking the transport of protons through the membrane-bound channel located in a hydrophobic region of the membrane

at or near the functional binding site for the coupling factor.

The ATPase activity of the isolated coupling factor was insensitive to DCCD [114]. If the ATPase was attached to the membrane, the DCCD did inhibit the enzyme. It was thought that DCCD did not inhibit ATP formation by interfering directly with the coupling factor protein, but rather by reacting with another tightly bound component within the membrane itself. It also seemed to act as an electron transport inhibitor.

It appeared that TPT behaved similarly to DCCD [114] but at much lower concentrations. It too inhibited electron transport as well as ATP formation.

Evans concluded [115] that the Mg^{2+}-Ca^{2+} ATPase of E. coli is tightly integrated in the cell membrane, and that the electrical phenomenon or conformational changes, or both, play important roles in the functioning and regulation of this enzyme in intact membranes.

Phlorizin, phloretin, and DES [116] are inhibitors of Na^+-dependent sugar transport, and they inhibit Na^+-K^+ ATPase activity but do not seem to affect the Mg^{2+} ATPase. In animal cells, the uphill sugar transport was dependent either directly or indirectly on the activities of the Na^+ pump. This pump could be inhibited by inhibiting the Na^+-K^+ ATPase activity or by inhibiting the metabolic generation of ATP itself. Other common inhibitors tried were ouabain, oligomycin and NEM.

The intracellular metabolic function was not permanently altered by PCMBS, and there was good evidence that the site of inhibition was the plasma membrane [12]. Another sulfhydryl group inhibitor, NEM, is known to act on the intracellular processes as well.

Phlorizin and NEM, like TPT, did not act on the coupling factor directly, but on a component either within or closely associated with the membrane [114].

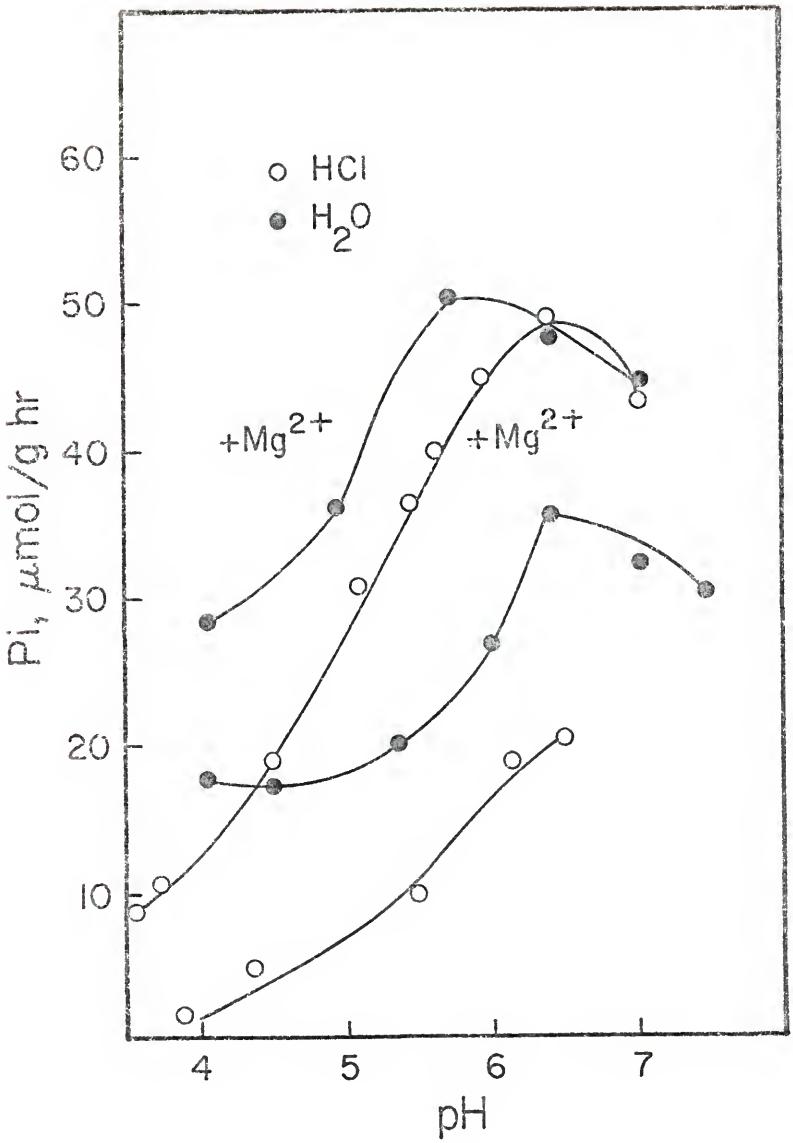
RESULTS

Cell-surface Phosphatase Activity of Fresh Scutellum Slices

Scutellum slices were incubated in distilled water for one hour, and then placed in a buffered ATP solution. Phosphatase activity of these slices was determined by measuring Pi in the bathing solution. The effect of pH on phosphatase activity is shown in Fig. 1. The addition of Mg^{2+} stimulated the activity at all pH's tested. The peak activity without Mg^{2+} was at pH 6.5, and with Mg^{2+} it was at pH 5.7.

Humphreys [117,118] found that maltase activity on fresh maize scutellum slices could be nearly eliminated by washing the slices for 30 to 60 min in 0.01N HCl. Such treatment did not interfere with sucrose synthesis, cellular respiration, or the transport of maltose or sucrose across the plasmalemma. When fresh scutellum slices were treated with 0.01N HCl prior to testing for phosphatase activity, there was a drop in the activity over the entire pH range from 4 to 7 (Fig. 1). When Mg^{2+} was added, however, the HCl-washed and H_2O-washed slices showed nearly equal phosphatase activities at pH 6.5. Acid treatment decreased phosphatase activity at the lower pH's (Fig. 1), but appeared to have no effect on the phosphatase having peak activity at pH 6.5 in the presence of added Mg^{2+}. The addition of Mg^{2+} to acid-treated scutellum slices greatly stimulated the phosphatase

Figure 1. Phosphatase activity of fresh scutellum slices. Fresh maize scutellum slices (0.5 g) were treated with H_2O or 0.01N HCl for 1 hr with a change of bathing solution after 30 min. Magnesium chloride (20 mM) was added for the + Mg^{2+} studies. Each vessel contained 50 mM MES (pH 3.5-6.5) or 50 mM MOPS (pH 6.5-7.5) and 3 mM ATP. The experiments were run at 30° for 60 min. Each point is an average of 5 values.


60
50
40
30
20
10
Pi, μmol/g hr
HCl
H2O
+Mg2+
+Mg2+
4
5
6
7
pH

activity especially between pH 6 and 6.5 (Fig. 1).

These results indicate that the phosphatases are at the surfaces of the scutellum cells. The activity was sensitive to bathing-solution pH, the substrate was added to the bathing solution and the product appeared in the bathing solution, the addition of Mg^{2+} to the bathing solution stimulated activity and HCl treatment destroyed part of the activity.

Phosphatase Activity of Frozen Scutellum Slices

Frozen scutellum slices (which allowed substrate access to both sides of the plasmalemma) were thawed quickly by the addition of either H_2O or HCl (0.01N), and then incubated at 30° for 30 or 60 min before measuring phosphatase activity. Frozen slices that were tested without pretreatment with either H_2O or HCl showed the highest phosphatase activity (Table 1). Phosphatase activity was decreased 25% after the slices were H_2O-treated for 30 min, and 65% after 1 hr. Frozen HCl-treated slices showed greater decreases in enzyme activity. After 30 min in HCl, the activity decreased from 153 µmol/g hr to 67 µmol/g hr. After 1 hr in HCl, the phosphatase activity was only 11 µmol/g hr. When Mg^{2+} was added to the reaction mixture, the phosphatase activity of the frozen, HCl-treated slices rose from 11 µmol/g hr to 40 µmol/g hr. The latter rate is nearly equal to that obtained with fresh slices at the same pH (Fig. 1).

The effect of pH on phosphatase activity of frozen, H_2O-treated slices is shown in Fig. 2. The activity in the pH 4 to 6 range remained relatively high, then decreased above pH 6. The addition of Mg^{2+} increased the activity

Table 1. Effect of H_2O or HCl treatment on phosphatase activity of frozen-thawed scutellum slices

