Reconstitution and properties of corn membrane ATPases

Daniel William Karl
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RECONSTITUTION AND PROPERTIES OF CORN MEMBRANE ATPASES

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Reconstitution and properties of corn membrane ATPases

by

Daniel William Karl

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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ABBREVIATIONS

$A_{254}$ Absorbance at 254 nm (Absorbance at other wavelengths is abbreviated similarly.)

ADP adenosine 5'-diphosphate

AMP adenosine 5'-phosphate

ANS 8-analino-1-naphthalenesulfonic acid

ATP adenosine 5'-triphosphate

$ATP_f$ uncomplexed ATP, regardless of its state of protonation

ATPase adenosine 5'-triphosphate phosphohydrolase

Bis-Tris N,N-bis(2-hydroxyethyl)-2-amino-2-hydroxymethyl-1,3-propanediol

CCCP carbonylcyanide m-chlorophenylhydrazone

DABS 4-diazobenzenesulfonic acid

$^{125}$I-DABS radioiodinated DABS

DCCD dicyclohexylcarbodiimide

DES diethylstilbestrol

di-0-C$_3$-(5) dipropyloxadicarbocyanine

DPH diphenylhexatriene

EDTA ethylenedinitrilotetraacetic acid

$Na_2$EDTA disodium salt of EDTA

FCCP carbonylcyanide-p-trifluoromethoxyphenylhydrazone

$g_{av}$ relative centrifugal force at the midpoint of a centrifuge tube

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
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<tr>
<td>$K_a$</td>
<td>formation constant</td>
</tr>
<tr>
<td>$K_{av}$</td>
<td>partition coefficient in gel filtration $\frac{(V_e-V_o)}{(V_t-V_o)}$</td>
</tr>
<tr>
<td>$K_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>MES</td>
<td>2(4-morpholino)ethanesulfonic acid</td>
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<tr>
<td>$Mg_f$</td>
<td>unchelated Mg$^{2+}$</td>
</tr>
<tr>
<td>NaDdso$_4$</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>octyl glucoside</td>
<td>octyl-$\beta$-D-glucopyranoside</td>
</tr>
<tr>
<td>$P_i$</td>
<td>inorganic orthophosphate, regardless of its state of protonation</td>
</tr>
<tr>
<td>Phosphate</td>
<td>phosphatase</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>$pMg$</td>
<td>$-\log (Mg_f)$</td>
</tr>
<tr>
<td>Polyclar AT</td>
<td>tradename for crosslinked polyvinylpyrrolidone</td>
</tr>
<tr>
<td>PTA-CrO$_3$</td>
<td>phosphotungstic acid - chromic acid staining reagent</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinylalcohol</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethyl-1,3-propanediol</td>
</tr>
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<td>$V_e$</td>
<td>elution volume</td>
</tr>
<tr>
<td>$V_o$</td>
<td>void volume: the elution volume of a solute completely excluded from a gel-filtration medium</td>
</tr>
<tr>
<td>$V_r$</td>
<td>elution volume of a low-molecular-weight solute which completely permeates a gel-filtration medium</td>
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<tr>
<td>$V_t$</td>
<td>total volume of a chromatographic bed</td>
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INTRODUCTION

The assimilation and transport of solutes is as important to the physiology of plants as to that of other complex organisms. Inorganic nutrients must be absorbed from the soil and delivered to cells throughout the plant. The products of photosynthesis must be transported to roots, storage tissues, and developing seeds. Moreover, higher plants use osmotic energy to control the opening of stomata, to move leaves (as in the retraction and extension of the leaves of *Mimosa* in response to touch), and quite possibly to drive long distance transport of solutes in the phloem. Understanding of the basic mechanisms of solute transport and accumulation would contribute toward a general understanding of plant physiology, and might be of immediate use in developing plants resistant to high salt environments or of plants highly efficient in assimilating scarce nutrients.

Although membrane transport has been studied by physiologists throughout this century (see for example Nystrom, 1973), progress in understanding membrane structure and function at the molecular level has come mostly in the last 10 or 15 years. This period has seen the development of biochemical techniques for solubilizing
and isolating membrane proteins without irreversible denaturation, for making artificial lipid membranes in planar or vesicular form, and for incorporating isolated membrane proteins into these model membranes. Simultaneously, the thermodynamic basis for the structure and stability of biological and model membranes has become much better understood (Tanford, 1973; Singer and Nicolson, 1972), and this understanding has contributed to the refinement of the techniques of membrane solubilization and reassembly (Helenius and Simons, 1975; Shamoo and Tivol, 1980).

Once a membrane protein has been isolated and reinserted into a lipid bilayer membrane, its properties can be determined separately from those of the rest of the membrane from which it came. The influence of the lipid environment on its activities can be determined, and most notably, the presence of transmembrane transport activities can be determined. Complex processes may be dissected by determining what proteins or protein complexes must be present in the membrane to allow the process to occur. This approach of resolution and reconstitution was pioneered by Racker and his co-workers (Kagawa and Racker, 1971; Racker, 1975) in their study of mitochondrial oxidative phosphorylation.
The chemiosmotic theory of oxidative phosphorylation, formulated by Mitchell (1961, 1979), has been very influential in guiding the study of membrane transport systems. Besides its utility in describing oxidative phosphorylation, the theory has provided a general scheme in which the coupling of transport to metabolic energy can be discussed. The challenge of testing this theory has led to the development of several elegant and ingenious techniques for measuring transmembrane pH and potential gradients in vesicle suspensions without having to insert electrodes into the interior of the vesicle.

Membranes of animal cells and bacteria, and of mitochondria and chloroplasts, have been studied extensively at both the physiological and the biochemical level. Some systems, including several ATPases, found in these membranes are understood in considerable detail. Although less is known about the cytoplasmic membranes of plants, the plasma membrane and the tonoplast are believed to be the sites of several systems which take up solutes and accumulate them against a concentration gradient. These systems are dependent on metabolic energy. There is good evidence that the energy for active transport in plants is supplied by ATP, presumably by the action of one or more ATPases. (General aspects of solute transport in plants are discussed by Luttge and Higinbotham, 1979;
I will discuss ATPases from plants and other organisms in more detail below.) Studies of plant ATPases will, it is hoped, clarify the processes of solute uptake and transport in plants, and aid in the design of physiological experiments. Conversely, physiological knowledge is an invaluable guide to biochemical studies of membrane transport processes.

I will discuss here experiments with K$^+$ ATPases isolated from corn roots and shoots, their relation to other ATPases, their reattachment to model membranes, and their relation to ion transport.
LITERATURE REVIEW

Kinetics of Ion Uptake

Systems 1 and 2

The rate of ion uptake into roots of various plants has been studied extensively as a function of the external concentration of the ion. Two systems generally can be distinguished: System 1 predominates at concentrations below 1 mM, while system 2 operates above 1 mM. In studies of $K^+$ uptake, system 1 is found to be highly specific for $K^+$; $Na^+$ and other cations do not compete with $K^+$, even when present at much higher concentrations. The rate of $K^+$ uptake by system 1 is independent of the anion present, and shows classical Michaelis-Menten saturation kinetics. The $K_m$ for $K^+$ is about 0.04 mM in corn (Luttge and Laties, 1966). At higher concentrations, the rate of uptake increases beyond the maximal rate of system 1; this additional contribution to the uptake rate is system 2. The $K^+$ uptake rate at high concentrations is dependent on the counterion, being much greater from KCl solutions than from $K_2SO_4$ solutions. $Na^+$ and other monovalent cations compete with $K^+$ for uptake by system 2, and are themselves taken up. The dependence of uptake rate on salt concentration does not obey the Michaelis-Menten equation, but instead suggests anticooperativity or the presence of
several cation uptake sites with decreasing affinity (Epstein, 1972). In this concentration range, K\(^+\) uptake appears to be driven by the membrane potential (see below). The dual uptake isotherm has been observed for several other solutes besides K\(^+\) (Epstein, 1972; Luttge and Higinbotham, 1979).

**Transport sites**

The nature and location of the K\(^+\) transport sites which are responsible for the dual isotherm of uptake and for the anticooperativity of its second phase (system 2) has been the subject of much controversy. The principal suggestions are those of Welch and Epstein (1969) that the two systems represent two transport mechanisms acting in parallel in the plasma membrane, of Nissen (1974) that only one carrier is involved but that its affinity for K\(^+\) is decreased by increased external K\(^+\) concentrations, and of Tori and Laties (1966), that the two systems act in series, one being located in the plasmalemma and the other in the tonoplast. The suggestion of Tori and Laties may appear implausible, but it is supported by a well-thought-out interpretation of the time dependence of ion uptake. The relative magnitudes of the two systems appear to depend on the length of the uptake period; system 2 is more pronounced in experiments involving long uptake.
periods and long rinses (Cram, 1969; Laties, 1964; Cram and Laties, 1971). Since ions taken up into the vacuole presumably must first pass through the cytoplasm, short uptake and rinse times emphasize uptake into the cytoplasm. Longer times emphasize uptake into the vacuole because, in most plant cells, the vacuole contains most of the intracellular volume.

The Tori-Laties hypothesis implies that at high salt concentration there is a change in the rate-limiting step of ion uptake. An increase in the nonspecific permeability of the plasma membrane to ions has been postulated (Tori and Laties, 1966) but this postulate has been vigorously criticized (Epstein, 1976).

Energetics of Ion Transport

Coupling of metabolism and transport

The terms active transport and facilitated diffusion require clarification in a discussion of the energetics of membrane transport processes involving ions. Active transport connotes transport coupled to metabolic energy and accumulation of a solute against a concentration gradient, while facilitated diffusion indicates a carrier-mediated process which tends to bring the solute into equilibrium across the membrane. When charged solutes are involved, or when the flux of one solute is coupled
to the flux of another by a carrier specific for both, this simple distinction does not hold; various degrees of coupling between transport and metabolism can exist. Mitchell (1970, 1979) and Harold and Altendorf (1974) have discussed such coupling in general, and Hanson (1978) has applied the ideas and terminology to the uptake and transport of ions by roots.

Four situations may be distinguished in discussing the coupling of solute uptake or efflux to metabolic energy, and certain predictions can be made. When an uncharged solute crosses a membrane by simple diffusion or by facilitated diffusion (uniport), its movement is coupled to the gradient of its concentration (or activity) across the membrane, but not to metabolic energy. Uniport of an ion, on the other hand, is coupled to the membrane potential as well as to its concentration gradient. Thus, it is coupled to metabolic energy to the extent that maintenance of the membrane potential requires expenditure of energy; ion movements which are basically passive may still be affected by anoxia or inhibitors of energy metabolism. Obligatory uptake of one solute along with or in exchange for another solute (symport or antiport) couples the fluxes of the two solutes. If the symport or antiport results in net charge movement across the membrane, the fluxes are also coupled to the membrane potential.
Symport and antiport mechanisms can effectively couple the flux of a solute to metabolic energy when the other solute (transported by the same transporter) is actively transported by some other mechanism. Finally, a solute may be moved across the membrane by an enzyme which couples the solute movement to a chemical reaction, such as hydrolysis of ATP.

If active transport is defined as the accumulation of a solute against its electrochemical potential gradient, it comprises transport directly coupled to chemical reaction and symport or antiport with another actively transported solute. Transport dependent on metabolism (and thus inhibited by poisons, anoxia, etc.) is a more inclusive category.