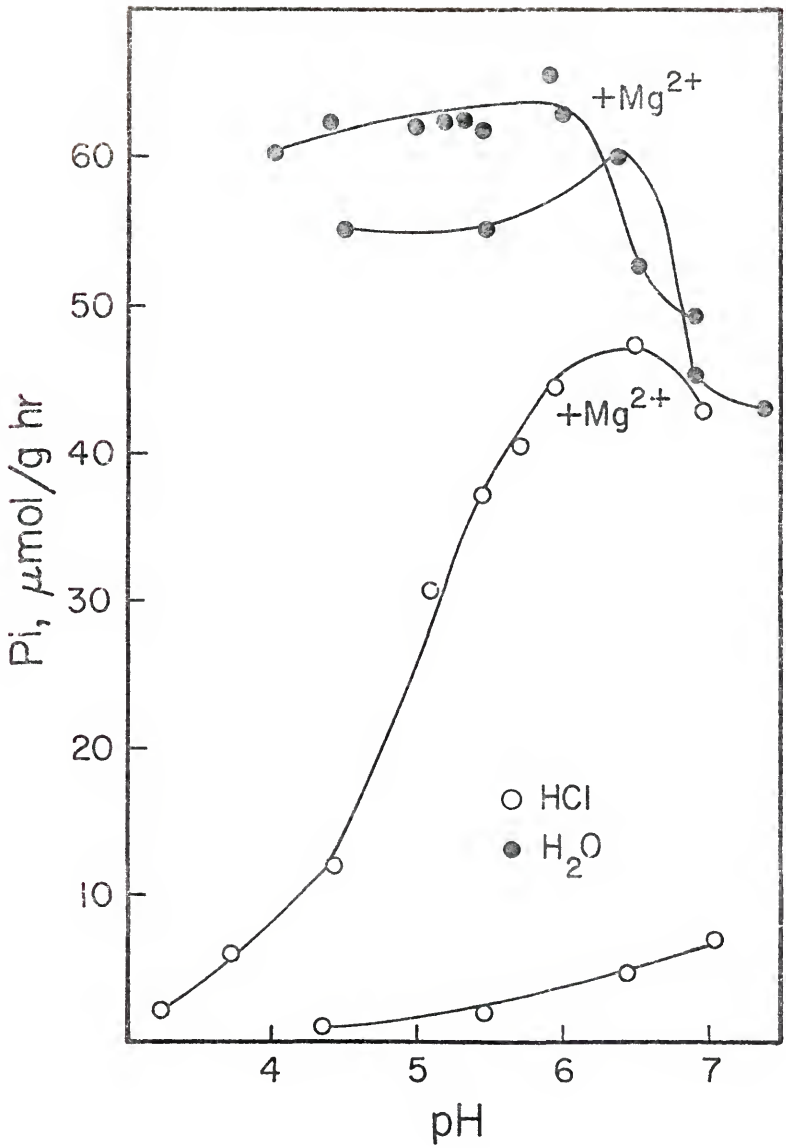
No.	Pretreatment	Pi, μmol/g hr
1	None	153
2	30 min in H_2O	114
3	60 min in H_2O	54
4	30 min in HCl	67
5	60 min in HCl	11
6	60 min in HCl; + Mg^{2+}	40
7	60 min in HCl; no ATP	0

Note: The substrate (3 mM ATP) was added to reaction mixture after the various pretreatments. Added Mg^{2+} was absent in the reaction mixture in all but #6. In #6, 20 mM of $MgCl_2$ was added to the reaction mixture.

The reaction was allowed to proceed for 60 min at 30° in 50 mM MES pH 6.5.

In the 60 min H_2O or HCl pretreatments, a change in bathing solution was made after 30 min.

Figure 2. Phosphatase activity of frozen scutellum slices. Frozen tissue (0.5 g) was thawed with 0.01N HCl or H_2O and treated for 1 hr with a change after 30 min. Magnesium chloride (20 mM) was added for the + Mg^{2+} studies. Each reaction vessel contained 50 mM buffer (MES pH 3.0-6.5 or MOPS pH 6.5-7.0) and 3 mM ATP, and was run at 30° for 60 min. Each point is an average of 5 experiments.


+Mg2+
+Mg2+
HCl
H2O
Pi, μmol/g hr
pH
60
50
40
30
20
10
4
5
6
7

less than 15% in the pH range of 4 to 6. The addition of Mg^{2+} shifted the peak phosphatase activity from pH 6.5 to pH 5.9. The addition of Mg^{2+} to fresh H_2O-treated slices also shifted the pH optimum to a lower value (Fig. 1).

The phosphatase activity of the frozen, HCl-treated slices in the absence of added Mg^{2+} was low at all pH's tested. In the presence of Mg^{2+}, however, there was a sharp rise in activity near pH 5 and a peak activity at pH 6.5.

Since Pi was measured as the indicator of phosphatase activity, the amount of Pi leakage and uptake by fresh acid-treated slices was measured. The results in Table 2 show only 1 to 2 µmol of Pi change in the bathing solution after 1 hr of incubation. When frozen acid-treated slices were tested for Pi leakage, only 1 µmol had leaked into the bathing solution after 1 hr. The results were nearly identical over the pH range of 4.5 to 8.0 (Table 2).

All the results in this study are presented in terms of fresh weight. Totals for protein, lipid and phosphorus were determined for 1 gm of fresh and frozen tissue to give an indication of the composition of the tissues. The results are given in Table 3. Small differences (13%) in total protein were observed between the fresh and frozen acid-treated tissues. The frozen tissue had a greater amount of extractable lipid (38%) than the fresh tissue, and the fresh tissue had 23% more total phosphorus than the frozen.

From Table 3 it is clear that after acid treatment there still remains a great deal of phosphorus in both fresh and

Table 2. Pi leakage and uptake

Tissue	Initial Pi in bathing solution, µmol	pH	ΔPi in bathing solution, µmol
Fresh	0	6.5	+ 1.0
	10	6.5	+ 2.0
	20	6.5	+ 1.0
	30	6.5	+ 1.0
Frozen	0	4.5	1.0
	0	5.5	1.0
	0	6.5	1.0
	0	8.0	0

Note: Each reaction vessel contained 0.5 g of HCl-treated scutellum slices, 50 mM buffer (MES pH 4.5-6.5, MOPS pH 8.0), added Pi, and 20 mM $MgCl_2$. The experiments were run for 60 min at 30°.

Table 3. Total phosphorus, nitrogen, and lipid

Tissue	Total Phosphorus/g	Total Nitrogen/g	Ethyl Ether Extractable Lipid, mg/g
Fresh	150.5 µmol	9.0 mg	89.2 mg
Frozen	126.0 µmol	8.0 mg	123.5 mg

Note: HCl-treated scutellum slices were analyzed for total phosphorus, nitrogen and lipid per g of fresh weight. The total lipid is the ethyl ether extractable lipid in the scutellum slices. More lipid was extracted from the frozen tissue, perhaps due to the disruption of the membranes during freezing.

frozen scutellum slices. This phosphorus may be bound in various organic compounds such as phytic acid [119]. These organically bound sources of phosphorus do not seem to be used as substrates by the phosphatase enzyme, nor do they leak from the tissue.

Electron micrographs of the frozen, HCl-treated slices showed few distinct organelles. There were no distinguishable mitochondria, ER, dictyosomes or nuclei. There were some lipid globules, and patches of cytoplasm. When these cells were specifically stained for plasmalemma, with the PTA-CrO_3 method [120], many vesiculated and nonvesiculated plasmalemma fragments were observed (Fig. 3).

From the above results, it is concluded that there is an acid-stable, tightly bound phosphatase located at the cell surfaces of the maize scutellum. This phosphatase is activated by Mg^{2+} and has a peak activity at pH 6.5. The nearly identical results obtained with acid-treated preparations of both fresh and frozen slices strongly suggest that the same phosphatase was being observed in both cases.

The following sections are concerned with the properties and location of this phosphatase.

Substrate Specificity

Both fresh and frozen acid-treated preparations were used to test various substrates (Table 4). Nucleoside monophosphates were poor substrates, as were sugar phosphates, PNPP, and P-Pi. The ADP and DCP were hydrolyzed at rates

Figure 3. Phosphotungstic acid stain for plant plasmalemma. Sections are of frozen, HCl-treated scutellum tissue stained with phosphotungstic-chromic acid, and showing plasmalemma (pl), cytoplasm (cy), and cell wall (CW). X 50,000

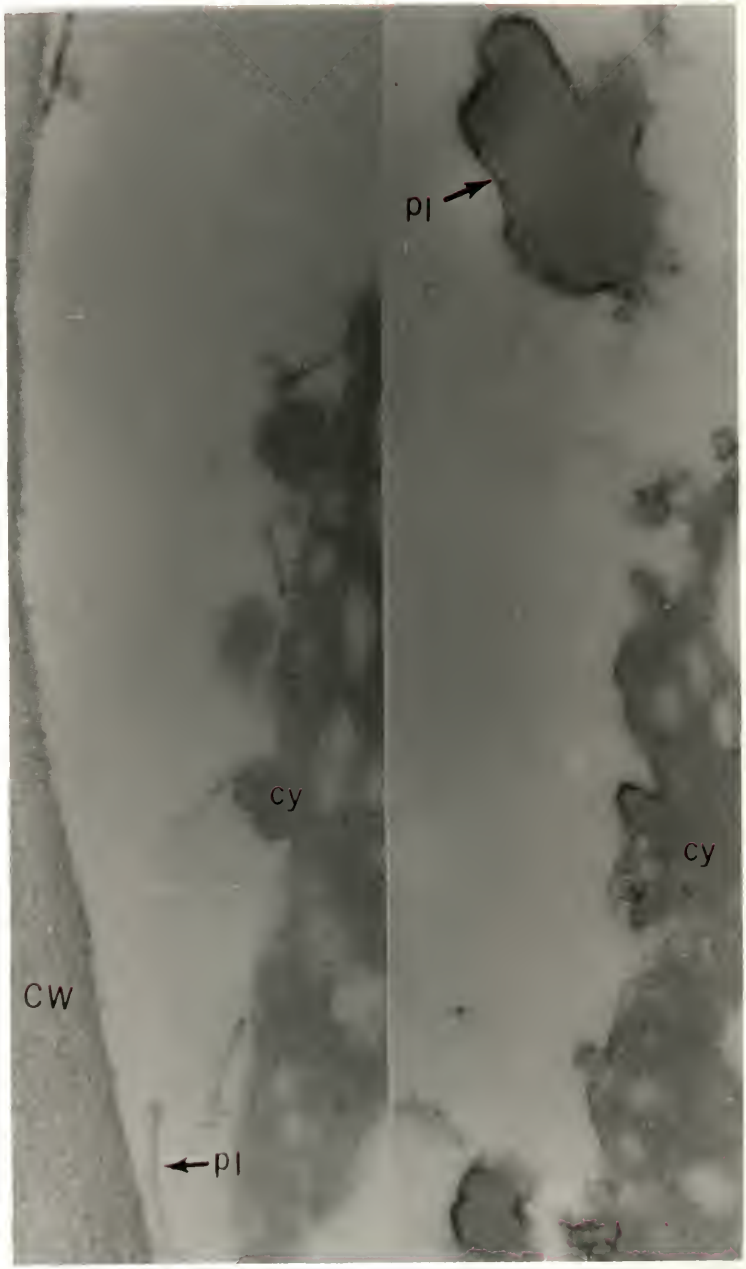

pl
cy
cy
CW
pl

Table 4. Substrate preference

Substrate	% of ATP Activity	
	Frozen	Fresh
UTP	73	83
GTP	53	60
CTP	100	103
TTP	82	86
ADP	67	77
UDP	7	5
CDP	78	86
AMP	13	23
GMP	11	25
CMP	9	13
TMP	9	15
P-Pi	34	10
Mannose-6-P	13	24
Glucose-6-P	14	27
Paranitrophenolphosphate	17	31

Note: Both fresh and frozen tissues were pretreated for 1 hr in 0.01N HCl. Each reaction vessel contained 3 mM substrate, 20 mM $MgCl_2$, 50 mM MES pH 6.5 and 0.5 g tissue, and was run for 1 hr at 30°. Each value is an average of 4-5 experiments.

between 67% and 86% of that of ATP. The UDP, however, gave only 5% to 7% of the ATP activity. The CTP was hydrolized at rates equal to that of ATP. The other nucleoside triphosphates were hydrolyzed at rates 53% to 86% of that of ATP.