In Mitchell's model (which he applies to cells as well as to mitochondria and chloroplasts), $H^+$ is directly extruded from the cell by an ATPase, creating a membrane potential and a pH gradient. Other solutes may then be transported by uniports or by symport or antiport with $H^+$. Energy for transport may be supplied by the membrane potential, the pH gradient, or both, and the direction of transport is determined by the nature of the carrier and the direction of the pH and potential gradients. Such antiports have been identified in mitochondria and in bacteria (see Brey et al., 1978 and Wiskich, 1977).
Active transport in plants

Sodium and chloride In general, plants actively accumulate Cl⁻ and actively extrude Na⁺ (Luttge and Higenbotham, 1979). It has been postulated that active uptake of Cl⁻, HPO₄²⁻, and other anions occurs by antiport with OH⁻ or, equivalently, by symport with H⁺. Lin and Hanson (1974) provide evidence that this is true of phosphate uptake. Barley roots actively extrude Na⁺ at the plasmalemma by a process which is stimulated by K⁺ (Jeschke and Stelter, 1973). The extrusion appears to involve exchange on Na⁺ and H⁺ (Ratner and Jacoby, 1976). This process is not detectable in corn roots (Jacoby and Ratner, 1974). There is evidence that some active Na extrusion process must occur in corn. Leigh and Wyn-Jones (1973) found that ²²Na⁺ influx was measurable during salt loading of corn roots (in the presence of CaCl₂, NaCl, and KCl) and the rate of Na⁺ influx would lead to a doubling of the Na⁺ content of the roots in 18 h. Since the Na⁺ content of the roots did not increase (although the electrochemical potential gradient of Na⁺ favored influx), active extrusion was presumed to compensate for the Na⁺ influx.

Accumulation in the vacuole Some plants, including barley, accumulate Na in the vacuole as well as extruding it at the plasmalemma. This accumulation may occur by a
Na⁺/H⁺ antiport. The internal pH of plant cell vacuoles is low, but the pH rises on isolation of intact vacuoles, even though they retain their contents (Lin et al., 1977a), suggesting that the low internal pH is maintained by metabolic energy. The vacuole is believed to be about 20 mV positive, relative to the cytoplasm (Lin et al., 1977b). Other ions, including Cl⁻ and K⁺, are commonly accumulated in the vacuole (Luttge and Higinbotham, 1979). The physiology of vacuoles from fungi and higher plants has been reviewed by Matile (1978).

**H⁺/K⁺ antiport** Lin and Hanson (1976) found that dithioerythritol and other thiol reducing reagents activate a K⁺ extrusion in corn roots which appears to be coupled to H⁺ uptake. Above pH6 (with fresh tissue) this extrusion appeared as a reduction in K⁺ uptake. It was not clear whether the K⁺ and H⁺ fluxes were coupled by a single carrier (an antiporter), but valinomycin did not mimic the effect. Sze and Hodges (1977) studied net uptake of K⁺ and other monovalent cations into plasma membrane vesicles from oat roots. These vesicles took up cations by a saturable, passive process. Uptake of Cl⁻ was much less than the uptake of cations, so electroneutrality was presumed to be maintained by efflux of H⁺. A H⁺/K⁺ antiport has been identified and studied in *E. coli* (Brey et al., 1978). The physiological role of H⁺ coupled
$K^+$ efflux is not clear, although it may be a means of regulating the membrane potential (Lin and Hanson, 1974) or the intracellular pH (Brey et al., 1978).

Potassium accumulation $K^+$ is accumulated in all plant cells (Luttge and Higinbotham, 1979). The energetics of the uptake appear to depend on the external $K^+$ concentration. At low concentrations, uptake is active, but at higher concentrations it is passive, driven by the membrane potential (Cheeseman and Hanson, 1979a, b; Cheeseman et al., 1980; Pitman, 1976). Cheeseman, Hanson, and co-workers (Cheeseman and Hanson, 1979a, 1979b; Cheeseman et al., 1980) have compared $K^+$ uptake into corn roots and the cell potentials of corn root cortical cells as functions of external $K_2SO_4$ concentration. Assuming the transport of $SO_4^{2-}$ (the influence of its concentration on the membrane potential) to be negligible, they developed semi-empirical equations describing the influence of $K^+$ on the membrane potential. Their results show that $K^+$ is taken up from solutions containing more than about 0.5 mM $K^+$ by a passive process driven by the membrane potential. At lower concentrations, $K^+$ is taken up actively. Valinomycin hyperpolarizes the cortical cells of roots in a low-$K^+$ (0.2 mM KHPO$_4$, pH6) medium (Lin and Hanson, 1976). This shows that the ratio of $K^+$ activities (inside/outside) is greater than the equilibrium ratio which could be
maintained by the membrane potential. Additions of valinomycin (a $K^+$ ionophore which acts as a uniport) let $K^+$ ions escape; the resulting diffusion potential increases the membrane potential (negative inside) of the cortical cells. Part of the membrane potential is dependent on metabolic energy, and thus is dissipated under anoxia. The anoxia-sensitive component is sensitive to DCCD, DES, and oligomycin below 0.2 mM $K^+$, but not at higher $K^+$ concentrations. Cheeseman and coworkers believe that these agents all inhibit the $K^+Mg^{2+}$ ATPase. These three inhibitors also block active $H^+$ secretion by the roots. FCCP (an $H^+$ ionophore which uncouples oxidative phosphorylation) also stops active $K^+$ uptake and eliminates the active component of the membrane potential. Its effects are not, however, identical with those of anoxia. Differences in the dependence of the membrane potential on extracellular $K^+$ suggest that the cytoplasmic $K^+$ concentration decreases when FCCP is present, perhaps due to equilibration of $K^+$ with the vacuole.