The greatest difference in phosphatase activity between fresh and frozen tissue preparations was obtained with P-Pi. In the frozen preparation, P-Pi gave 34% of the ATP activity, but only 10% with the fresh preparation.

Stoichiometry of the Phosphatase Reaction with ATP as the Substrate

End point stoichiometric studies were run in triplicate with fresh HCl-treated slices. At the end of 60 min, the ΔATP was 24.2 μmol/g, ΔPi was 44.0 μmol/g, ΔADP was 6.3 μmol/g and ΔAMP was 13.8 μmol/g. (Each value is an average of 3 replicates.) Thus, 83% of the ATP lost from the bathing solution was recovered as ADP and AMP.

Time course stoichiometric studies were done with both fresh and frozen acid-treated slices. In the fresh preparation, as the level of ATP in the bathing solution decreased with time, the Pi and the ADP levels increased (Fig. 4). The ADP level reached a plateau after about 10 min, and remained nearly constant over the remainder of the 60-min period. The Pi level, however, continued to increase. After about 20 min, the rate of Pi production rose and then remained linear. After 60 min, 46 μmol of Pi were found in the bathing solution, along with 11 μmol of ADP and 25 μmol of ATP. There was little difference in Pi production between fresh and frozen preparations. Initially 51 μmol of ATP were present (Table 5). These values are close to the theoretical values. The total Pi as a result of ATP hydrolysis and the

Figure 4. Time-course stoichiometry with fresh and frozen HCl-treated tissue. Fresh scutellum slices (0.5 g) were HCl-treated for 1 hr with a change after 30 min. The reaction mixtures contained 20 mM $MgCl_2$, 50 mM MES pH 6.5, and 2.5 mM ATP, and were run at 30° for 60 min. All points are an average of 3 experiments.

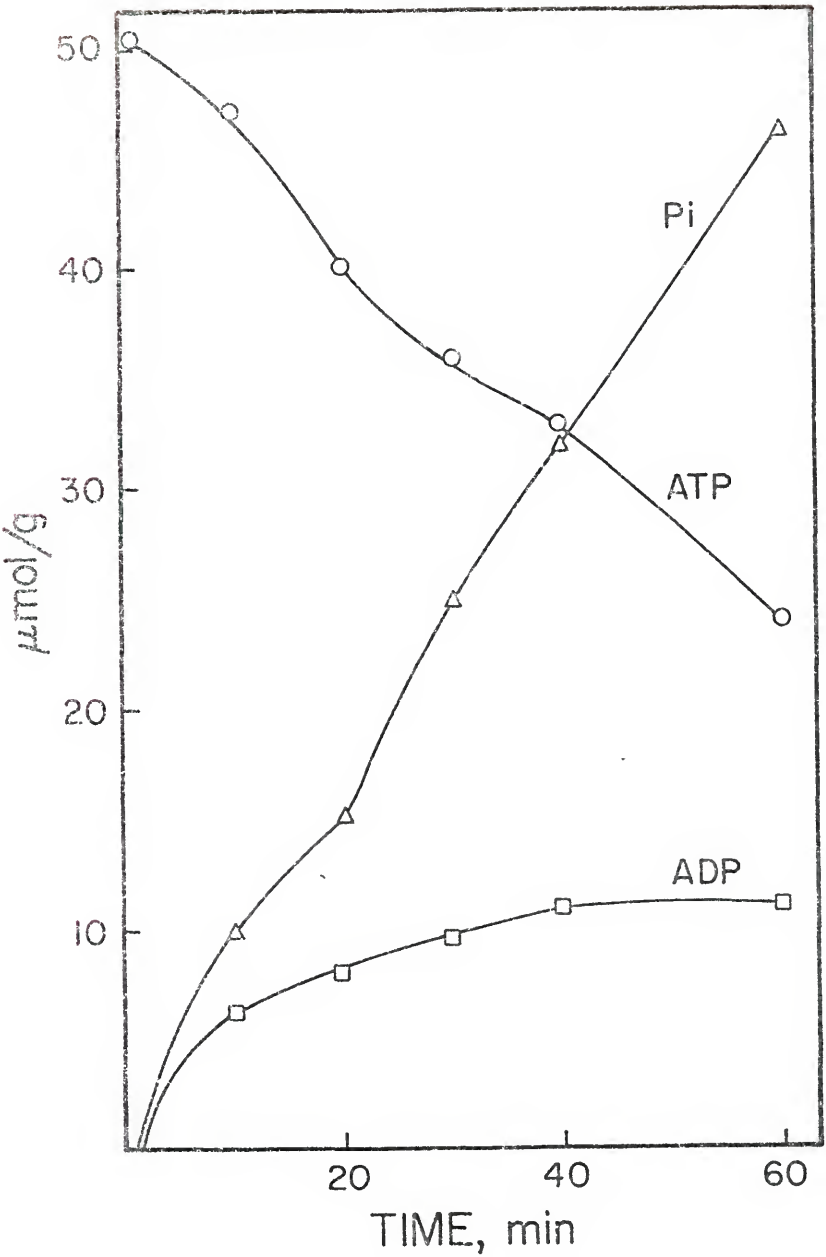

50
40
30
20
10
μmol/g
Pi
ATP
ADP
20
40
60
TIME, min

Table 5. Stoichiometry with fresh and frozen HCl-treated tissue

	ATP, µmol		ADP, µmol		Pi, µmol	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
Initial	51	50	0	0	0	0
60 min	24	26	11	11	46	38

Note: HCl-treated scutellum slices (0.5 g) were placed in a reaction mixture containing 2.5 mM ATP, 50 mM MES pH 6.5, and 20 mM $MgCl_2$, and was run at 30°. Each value is an average of 3 experiments.

hydrolysis of ADP subsequently formed should total 43 µmol. the amount measured was 46 µmol.

The results with acid-treated, frozen slices were similar to those of the fresh preparations (Table 5 and Fig. 5).

Enzyme Kinetics

The kinetic studies for ATP were run for 10 min with fresh and frozen acid-treated preparations. The substrate concentration curve is shown in Fig. 6.

The Lineweaver-Burke plot of the combined data from fresh and frozen tissue preparations is shown in Fig. 7. The K_m was calculated to be 0.65 mM.

The kinetic studies with ADP in fresh, HCl-treated slices resulted in a K_m of 5.0 and a 1/Vmax identical to that of ATP (Fig. 7).

Effect of Divalent Cations on the Phosphatase Activity

Most ATPases require Mg^{2+} for activity. Magnesium ions have been shown to combine with ATP to form MgATP, which then acts as the true substrate for the enzyme [121]. The phosphatase activity of both the fresh and the frozen HCl-treated preparations was stimulated by the addition of Mg^{2+} (Figs. 1 and 2). The effect of Mg^{2+} concentration on phosphatase activities of these preparations is shown in Fig. 8. The rate of phosphatase reaction increased with increasing Mg^{2+} concentration at least up to 20 mM. This is far in excess of the ATP concentration of 3 mM. Storer and Cornish-Bowden [121] have shown that the true MgATP concentration cannot be

Figure 5. Pi formation in fresh and frozen scutellum slices. Fresh and frozen HCl-treated scutellum slices (0.5 g) were placed in a reaction vessel containing 20 mM $MgCl_2$, 50 mM MES pH 6.5, and 2.5 mM ATP and run for 60 min at 30°. Each point is an average of 3 experiments.

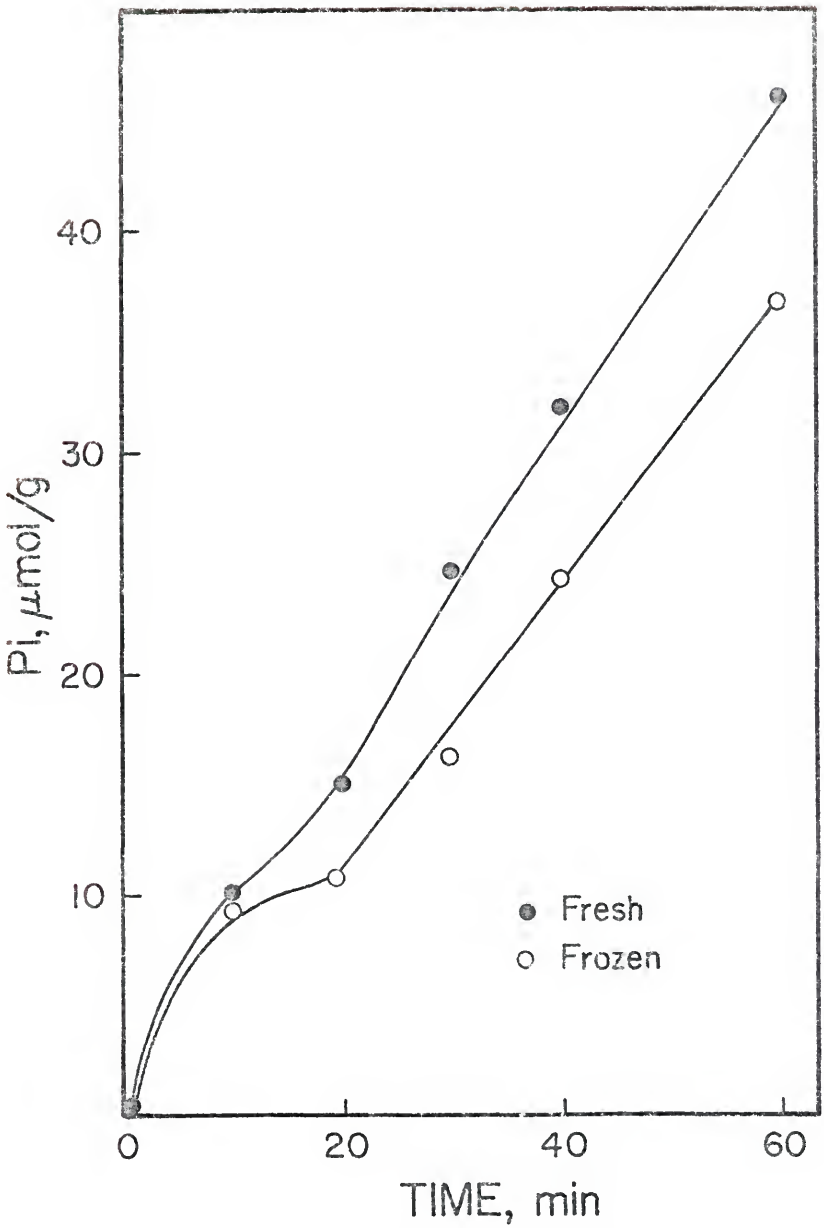

40
30
20
10
Pi, μmol/g
0
20
40
60
TIME, min
Fresh
Frozen

Figure 6. Rate of phosphatase activity with increasing ATP concentration. Fresh and frozen HCl-treated scutellum slices were placed in a reaction vessel containing various concentrations of ATP, 20 mM $MgCl_2$ and 50 mM MES pH 6.5 and run at 30° for 10 min. Each point is an average of 2 experiments.

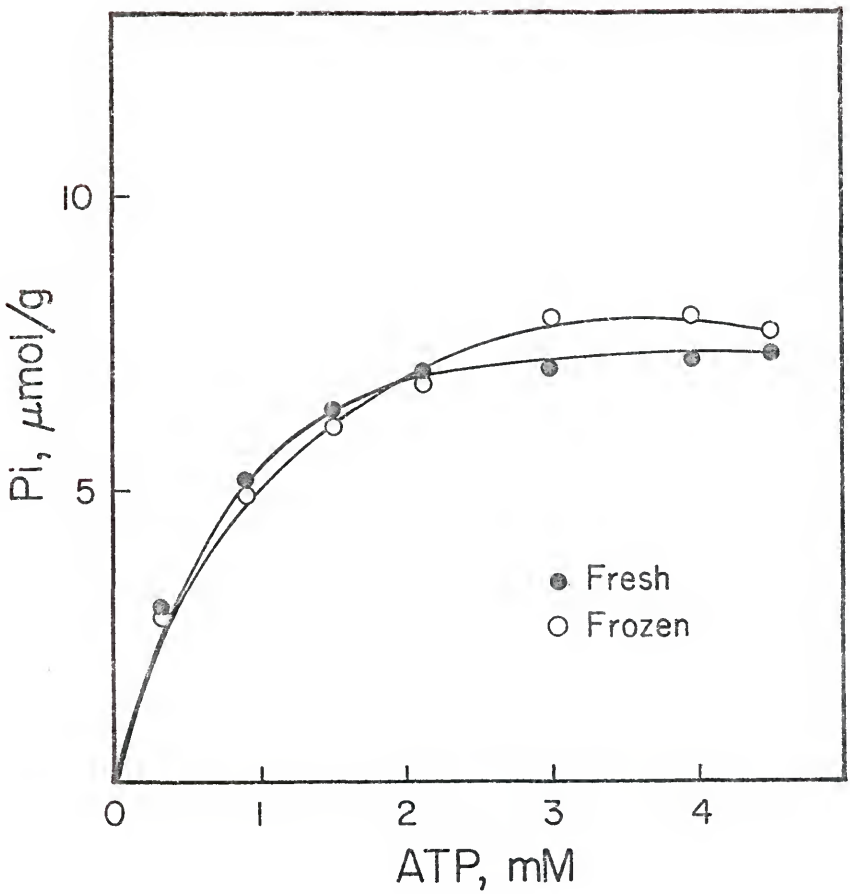

10
5
Pi, μmol/g
Fresh
Frozen
0
1
2
3
4
ATP, mM

Figure 7. Kinetics studies with ATP and ADP. Fresh and frozen scutellum slices were HCl-treated and placed in a reaction vessel with 20 mM $MgCl_2$ and 50 mM MES pH 6.5, and run at 30°. The experiments with ATP were run for 10 min, and those with ADP were run for 30 min. The graph is expressed in terms of 10-min values. Each point is an average of 4 experiments.
v = µmol Pi/g/10 min; s = mM ATP or ADP.

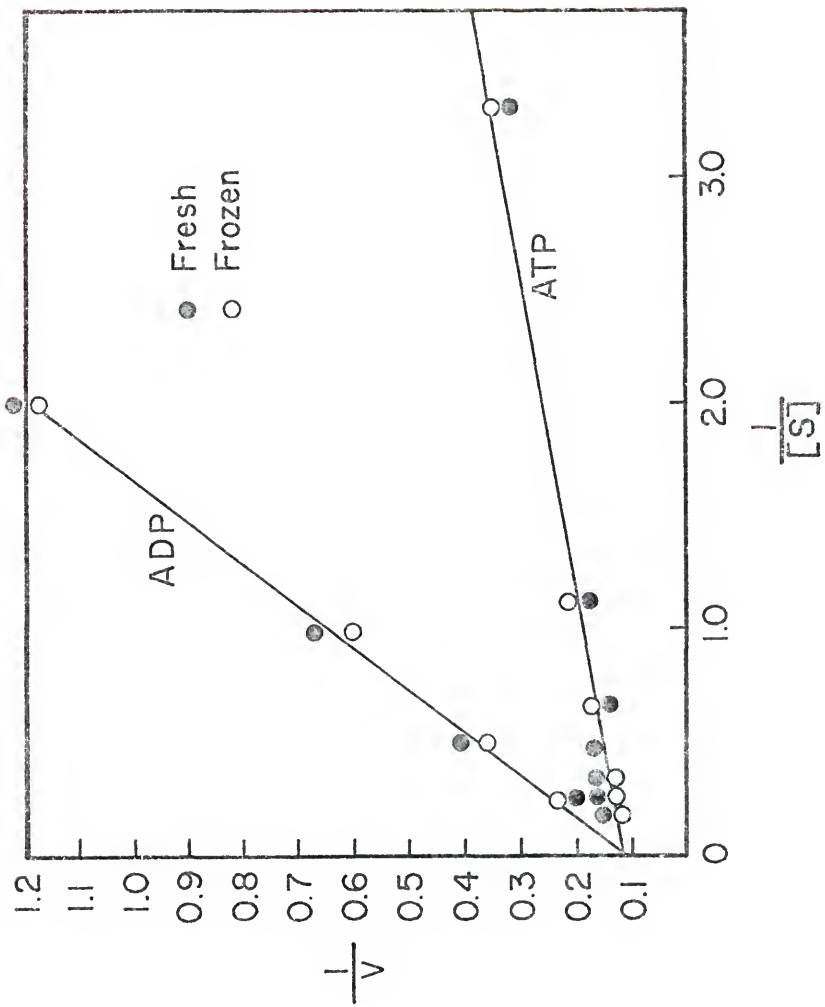
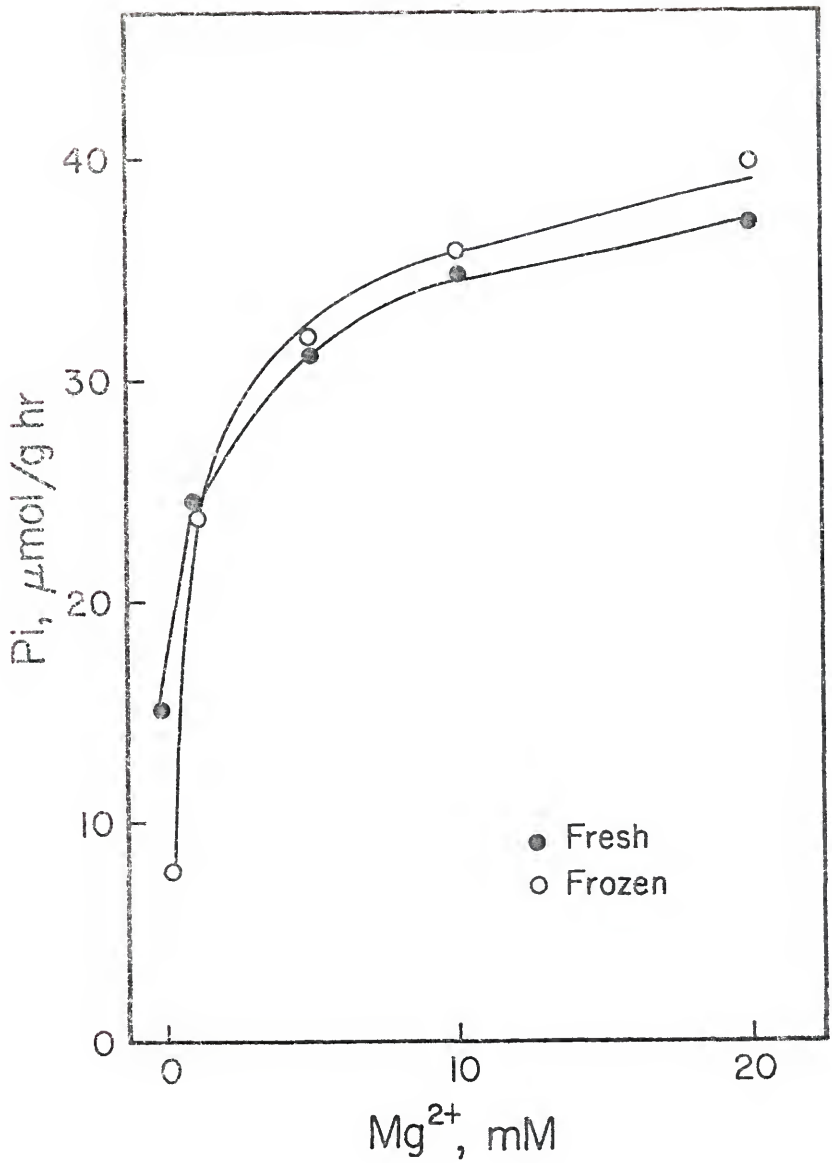