ATP as the energy source for transport

It is believed that ATP provides the energy for active transport in plants, at least partly by driving the electrogenic extrusion of $H^+$ from the cell by a membrane ATPase (Poole, 1978; Marrè, 1979). Cation uptake is
accompanied by H\(^+\) extrusion when readily translocated anions such as Cl\(^-\) and NO\(_3^-\) are not available for uptake (Osmond, 1976; Smith and Raven, 1976). Inhibitors of H\(^+\) extrusion, such as DCCD, DES, and vanadate, are also inhibitors of a K\(^+\)Mg\(^{2+}\)ATPase found in plasma membrane preparations (Balke and Hodges, 1979; Cheeseman et al., 1980; Cocucci et al., 1980a, b). The rate of ion uptake is closely correlated with the level of ATP in the cell when ATP synthesis is reduced by inhibitors or anoxia. Restoration of ATP levels by fusion of protoplasts with ATP-loaded liposomes restores the ion uptake rate (Petraglia and Poole, 1980a, 1980b; Leonard and Mettler, 1977).

**K\(^+\)ATPase Activities of Plants**

Hodges (1976) discusses most of the earlier evidence that a K\(^+\)Mg\(^{2+}\)ATPase located on the plasma membrane is involved in ion uptake. One of the most striking early results was the close correlation found by Fisher and Hodges (1969) between K\(^+\) uptake rates of oat roots and the total K\(^+\)-stimulated ATPase found in membrane fractions of oat roots at various KCl concentrations. Both sets of data showed anticooperative kinetics when K\(^+\) was varied, and the K\(^+\)-stimulated ATPase activity was sufficient to account for the rate of K\(^+\) uptake by the roots. Hodges, Leonard, and coworkers extended this correlation
to other species, including corn and barley, although the agreement between transport and ATPase kinetics was not always as good as with oats (Fisher et al., 1970; Leonard and Hotchkiss, 1976).

Sze and Hodges (1977) compared the rate of uptake of several alkali metal cations with their effectiveness in stimulating the Mg\(^{2+}\) ATPase of oat root plasma membranes. They studied a range of cation concentrations, from 1 to 50 mM, and concluded that the specificity of the ATPase did not fully account for the specificity of ion uptake.

Sze (1980a) has studied the ATPase activity of membrane vesicles from tobacco callus cells. She selects sealed vesicles by flotation on a dextran layer, and tests for ion transport by measuring stimulation of ATPase activity by ionophores. Assaying in the presence of K\(^+\) and Mg\(^{2+}\), she finds that the ionophores CCCP, valinomycin, and nigericin stimulate ATPase activity. Nigericin (which can exchange K\(^+\) for H\(^+\)) and the combination of valinomycin and CCCP are more effective than CCCP or valinomycin alone, suggesting that the enzyme drives a K\(^+\)/H\(^+\) exchange. This would lead to development of concentration gradients which would oppose further transport and thus slow the ATPase. Dissipation of the concentration gradients by ionophore action would stimulate the ATPase in the same way that uncouplers (H\(^+\) ionophores) stimulate mitochondrial ATPase.
Sze and Churchill (1981) have also found a membrane potential generated in these vesicles by ATP hydrolysis.

**Location of the K\(^{+}\)Mg\(^{2+}\)ATPase**

The K\(^{+}\)Mg\(^{2+}\)ATPase has been found in a variety of plants, and is believed to be located on the plasmalemma. Earlier evidence for this belief was all derived directly or indirectly from the PTA-CrO\(_3\) stain for plasma membranes (Roland et al., 1972; Nagahashi et al., 1978), which is of questionable reliability (Quail, 1979). Recently, Perlin and Spanswick (1980) have shown that the K\(^{+}\)Mg\(^{2+}\)ATPase coincides in density gradients with labeled plasma membranes from corn protoplasts. The plasma membranes were labeled with the non-permeating reagent \(^{125}\)I-DABS before lysis of the protoplasts. The density, protein content, and lipid composition of the plant plasma membrane fraction are similar to properties of other eukaryote plasma membranes, including those of yeast (Kramer et al., 1978) and Neurospora (Scarborough, 1976). Thus, the assignment of the K\(^{+}\)Mg\(^{2+}\)ATPase to the plasma membrane is probably correct.

It is not clear, however, that the plasma membrane K\(^{+}\)Mg\(^{2+}\)ATPase is the only membrane ATPase in corn. Leigh et al. (1975) found two peaks of K\(^{+}\)ATPase from corn roots in sucrose-Ficoll density gradients. One peak was associated
with plasma membranes and the other with a less dense, smooth membrane fraction. In a later paper (Leigh and Wyn Jones, 1975), they attempted to correlate the activities on the two membranes with ion uptake into the cytoplasm and into the vacuole. They found such a correlation, but also found strong cross correlations between plasma membrane ATPase and uptake into the vacuole and between ATPase of the light membrane fraction and uptake into the cytoplasm. Leonard and VanDerWoude (1976) have criticized the finding of two $K^+$ATPase bands, saying that the use of Ficoll altered the centrifugation properties of the membranes. Given that the distribution of the membrane fragments in a density gradient depends on the gradient material, the question of which result is artifactual is moot; there is no reason to discard either result out of hand. That Ficoll would drastically alter the properties of only part of a homogeneous membrane preparation seems a little implausible.

Davis (1979) has discussed the literature describing a variety of reported plant ATPases with different properties. Hendricks (1977) and Davis (1979) have found that $K^+$ATPase in corn coleoptiles and three-day-old shoots is associated with at least two different membrane fractions, one of which may be the plasma membrane.
A magnesium-requiring Ca\(^{2+}\)ATPase has been detected in corn microsomes by Gross and Marme (1978), who showed ATP-driven Ca\(^{2+}\) accumulation in the microsomes.