Fresh
Frozen
ADP
ATP
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/V
0
1.0
2.0
3.0
1/[S]

Figure 8. Effect of Mg^{2+} on phosphatase activity. Fresh and frozen HCl-treated scutellum slices were placed in a reaction vessel containing 3 mM ATP, varying concentrations of $MgCl_2$, and 50 mM MES ph 6.5, and run at 30° for 60 min. Each point is an average of 3 experiments.


40
30
20
10
0
Pi, μmol/g hr
0
10
20
Mg2+, mM
Fresh
Frozen

derived by merely adding equimolar concentrations of the ion and ATP. They suggested that in cases where the enzyme being studied was not inhibited by high metal ion concentrations, 5 mM or more excess of Mg^{2+} over ATP was likely to give a better stimulatory effect. It is assumed in calculating the K_m's for ATP and ADP (Fig. 7) that in 20 mM $MgCl_2$ all of the nucleoside was present in solution as the Mg^{2+} complex.

Phosphatase activity in both fresh and frozen slice preparations was also stimulated by Mn^{2+}, Ca^{2+} and Co^{2+} (Fig. 9). The relative effectiveness of the cations was $Mn^{2+} > Mg^{2+} \geq Ca^{2+} > Co^{2+}$. At high Mn^{2+} concentrations, the stimulatory effect was sharply decreased; at 20 mM Mn^{2+}, phosphatase activity was only one-half of that obtained with 5 mM Mn^{2+} (Fig. 9). Calcium ions gave maximum stimulation at 10 mM, but at higher concentrations of Ca^{2+} the stimulatory effect decreased. Cobalt ions were much less effective than the other divalent ions tested, and maximum stimulation was obtained with 5-10 mM Co^{2+}.

Effect of Na^+ and K^+ on the Phosphatase Activity

The stimulatory effect of Na^+ and K^+ was tested in the fresh and frozen preparations with and without Mg^{2+}. When various ratios of Na^+ and K^+ were added in the presence of Mg^{2+}, the additional stimulation was very small (Table 6) in fresh tissue preparations. With a Na^+/K^+ ratio of 0.25 the additional stimulation was only 10% more than with Mg^{2+} alone. The other ratios gave smaller amounts of stimulation. In the frozen tissue preparation, the addition of Na^+ and K^+ gave

Figure 9. Effect of divalent cations on phosphatase activity. Fresh and frozen HCl-treated scutellum slices were placed in a reaction vessel containing 3 mM ATP, varying ion concentrations and 50 mM MES pH 6.5 and run at 30° for 60 min. Each point is an average of 3 experiments.

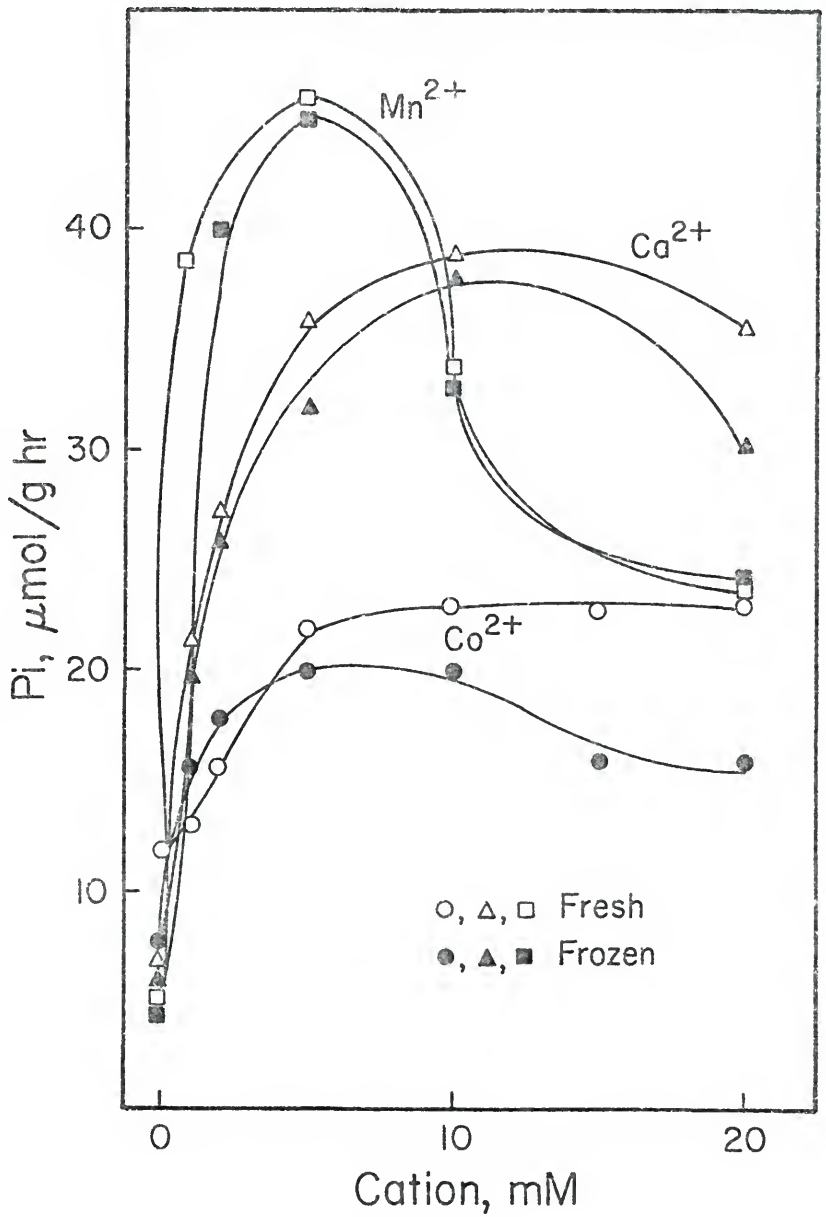

Mn2+
Ca2+
Co2+
40
30
20
10
Pi, μmol/g hr
○, △, □ Fresh
●, ▲, ■ Frozen
0
10
20
Cation, mM

Table 6. Effect of Na^+ and K^+ on phosphatase activity

Na^+/K^+ ratio	Mg^{2+}	Pi, µmol/g hr	
		Fresh	Frozen
No Na^+ or K^+	+	45 ± 2.2	30 ± 2.0
	-	25 ± 5.6	6 ± 2.5
Na^+ only	+	46 ± 0	35 ± 0.8
	-	35 ± 0.6	9 ± 0.6
4	+	48 ± 0.8	34 ± 1.0
	-	35 ± 0.6	---
1.5	+	48 ± 0.8	36 ± 1.0
	-	36 ± 0.8	9 ± 0.6
0.66	+	48 ± 0.6	36 ± 0.6
	-	36 ± 0.6	---
0.25	+	50 ± 0.6	36 ± 0.6
	-	36 ± 1.0	9 ± 0
K^+ only	+	50 ± 0.6	36 ± 1.0
	-	37 ± 0.6	---

Note: Total monovalent ions equalled 50 mM. Mg^{2+} concentration was 10 mM. Only Na^+- and K^+-free ATP was used in this study. Each value is an average of 3-4 experiments followed by the standard deviation.

from 13% to 20% additional stimulation over Mg^{2+} alone.

In the absence of Mg^{2+}, Na^+ alone at 50 mM stimulated the phosphatase activity by 40%, and K^+ alone at the same concentration by 48%, in the fresh tissue preparations. The various Na^+/K^+ ratios gave amounts of stimulation very similar to Na^+ alone or K^+ alone. The phosphatase activity in the frozen tissue preparations was stimulated 50% by the addition of various ratios of Na^+/K^+.

Effect of DNP on the Phosphatase Activity

In mitochondria [97], chloroplasts [98], bacteria [122], and intact ascites tumor cells [123], DNP stimulates ATPase activity. In fresh HCl-treated scutellum slices, DNP increased the phosphatase activity at pH's below 5 (Fig. 10). At pH 3.8, DNP caused a fourfold increase in phosphatase activity. In contrast to the results with fresh tissue, DNP had no effect on phosphatase activity in frozen tisssue. With fresh H_2O- or HCl-treated scutellum slices, DNP also causes a proton influx and sucrose efflux at pH's below 5 [99,103]. Although the pH of the bathing solution affects the amount of DNP that enters the cells [103], it has a much greater effect on DNP-induced proton influx [103] and sucrose efflux [99], and on DNP stimulation of the phosphatase activity (Fig. 10).

Inhibitors

Several enzyme inhibitors, some specific for ATPase, were tested with fresh and frozen acid-treated preparations (Table 7). In most cases, responses of fresh and frozen preparations to the added inhibitors were quite similar.

Figure 10. Effect of DNP on phosphatase activity of fresh scutellum slices. Fresh and frozen HCl-treated slices were placed in a reaction vessel with or without 5×10^{-4} M DNP, and with 3 mM ATP, 20 mM $MgCl_2$ and 50 mM MES (pH 3.5-6.0), and run at 30° for 60 min.

In the absence of substrate, DNP caused a small amount (7 µmol/g) of Pi efflux, but the data reflect a corrected value. Each point is an average of 3-4 experiments.

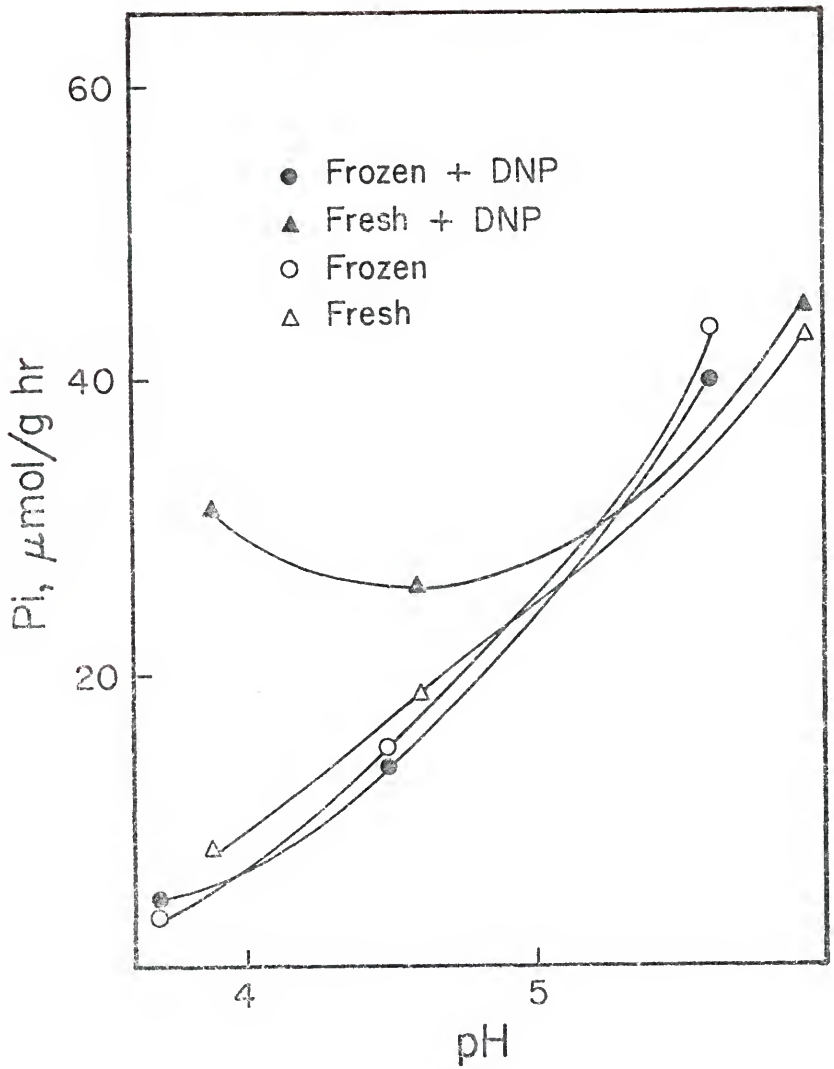

60
40
20
Pi, μmol/g hr
Frozen + DNP
Fresh + DNP
Frozen
Fresh
4
5
pH

Table 7. Inhibitors

		% Inhibition	
		Fresh	Frozen
Oligomycin	1 µg/ml	0	0
	10 µg/ml	0	0
DCCD	10 µM	17	20
	100 µM	9	10
	1 mM	4	7
EDCD	200 µM	11	4
	5 mM	29	22
	10 mM	33	30
NEM	10 µM	7	0
PCMB	0.5 mM	10	0
PCMBS	0.1 mM	9	2
	1.0 mM	14	9
	2.5 mM	18	13
	5.0 mM	32	---
Na Azide	5 mM	4	2

Note: Fresh and frozen HCl-treated slices were placed in a vessel with 3 mM ATP, 20 mM $MgCl_2$, 50 mM MES pH 6.5 and various concentrations of inhibitors. Since oligomycin and DCCD have low water solubilities, they were made up in 95% EtOH. The controls received equal quantities of 95% EtOH. Since the DCCD, at higher concentrations, precipitated when added to the bathing solution, the true concentration of DCCD in these cases was not known. The data are presented as a % of the controls. The reaction was run at 30° for 60 min.

Although DCCD is a specific and potent inhibitor of some plant and animal ATPases, it did not greatly inhibit the phosphatases of the scutellum preparations. The maximum inhibition by DCCD was only 20%. A more water-soluble analogue of DCCD (EDCD) caused a 30% to 33% inhibition, but only at relatively high concentrations (10 mM). A 5 mM concentration of PCMBS also gave 32% inhibition. Both PCMB and sodium azide gave little or no inhibition, and oligomycin did not inhibit the phosphatase.

Cytological Studies

To establish the localization of the phosphatase reaction within the cell, a cytological study was done. Fresh HCl-treated slices were fixed in glutaraldehyde and then placed in a reaction mixture containing buffered ATP, Mg^{2+} and lead nitrate.

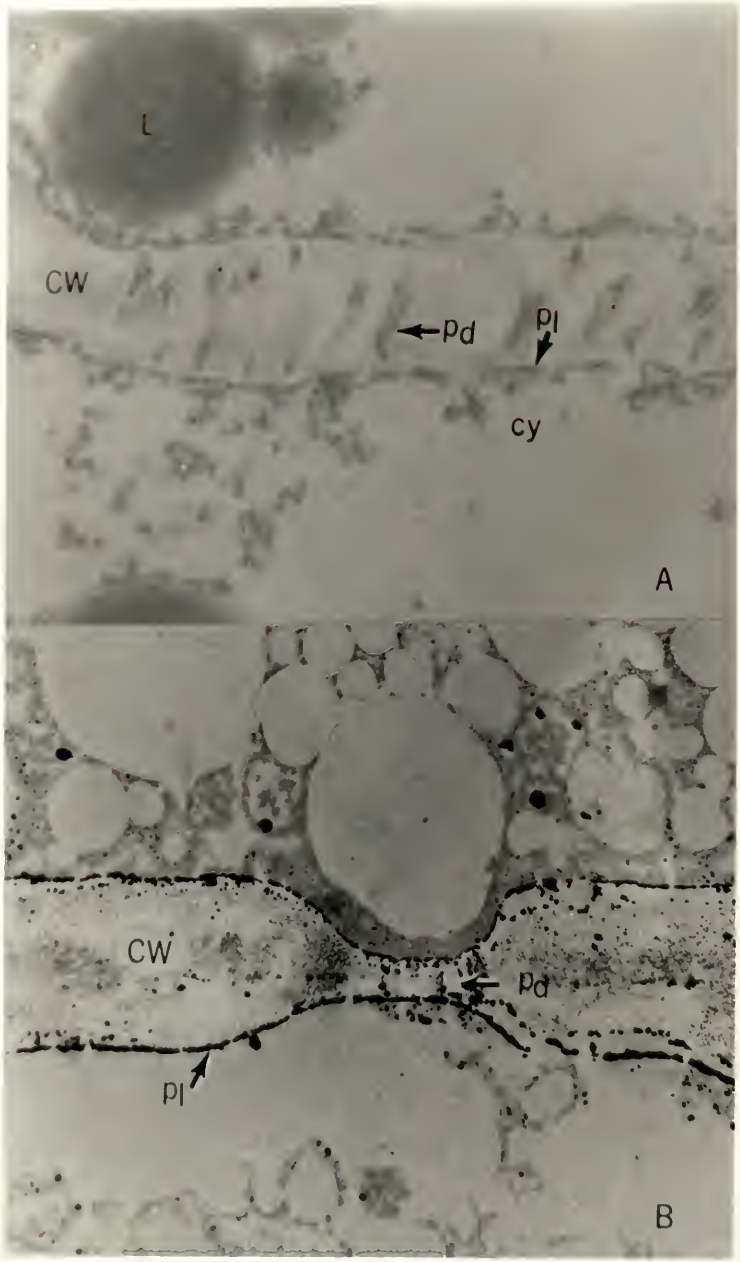
A 1% glutaraldehyde pretreatment for 30 min inhibited the phosphatase activity 56%. When glutaraldehyde treatment was followed by a 1% lead nitrate treatment for 30 min, the enzyme activity was inhibited 72%. Thus, there remained sufficient enzyme activity for a cytological localization study.

Precipitates of lead phosphate were deposited at the site of the ATPase activity (Fig. 11). The activity was localized on the plasmalemma. There were a few scattered lead phosphate deposits in the cytoplasm, but they were not associated with any particular organelle. The control without ATP showed no such localization.

Figure 11. Electron micrograph of ATPase localization in fresh scutellum slices.