There have been several reports of ATPase activity associated with membranes from isolated vacuoles from several plant species (Leigh and Walker, 1980; Doll et al., 1979; Lin et al., 1977a). Briskin and Leonard (1980) have disputed the results of Lin and Wagner. They find no ATPase on vacuole membranes from tobacco protoplasts. Part of the disagreement depends on the properties assumed for the ATPase, particularly its substrate specificity. The vacuole enzyme hydrolyzes several nucleoside triphosphates besides ATP. Briskin and Leonard contend that any activity which is not highly specific for ATP is due to phosphatases. Although the K\(^{+}\)Mg\(^{2+}\)ATPase from corn plasma membranes is fairly specific for ATP, some transport ATPases from animals have a broad substrate specificity. Adenylate kinase, which has been detected in plant membrane preparations (Gross and Marme, 1978), could account for some of the activity observed with nucleoside diphosphates (Leigh and Walker, 1980).

Balke and Hodges (1975) have shown conclusively that MgATP is the true substrate of the K\(^{+}\)Mg\(^{2+}\)ATPase from oat root plasma membranes. MgATP is generally believed to be the substrate of the K\(^{+}\)Mg\(^{2+}\)ATPases of other species as well.
Davis (1979) found a K⁺ stimulated ATPase in corn shoots which did not require Mg²⁺ as a cofactor. It is specific for free nucleoside triphosphates at low concentrations, but will hydrolyze nucleoside diphosphates at higher concentrations. The Km for ATP is 150 μM. Davis purified this enzyme and found it could be separated from acid phosphatase and 5'-nucleotidase activities. The purified enzyme hydrolyzes ATP (Km 30 μM) and ADP (Km 0.43 mM) about equally well when saturated (Vm about 700 μmol mg⁻¹ h⁻¹). p-Nitrophenylphosphate, pyrophosphate, and tri-polyphosphate are hydrolyzed less rapidly, and activity is negligible with alkyl phosphates.

Benson (1977) has isolated a K⁺ATPase from corn roots which is very similar to the enzyme studied by Davis (1979). Benson's assays contained MgCl₂ at the same molar concentration as ATP. Maslowski and Komoszynski (1974) extracted corn shoot microsomes with deoxycholate and KI. Their extract showed Mg²⁺-independent ATP hydrolysis, stimulated by KCl and NaCl, with pH optima at 6.0 and 7.5.
Purified Transport ATPases

Properties of membrane proteins

Membrane proteins, including transport ATPases and their subunits, may be grouped into three classes based on their physical properties and their manner of association with the membrane (Singer and Nicolson, 1972; Singer, 1974; Tanford, 1973).

Members of one class are known as extrinsic proteins. These proteins appear to be held to the membrane by hydrogen bonds and by electrostatic interactions; they are not believed to penetrate deeply into the lipid bilayer. They can be released from the membrane by changes of ionic strength, by sonication, by treatment with chelating agents, or by changes of pH. Once separated from the membranes they behave as ordinary soluble proteins and may be isolated and studied by the conventional procedures of protein chemistry. They do not bind large amounts of lipid or detergent in cooperative fashion, except for denaturing detergents such as SDS.

Members of a second class are known as intrinsic proteins. These proteins are firmly associated with the membrane, embedded in the lipid bilayer and held by hydrophobic interactions. They may be solubilized by dissolving the bilayer with detergents (Helenius and Simons, 1975; Stubbs and Litman, 1978), yielding mixed micelles of
detergent, lipid, and protein. The lipid may be separated from the protein in the presence of excess detergent. In the absence of detergent and phospholipid, intrinsic proteins tend to aggregate or to be insoluble. They may be unstable under such circumstances.

A third class of membrane proteins is the proteolipids. These are small, very hydrophobic proteins which have the solubility properties of lipids - that is, they are soluble in organic solvents such as chloroform-methanol mixtures or in acidified butanol (Fillingame, 1980; Folch-Pi and Stoffyn, 1972). In detergent extraction experiments, they behave as intrinsic proteins.

Classes of ATPases - the mitochondrial type

Several ATPases from various sources have been studied extensively. Two general types of ATPases have been shown to be active in transmembrane transport of ions. The first type consists of the chloroplast, mitochondrial, and bacterial ATPases, which have been recently reviewed by Shavit (1980), Fillingame (1980), and Downie et al. (1979), respectively. These enzymes have numerous similarities. They are multi-subunit proteins of high molecular weight, comprising an $F_0$ portion, which is firmly embedded in the membrane, and an $F_1$ portion, which contains the ATP hydrolyzing site and behaves as an
extrinsic protein. The $F_1$ portion is released from the membrane by osmotic shock, EDTA, or chaotropic salts (Kagawa, 1974). The $F_0$ portion or the intact $F_1-F_0$ complex can be solubilized only with detergents. All three of these ATPases have been shown in situ and in reconstituted systems to generate an electrogenic flux of protons across the membrane.

**Catalytic properties** All are inhibited by DCCD at low concentration, and the mitochondrial ATPase is also inhibited by oligomycin. These inhibitors are believed to act by blocking a $H^+$ channel formed by a proteolipid in the $F_0$ portion of the enzyme (Fillingame, 1980; Criddle et al., 1977; Nelson et al., 1977). None of these ATPases is inhibited by vanadate. MgATP is the preferred substrate, but they will hydrolyze other nucleoside triphosphates (with Mg$^{2+}$). The chloroplast and mitochondrial ATPases are believed to function in vivo in ATP synthesis driven by a $H^+$ gradient (Racker, 1975; Mitchell, 1979) while the bacterial ATPase operates in either direction (Wilson, 1978) depending on the energy metabolism of the cell.

**Structure** Both the $F_1$ and $F_0$ portions consist of several polypeptides. The $F_1$ portion consists of $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$ subunits of molecular weights 59,000, 56,000, 37,000, 17,500, 13,000 (values are for chloroplast $F_1$—the others are similar). The stoichiometry of the
subunits is still a matter of dispute. There is support for $a_2^3 b_2 y_1-2 s_1-2 e_1-2$ and for $a_3 b_3 y s e$. The composition and stoichiometry of the $F_0$ portion is not known but it includes the proteolipid. The mitochondrial ATPase has two soluble subunits in addition to $F_1$ which are believed to be involved in binding $F_1$ to the membrane, and a soluble inhibitor protein associated with it. The $\beta$ subunit appears to carry the ATP-hydrolyzing site (Baird and Hammes, 1979; Hobbs and Albers, 1980).