A. Control, without ATP, showing plasmalemma (pl), cytoplasm (cy), plasmodesma (pd), lipid (L), and cell wall (CW).
X 20,400

B. Enzyme localization, showing lead phosphate on the plasmalemma (pl) and along the plasmodesma (pd).
X 18,000


L
CW
pd
pl
cy
A
CW
pd
pl
B

Phosphatase localization in the frozen slices was not attempted, because freezing and thawing releases the vacuolar contents, and disrupts other organelles (Fig. 3), causing the appearance of electron-dense granules throughout the cell. Presumably, these granules are Mg and K salts of phytic acid [119]; they resemble the lead phosphate deposits. Such vacuolar interference would have made localization studies in the frozen tissue extremely difficult.

Mitochondrial ATPase

In the frozen-thawed slices, a possible source of phosphatase activity may be the mitochondrial ATPase.

Mitochondrial preparations from fresh maize scutella were tested for ATPase activity with the same concentrations of Mg^{2+} and ATP that were used in whole-tissue experiments. The ATPase activity at pH 8.5 was 40% greater than at pH 6.5. This is in agreement with pH optima for mitochondrial ATPases [11].

Equivalent aliquots from the same initial mitochondrial preparations were frozen for 48 hours at -5°, thawed, and washed with 0.01N HCl for 30 min. The mitochondrial preparation was then rinsed with buffer and tested for ATPase activity at pH 6.5 and 8.5. There was no phosphatase activity at either pH.

DISCUSSION

Acid phosphatases (E.C. 3.1.3.2) and alkaline phosphatases (E.C. 3.1.3.1) have wide specificity and are not stimulated by metal ions [13,124]. By contrast, ATPases (E.C. 3.6.1.5) show a more narrow specificity, primarily for nucleoside 5'-triphosphates [124], and are activated by certain cations, usually Mg^{2+} and Ca^{2+}. Some ATPases are additionally stimulated by Na^{+} and K^{+}.

The results of this study strongly suggest that the phosphatase activity observed in the HCl-treated preparations of both fresh and frozen scutellum slices is a plasmalemma-bound ATPase. Evidence in support of this conclusion is summarized below under four headings:

Fresh scutellum slices

Frozen scutellum slices

Effect of DNP

Enzyme localization

Fresh Scutellum Slices

1. Fresh HCl-treated slices have a phosphatase activity with a sharp pH optimum at 6.5 (Fig. 1). HCl treatment causes considerable loss of phosphatase activity below this optimum pH, but very little loss in activity at pH 6.5. This is similar to the pH optima for ATPases from plasmalemma fractions of various plant sources [10,11,39,40,50,51].

2. The addition of Mg^{2+} stimulated the phosphatase activity in both the H_2O- and HCl-treated slices. With added Mg^{2+}, the HCl-treated preparations showed nearly identical activity at pH 6.5 as the H_2O-treated preparations (Fig. 1).

3. Adding Ca^{2+} was as effective as adding Mg^{2+}, and the enzyme could, perhaps, be called a Mg^{2+}-Ca^{2+} ATPase. Other divalent cations were also effective stimulators of the enzyme (Fig. 9). With Mn^{2+}, the enzyme was maximally stimulated at 5 mM, then the stimulatory effect decreased sharply. This is similar to the results reported by Jolliot et al. [40] on potato tuber plasmalemma ATPase. They found the greatest stimulation with Mn^{2+} occurred at 2 mM, and dropped sharply as the concentration of Mn^{2+} increased. With Mg^{2+} and Ca^{2+}, peak stimulation was obtained at 4 mM and decreased with increasing concentration, but the drop was more gradual. At 20 mM, Mn^{2+} gave only 50% of the stimulation of Mg^{2+} or Ca^{2+}.

4. The monovalent cations Na^+ and K^+ in the presence of Mg^{2+} gave a small additional stimulation, but in the absence of Mg^{2+} both Na^+ and K^+ gave 40% to 50% stimulation. Leonard and Hodges [51] and Balke and Hodges [49] reported only slight stimulation of the basal ATPase activity of oat root plasmalemma by Na^+ or K^+ in the absence of Mg^{2+}. However, bean cotyledon plasmalemma ATPase was reported to be stimulated by Na^+ or K^+ to a greater amount in the absence of added Mg^{2+} than in its presence [6].

5. The scutellum phosphatase showed a clear preference for ATP and CTP over the other substrates tested. The

substrate PNPP, which is commonly used to detect acid and alkaline phosphatases [88], gave low activity with the scutellum preparations. Although UTP gave 73% to 83% of the activity of ATP, the diphosphate, UDP, gave only 5% to 7%. However, ADP gave 67% to 77% of the activity of ATP (Table 4). The high activity obtained with ADP is similar to results obtained with other isolated plant plasmalemma preparations [7,40]. It is possible that the high activity with ADP may be a result of a nucleoside diphosphatase. This enzyme has been reported [125] to hydrolyze nucleoside diphosphates, but not tri- or monophosphates. In the maize scutellum slices, however, very low activity was observed with UDP. The Vmax (Fig. 7) could indicate that ADP is being acted on by the same enzyme which is acting on ATP. This relatively high specificity for ADP has been reported with other plant plasmalemma ATPases [7,40,47].

6. The K_m for the phosphatase from fresh and frozen HCl-treated slices was 0.65 with ATP as the substrate and 5.0 with ADP as the substrate (Fig. 7). The K_m value for ATP is similar to other published values for higher plant plasmalemma ATPases [49,51,124].

Frozen Scutellum Slices

The results indicate that the enzyme activity observed in the frozen-thawed, HCl-treated scutellum slices is the same as that observed with fresh HCl-treated slices.

1. The pH optimum (6.5) of the frozen HCl-treated preparation was identical to that of the fresh slice preparation

(Figs. 1, 2). In the absence of Mg^{2+}, the frozen HCl-treated slices gave very low activity over a pH range from 4.4 to 7.0 (Fig. 2). Acid treatment of the frozen slices decreased the phosphatase activity to 11 μmol/g hr without added Mg^{2+} at pH 6.5. Addition of Mg^{2+} resulted in a 364% rise in phosphatase activity (Table 1) at pH 6.5. This level is comparable to that obtained with fresh HCl-treated slices with added Mg^{2+} at pH 6.5.

2. The stoichiometry, kinetics, monovalent and divalent stimulations, effects of inhibitors, and substrate specificity of the phosphatase in the HCl-treated frozen preparations were similar to or identical with those of the HCl-treated fresh preparations.

Effect of DNP

The addition of DNP to fresh HCl-treated preparations stimulated the rate of ATP hydrolysis. In contrast, DNP had no effect on the phosphatase activity in frozen tissue (Fig. 10). It seems that the DNP effect can only be seen if the plasmalemma is intact either as whole cells, organelles or sealed vesicles, thus being relatively impermeable to H^+ [97,123,126,127]. Adding DNP to reaction vessels containing intact ascites tumor cells stimulated the ATPase activity by 177% [123]. Mitchell and Moyle [97] have proposed that in mitochondria, DNP acts as an uncoupler of ATPase, by increasing the permeability of the membrane to protons [97]. When the ATPase was purified from rat liver mitochondria, Lambeth and Hardy [104] reported that DNP decreased the activity of this purified enzyme.

Since DNP is a lipid-soluble weak acid with a pK of 4.0, the amount that enters the cells increases as the pH is lowered. However, previous studies with the maize scutellum have shown that below pH 5, DNP induced a proton influx, and that the bathing solution pH had a much greater effect on proton influx than it did on DNP content in the tissue [103]. The DNP was reported to cause an efflux of sucrose from maize scutellum slices and an influx of H^+ across the plasmalemma, and both fluxes increased as the pH was lowered [99]. The effect of DNP on sucrose efflux and the stimulation of the ATPase in the maize scutellum slices may both result from the proton influx and the resulting loss of membrane potential.

Enzyme Localization

The following studies support the idea that the ATPase is located in the plasmalemma.

1. The cytological localization (Fig. 11) clearly showed that the areas of ATPase activity were localized on the plasmalemma. There are several reports of similar results obtained with phloem sieve cells [23] and sieve tube members [25,26].

2. In both the fresh and the frozen tissue preparations, HCl treatment for 1 hr did not destroy the enzyme activity. This rigorous treatment should denature most proteins. However, the plasmalemma proteins of the scutellum cells apparently are not affected, since after HCl treatment rates of sucrose and maltose uptake [118], sucrose synthesis from fructose [99], and respiration [99] are not affected.

The levels of enzyme activity at pH 6.5 in fresh and frozen tissue preparations were nearly identical (Figs. 1, 2). However, HCl treatment decreased the activity in the pH 4-5 range (Fig. 1), presumably by destroying the acid phosphatases.

3. In studies with Pi leakage in both fresh and frozen tissue preparations, after 1 hr less than 2 μmol of Pi leaked into the bathing solution (Table 2). In the fresh tissue, there was no uptake of Pi from the bathing solution. It is unlikely, therefore, that the ATP entered the cell, was hydrolyzed and the Pi leaked out to the bathing solution. The results indicate that the reaction occurred in or on the cell surface (membrane), the ATP was hydrolyzed from the outside of the cell, and the Pi remained in the bathing solution.

The stoichiometric studies confirm that the substrate and products remain in the bathing solution (Table 5). This enzyme seems to be capable of recognizing substrate coming from outside the cell. Similar results were reported with intact ascites tumor cells [123]. These tumor cells hydrolyzed ATP to produce AMP and Pi, and both products were found in the bathing solution. Under normal *in vivo* conditions, the ATPase would only encounter the substrate from the inside.

Nurminen *et al.* [46] reported various enzymes on the cell wall and plasmalemma of yeast. When the wall was enzymatically digested and the released enzymes assayed, saccharases and acid phosphatases were released, but the Mg^{2+} ATPase was not. This was interpreted to mean that this enzyme was bound to the plasma membrane.