**ATPases of the plasma membrane type**

The other general type of transport ATPase includes several enzymes with different transport activities, including the animal $Na^+$, $K^+$ ATPase, the $Ca^{2+}$ ATPases of sarcoplasmic reticulum and plasma membrane, gastric mucosal $H^+$, $K^+$ ATPase, and the $H^+$ ATPases of yeast and *Neurospora* plasma membranes (see below, and reviews by Sachs (1977), Hobbs and Albers (1980), DeMeis and Vianna (1979)).

**Structure** These enzymes are intrinsic proteins of molecular weight 100,000; they may or may not be accompanied by other polypeptides. A glycoprotein ($M_r = 55,000$) copurifies with and is a functional part of the $Na^+$, $K^+$ ATPase. The sarcoplasmic reticulum ATPase is associated with a proteolipid ($M_r = 12,000$), the presence
of which enhances Ca\(^{2+}\) uptake in reconstituted vesicles (Racker and Eytan, 1975). The Na\(^+\),K\(^+\) ATPase copurifies with a proteolipid of similar size, which may be involved in binding the inhibitory glycoside ouabain (Rogers and Lazdunski, 1979). The Kdp high affinity K\(^+\) transport system of *E. coli* may also be a member of this group. It consists of three polypeptides (\(M_r = 96,000, 47,000, \text{ and } 22,000\)), and has K\(^+\)Mg\(^{2+}\) ATPase activity (Epstein et al., 1978). The H\(^+\)ATPase of *Neurospora* membranes has not been purified, but a polypeptide of \(M = 100,000\) is labeled when plasma membranes are labeled with \(^{32}\)P-ATP in the concentration range wherein the ATPase is active. Destruction of ATPase activity by digestion of the membranes with trypsin prevents labeling and eliminates the \(M = 100,000\) band from gels (Dame and Scarborough, 1980). The plasma membrane ATPase of the yeast *Schizosaccharomyces pombe* has been purified as an aggregate (\(M_r = 800,000 \text{ to } 1,000,000\)) of 100,000-dalton monomers (Dufour and Goffeau, 1980a).

**Catalytic properties** All of the ATPases of this class are inhibited by vanadate (O'Neal et al., 1979; Cantley et al., 1977; Bowman and Slayman, 1979; Dufour et al., 1980). All require a divalent cation cofactor. Their substrate specificities vary considerably. Ca\(^{2+}\) ATPase will hydrolyze a wide variety of high-energy phosphate compounds, and this hydrolysis will drive Ca\(^{2+}\) transport
(DeMeis and Vianna, 1979). Na\(^+\), K\(^+\) ATPase is more selective, and largely specific for ATP (Dahl and Hokin, 1974); the enzyme also has K\(^+\)-stimulated p-nitrophenylphosphatase activity, but this activity does not drive ion transport ( unlike the PNPPase of sarcoplasmic reticulum ATPase - Inesi, 1971). The fungal H\(^+\) ATPases are highly specific for ATP (Dufour and Goffeau, 1980a, b; Scarborough, 1977) and the E. coli K\(^+\)Mg\(^{2+}\) ATPase is completely so. The fungal and E. coli ATPases function well with a variety of divalent cations; Mg\(^{2+}\) is highly effective, but Co\(^{2+}\) is as active or more so (Dufour and Goffeau, 1980a; Scarborough, 1977; Epstein et al., 1978).

**Comparison with the plant K\(^+\)Mg\(^{2+}\)ATPase**

Dupont and Leonard (1980) and Dupont et al. (1981) have solubilized the K\(^+\)Mg\(^{2+}\)ATPase from corn root microsomes with octyl glucoside and have made some progress in purifying it. The enzyme is unstable in the detergent and must be precipitated promptly to retain good activity. The (NH\(_4\))\(_2\)SO\(_4\) precipitate is about fourfold enriched in ATPase. The partly purified enzyme is about 20% stimulated by KCl; the stimulation approximates a simple Michaelis-Menten isotherm with a \(K_a\) of 4 mM for KCl. This contrasts with the anticooperativity characteristic of the membrane bound ATPase. ATP is the preferred substrate, with a \(K_m\)
of 0.7 mM at 50 mM KCl and a $K_m$ of 1.3 mM without KCl. The purified enzyme is inhibited by vanadate and by diethylstilbestrol (DES). A divalent metal ion is required for ATP hydrolysis; the order of effectiveness is $Co^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+} > Ca^{2+}$. Properties of the purified ATPase resemble in many ways those of the $H^+\text{ATPases}$ of fungal plasma membranes. A 100,000-dalton polypeptide is present in the partly purified preparation, which appears to consist of vesicular aggregates. It is reasonable to consider the corn $K^+\text{Mg}^{2+}\text{ATPase}$ a member of this second broad group of transport ATPases.

Reconstitution of Membrane ATPases

Methods of membrane reconstitution

A number of procedures have been developed which employ the tendency of membrane proteins to associate with phospholipids, and the tendency of phospholipids to form bilayers, to reconstitute protein-containing membranes. These methods have been reviewed by Montal (1976), Korenbrot (1977), Racker (1973, 1979), Racker and coworkers (1979), and Shamoo and Tivol (1980). The most widely applicable procedures are those which depend on removal of detergent from a mixture of detergent, phospholipid, and protein. Detergent may be removed from the mixed micelles by dialysis (Kagawa and Racker, 1971), by dilution (Racker et al., 1975,
1979) of the mixture so that the detergent is below its critical micelle concentration or by adsorption (Giraudat et al., 1980). Octyl glucoside and sodium deoxycholate are the detergents usually employed; they have high critical micelle concentrations (which facilitates dialysis) and tend not to denature proteins. Other procedures include sonication of protein and lipid together (Racker, 1973) and direct incorporation of protein into preformed vesicles. Such incorporation may take place spontaneously (Racker, 1979) or in response to a cycle of freezing and thawing (Kasahara and Hinkle, 1977; Dixon and Hokin, 1980).

Octyl glucoside (n-octylβ-D-glucopyranoside) was introduced as a membrane solubilizer by Baron and Thompson (1975). It is a gentle nonionic detergent with a high critical micelle concentration. Its use has permitted the solubilization of several membrane proteins which are intolerant of cholate, including rhodopsin (Stubbs and Litman, 1978) and corn K⁺ATPases (Davis, 1979; DuPont and Leonard, 1980). The elegant experiments of Stubbs and Litman (1978) illustrate the stages of solubilization of membranes treated with octyl glucoside.

Racker et al. (1979) have discussed the use of octyl glucoside in the reconstitution of membrane proteins by the detergent dilution method. They applied the method to several proteins which had previously been reconstituted.
by other methods, and obtained good results when the concentration of octyl glucoside was adjusted to the best value for each system.

The mitochondrial, chloroplast, and bacterial coupling factor ATPases, the Ca\(^{2+}\) ATPases of sarcoplasmic reticulum and of erythrocyte plasma membrane, and Na\(^{+}\), K\(^{+}\) ATPases from several sources have all been incorporated into vesicles by one or more of these procedures, and have been shown to pump ions across the membranes of the resulting vesicles. The ion pumping activity of the gastric H\(^{+}\), K\(^{+}\) ATPase and the Neurospora H\(^{+}\) ATPase have been demonstrated only in vesicles of the native membrane (Scarborough, 1980; Chang et al., 1977).

There have also been many attempts (several of them successful) to reconstitute proteins into planar bilayer membranes. These have been reviewed by Montal (1976) and by Shamoo and Tivol (1980) and will not be discussed here. Successful experiments of this type would permit direct measurement of electrogenic transport, but the development of methods for measuring potentials across vesicle membranes with permeant ions has made the need for direct measurements less pressing.
Examples of Reconstitution and Demonstration of Ion Transport

Kagawa and Racker (1971) reconstituted the mitochondrial oligomycin-sensitive ATPase into membranes made with soy phospholipids by cholate dialysis, starting with a hydrophobic protein fraction from mitochondria, and isolated $F_1$ ATPase. They measured ATP-$P_4$ exchange and MgATP-dependent ANS fluorescence enhancement to show reconstitution of the energized state of the membrane, as seen with submitochondrial particles. Vesicles made this way accumulate $H^+$ in the presence of MgATP, valinomycin and intravesicular $K^+$, indicating that they accumulate protons electrogenically (Kagawa et al., 1973). The intact $F_1$-$F_0$ complex has been isolated and reconstituted by Serrano et al. (1976), who found similar evidence for $H^+$ transport driven by ATP hydrolysis. This shows that the $H^+$ transport is a property of the ATPase rather than of some other protein in the crude hydrophobic protein fraction.

Racker and Stoeckenius (1974) showed that when mitochondrial ATPase and bacteriorhodopsin (which acts as a light-driven $H^+$ pump) were reconstituted together, by the cholate dialysis method, illumination of the vesicles in the presence of ADP and $P_4$ led to ATP synthesis. Racker et al. (1979) later showed that reconstitution by the octyl glucoside dilution method gave higher rates of
light-driven ATP synthesis than the cholate dilution, sonication, or freeze-thaw-sonication methods.

Kagawa and co-workers have isolated and reconstituted the DCCD-sensitive ATPase from the thermophilic bacterium PS3. They found it capable of pumping $H^+$ with hydrolysis of ATP and of synthesizing ATP from ADP and $P_i$ using energy from a pH or potential gradient across the membrane. $H^+$ transport was electrogenic; net uptake was observed when the membranes were made permeable to $K^+$ with valinomycin (Kagawa, 1978). Reconstitution of the ATPase-containing membranes was done by the detergent dialysis procedure using a mixture of cholate and deoxycholate. Reconstitution was best in buffers of low ionic strength, at pH 8, in the presence of 7.5 mM MgSO$_4$ (Sone et al., 1977).

Pick and Racker (1979) isolated the DCCD-sensitive ATPase complex from spinach chloroplasts and reconstituted it by the freeze-thaw-sonication method, inserting it into vesicles made by sonicating soy phospholipids. They demonstrated ATP synthesis driven by a pH gradient created by an acid-base transition. Racker et al. (1979) found that dilution of octyl glucoside was the best method for reconstituting this enzyme. ATP synthesis coupled to illumination of vesicles of chloroplast ATPase and bacteriorhodopsin was shown by Winget et al. (1977), who reconstituted the proteins by cholate dialysis.
Further evidence that these enzymes drive or are driven by H⁺ fluxes comes from studies of the proteolipids which form part of the F₀ portion of these enzymes. DCCD inhibits all three of these enzymes and has been shown to react with the proteolipids, forming in each case a covalent bond with a single aspartate residue (Fillingame, 1980). The mitochondrial proteolipid has been reconstituted and shown to behave as an H⁺ ionophore in vesicle membranes. One experiment measured net uptake of H⁺ into vesicles when valinomycin was added and a K⁺ gradient was present across the vesicle membrane (Konishi et al., 1979). Another experiment measured the potential generated across a lipid-impregnated membrane filter to which the vesicles were adsorbed, when bacteriorhodopsin reconstituted with the proteolipid was illuminated. Bacteriorhodopsin alone generated a potential, which was reduced in the presence of proteolipid. In both experiments, the effect of the proteolipid was abolished by oligomycin, suggesting that oligomycin blocks the H⁺ channel formed by the proteolipid (Criddle et al., 1977; Konishi et al., 1979).