ATPase Models

Most ATPases are considered to be composed of two functional parts, each with several globular proteins precisely arranged in some specific functional quaternary structure. The portion of the enzyme which is associated with the actual synthesis or hydrolysis of ATP is referred to as the F_1 portion, and the portion thought to be involved in proton transport as the F_o portion [74,97]. The F_1 portion of the enzyme is attached to the F_o portion, and the latter is tightly embedded in the lipid bilayer, whereas the former protrudes from the membrane. Thus, most membrane-bound ATPases are thought to be vectorially oriented. Models of ATPases are often drawn with the substrate recognition portion (F_1) sticking into the cytoplasm, matrix of the mitochondria, or stroma of the chloroplast [74,97].

Much of the information upon which ATPase models are based comes from inhibitor studies. The compound DCCD is an inhibitor of some ATPases from plant and animal sources, acting on the membrane-bound portion (F_o) of the ATPase but not on the portion (F_1) that is known to catalyze the hydrolysis of ATP [83,109,110,114]. Intact ATPases attached to the membrane are inhibited by DCCD in many instances; however, the purified enzyme which contains only the F_1 portion is not susceptible to inhibition by DCCD [107,114).

The maize scutellum plasmalemma ATPase was not inhibited by DCCD, although the membrane was intact. ATPase from maize root plasmalemma has been reported to be inhibited by 50%

when 50 to 100 μM DCCD was present [7]. This was not as large an inhibition as was seen with other ATPases where 90% to 95% inhibition was reported [109,110]. There are reports of bacterial ATPases which seem to be resistant to DCCD [110] and an ATPase on the tonoplast of Hippeastrum [111] which was not inhibited by DCCD.

It is possible that the ATPase on the maize scutellum slices is placed in the membrane in such a way that it is inaccessible to the added inhibitors. The water-soluble analogue of DCCD (EDCD) did inhibit the enzyme activity by 33%, but at a relatively high (10 mM) concentration.

At this time, the vectorial model described is considered to be the best model for the mitochondrial and chloroplast ATPase [74,97]. Not as much is known about the ATPase in the plasmalemma of higher plants. Most of the information comes from work with partially purified plant plasma membranes, and the structure of the enzyme is not known [40,51, 52,55].

The sarcoplasmic reticulum, Na^+-K^+ ATPase has been shown to span the membrane, and the Ca^{2+} ATPase from the same source is thought to be predominantly membrane-bound with a small portion sticking out into the cytoplasm [97]. Both are thought to be functionally vectorial. Most ATPases are relatively large proteins and could easily span the lipid bilayer [74].

The Na^+-K^+ ATPase from the microsomal fraction of canine renal medulla [75] is thought to be composed of two subunits,

the large and small polypeptides. From studies with cardiac glycoside binding experiments, it was found that the larger of the two subunits spans the membrane, and is seen from the outside as well as the inside of the cell. This large polypeptide is capable of binding with the cardiac glycoside from the outside, and is also responsible for the hydrolysis of ATP, presumably from the inside.

Bacterial membranes obtained by osmotic lysis [16] yield nearly all right-side-out vesicles. Such vesicles showed a large ATPase activity when the substrate was introduced from the outside. The high rate of ATP hydrolysis was not expected, since the model presumed a vectorial orientation with the substrate recognition portion of the enzyme on the inside. This model is supported by results using spheroplasts of E. coli, indicating that the ATPase was localized on the inner part of the cytoplasmic membrane [66].

Penefsky [95] reported that an analogue of ATP (AMP-PNP) was a potent competitive inhibitor of mitochondrial ATPase, but did not inhibit oxidative phosporylation. He concluded that the hydrolytic site of the enzyme was not identical to the synthetic site.

It is possible that plant plasmalemma ATPases are different in orientation to mitochondrial or bacterial ATPases. The hydrolytic site may respond to substrate levels, as well as other factors such as ion concentrations and pH, and undergo some conformational changes accordingly. Stein et al. [22] presented a model to explain the transport of ATPase-

mediated Na^+ and K^+. They suggested a tetrameric model which depends on conformational changes to bring about transport.

In the maize scutellum tissue studies, the results with fresh and frozen tissue preparations are very similar. It appears, then, that the scutellum plasmalemma ATPase is tightly bound to the lipid bilayer, and is capable of substrate recognition from either side of the plasmalemma.

There is evidence for the existence of a proton pump in the maize scutellum plasmalemma [103]. The ATPase on the plasmalemma may be acting as the pump. The ATPase could set up a proton gradient which would drive cation and sugar transport [10,11,12,13,14,99] across the membrane.

EXPERIMENTAL

Preparation of the Scutellum Slices

Corn grains (*Zea mays* L. c.v. McNair 508) were soaked in running water for 24 hr, then placed on moist paper towels and grown in the dark at 24°-25° for 72 hr. The scutella were excised, and cut transversely with a razor blade into slices of 0.5 mm or less in thickness.

The scutellum slices were washed in deionized water several times, until the wash water remained clear. They were then blotted dry on filter paper, and weighed into groups of 0.5 g each (80-90 slices). Scutellum slices to be frozen were similarly prepared, weighed, and placed in the freezer at -4° to -5°. The frozen slices could be stored at least 27 days with no loss of phosphatase activity.

The thickness of the scutellum slices, in one sampling, was measured under a dissecting scope fitted with an ocular micrometer. A single slice, so measured, was then sectioned and observed under a light microscope, and the number of cells that made up the thickness was counted. The cells of the scutellum are nearly uniform, and each cell measured approximately 50 μm in diameter. The thickness of a random number of slices was then measured. The results showed that the slices were made up of from 3 to 7 cells in thickness. There were many intercellular spaces.

Unless otherwise noted, all incubations were carried out with 0.5 g of scutellum slices in 10 ml bathing solution at 30° in a "Gyrotory" water bath (New Brunswick Scientific Co., New Brunswick, N. J.).

HCl- or H_2O-Treatment

Slices (0.5 g) were incubated in 0.01N HCl at 30° for 1 hr. The bathing solution was replaced with fresh HCl after 30 min. The slices were then rinsed twice with distilled H_2O.

Groups of slices to be H_2O-treated were similarly handled, with glass-distilled H_2O in place of HCl.

Analysis of Pi

Pi was measured as the product of hydrolysis of the various substrates. The modified Fiske-Sabbarow method of Bartlett was used [128].

ATP Analysis

The method described by Lamprecht and Trautschold was followed for the ATP determinations [129].

ADP and AMP Analysis

The method described by Adam [130] was followed for ADP and AMP determinations.

Preparation of Na^+- and K^+-Free ATP

To a certain volume of ATP (∿ 50 mM) was added 4 g of Dowex-50. After stirring for 1 min the solution was filtered and neutralized with Tris-base. The concentration of ATP was then determined spectrophotometrically [129]. The final concentration was determined to be 44.7 mM.

Mitochondrial Preparation

Mitochondria were isolated from fresh, whole scutella according to the method of Hanson et al. [131]. The purity of the preparation was checked by electron microscopy. Many intact as well as partially broken mitochondria were seen.

Total Lipids

Total lipids were determined by a method outlined in the AOAC Method of Analysis [132]. One g of tissue was used in this study.

Total Nitrogen and Phosphorus

Scutellum slices (0.5 g) were digested in H_2SO_4. The digest was diluted to 35 ml with H_2O and aliquots were taken for total nitrogen, determined by Nesselerization [133], and for total phosphorus, determined by the method of Bartlett [128].

ATP Product Localization

The method of Wachstein and Meisel was modified for the ATPase localization study [134]. Fresh scutellum slices were HCl-treated and then incubated in buffered 1% glutaraldehyde (50 mM MES pH 6.5) for 30 min. The slices were then rinsed twice with buffer and treated with:

1. Lead nitrate (1%) + Mg^{2+} (20 mM) + buffer (50 mM MES pH 6.5) + ATP (3 mM);
2. Controls consisted of: Mg^{2+} alone; lead nitrate alone; buffer alone; and lead nitrate + Mg^{2+} + buffer.

The reactions were allowed to proceed for 30 min at 30°. The

slices were then rinsed with buffer and fixed in 4% buffered OsO_4 for 30 min. They were then dehydrated in a series of ethanol solutions, and finally in 100% acetone. The slices were embedded in an Epon-Araldite plastic mixture, sectioned, and observed under a Hitachi HU-11C electron microscope.

Phosphotungstic-Chromic Oxide Stain for Plasmalemma of Frozen Scutellum Slices

Frozen slices were prepared for EM as described above, without the incubation in lead nitrate and ATP. Following dehydration steps, the frozen slices were embedded as before and sectioned. The specific plant plasmalemma stain with phosphotungstic acid and chromic oxide stain was carried out as described by Roland et al. [120].

Biochemicals

All biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. The adenosine 5'-triphosphate, Sigma grade II was used throughout this study [135].

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BIOGRAPHICAL SKETCH

Heijia Lee Wheeler was born on June 6, 1942, in Seoul, Korea; however, her father did not officially record her birth until 20 days later, so that the official date of birth is listed as June 26, 1942.

She received her B.A. in botany from The George Washington University in June of 1964. She then received her M.S. in botany from The Pennsylvania State University in June of 1966. She came to Gainesville, Florida, in July of that same year, and worked for a short time at the J. Hillis Miller Health Center as a research assistant. She then accepted a faculty position at Santa Fe Community College in January of 1967. She took a short leave from the college to finish the work on the degree of Doctor of Philosophy, and has subsequently returned on a full-time basis.

Heijia Lee Wheeler is married to Willis B. Wheeler and is the mother of two lovely children.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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