ATPases of the other class have also been reconstituted and shown to be ion pumps. The sarcoplasmic reticulum ATPase has been reconstituted by the cholate dialysis and sonication techniques. The reconstituted vesicles accumulate Ca²⁺ in the presence of MgATP (Racker, 1972;
Racker, 1973; Racker and Eytan, 1973, 1975; Meissner and Fleischer, 1974). Racker and Eytan (1975) found that the presence of a proteolipid in the reconstituted preparations led to greater Ca\(^{2+}\) accumulation. They suggested that the proteolipid had ion-conducting properties similar to the proteolipids of mitochondrial and related ATPases. The isolated proteolipid has been found to reduce the conductivity of bilayer membranes and to increase the ordering of phospholipids, suggesting a structural role (Laggner and Graham, 1976).

\[\text{Na}^{+},\text{K}^{+}\text{ATPases from several sources have been reconstituted (see review by Wallick et al., 1979) and have been found to transport } \text{Na}^{+} \text{ and } \text{K}^{+} \text{ in the ratio } 3 \text{Na}^{+}:2\text{K}^{+}:1\text{ATP.} \]

The reconstituted preparations possess sufficient permeability to Cl\(^{-}\) to allow net transport to occur with Cl\(^{-}\) movement providing electroneutrality. The observed membrane potential in the reconstituted system has been shown to be partly electrogenic in origin, and partly due to a diffusion potential established by the K\(^{+}\) gradient (generated by K\(^{+}\) transport) (Dixon and Hokin, 1980).

These authors used the freeze-thaw-sonication method to reconstitute the ATPase. Cholate dialysis and sonication methods have also been used successfully (Hilden and Hokin, 1975; Racker et al., 1975). An early report that the enzyme from canine renal medulla transported NaCl instead of Na\(^{+}\) and K\(^{+}\) in a reconstituted system (Golden
and Tong, 1974) has been ascribed to alteration of the properties of the enzyme during purification and reconstitution process (Shamoo and Tivol, 1980), perhaps due to lipid oxidation during the rather long period of dialysis.

A Ca\(^{2+}\)Mg\(^{2+}\) ATPase from pig erythrocytes was purified and reconstituted by Haaker and Racker (1979) using the freeze-thaw-sonication procedure. This procedure has the advantage of being relatively insensitive to the detergent Triton X-100, which remains in the preparation following purification of the enzyme by the mixed micelle gel filtration method of Wolf et al. (1977). Furthermore, it does not require a concentrated protein solution. ATPase activity was activated by Ca\(^{2+}\) and calmodulin; it was not sensitive to inhibition by Ca\(^{2+}\) accumulated in the vesicles. Nevertheless, the calcium ionophore A-23187 stimulated the activity, suggesting that Ca\(^{2+}\) accumulation is electrogenic and is limited by a membrane potential. The Ca\(^{2+}\)Mg\(^{2+}\) ATPase of plants is also stimulated by calmodulin (Dieter and Marme, 1980) and has been shown to drive accumulation of Ca\(^{2+}\) in microsomal vesicles (Gross and Marme, 1978); it has not yet been purified and reconstituted.

An H\(^{+}\) ATPase of bovine adrenal chromaffin granuole membranes has been solubilized with deoxycholate and reconstituted by dilution and adsorption of the detergent with Biobeads SM2 (macroreticular polystyrene beads).
It was shown to be an electrogenic $H^+$ pump (Giraudat et al., 1980).

The $K^+$ATPase of gastric mucosa is virtually the only protein in the membrane in which it is found; it is present in sufficient quantity that binding of ATP to the enzyme alters the membrane surface charge dramatically. Vesicles have been isolated by centrifugation followed by free flow electrophoresis in the presence of ATP. The resulting purified vesicle preparation was used in experiments which showed an electroneutral $H^+/K^+$ exchange across the membrane driven by hydrolysis of ATP (Chang et al., 1977; Sachs, 1977).

The plasma membrane ATPase of Neurospora has not been solubilized, but it has been shown to be an electrogenic $H^+$ pump by experiments with isolated plasma membrane vesicles. Yeast plasma membrane ATPase has been solubilized and purified. Experiments involving recombination of this enzyme with phospholipids have been reported (Dufour and Goffeau, 1980a, 1980b) but the authors did not attempt to measure ion transport activity. ATP-$P_i$ exchange activity of a yeast ATPase has been reconstituted (Malpartida and Serrano, 1981).
MATERIALS AND METHODS

Materials

Fluorescent probes

Carboxyfluorescein (Eastman) was purified by Hagin's procedure as described by Socoler and Lowenstein (1979). This entails treatment of an ethanolic solution of the dye with decolorizing carbon, followed by recrystallization from ethanol/water.

Diproplyoxadycarbocyanine (abbreviated diO-C₃-(5)) was synthesized in this laboratory by Jack Dillon, according to the procedure of Sims et al. (1974).

Phospholipids

Refined soy lecithin (ICN) was dissolved in CHCl₃ and precipitated with acetone and MgCl₂ by Kates' procedure (1972). The precipitate was dissolved in CHCl₃ and then was washed three times by partition in the two-phase system CHCl₃:MeOH:H₂O 8:4:3. The second washing included 100 mM NaCl and 10 mM sodium EDTA, pH 7, in the H₂O to make sure the phospholipids were present as the Na⁺ salts rather than the Ca²⁺ or Mg²⁺ salts. Lecithin is a misnomer for this material, which is really a complex mixture of polar lipids isolated from crude soybean oil; it will be referred to as soy phosphatides.
Egg yolk phosphatidylcholine (egg PC) was prepared by extracting fresh egg by the method of Wells and Hanahan (1969), and fractionating the resulting acetone precipitate on columns of alumina (Woelm) and silicic acid (Mallinkrodt) as described by Bangham et al. (1974). It was homogenous by thin layer chromatography on silica gel G (CHCl₃:MeOH:H₂O 65:25:4) and by ³¹P NMR (London and Feigenson, 1979). For a few experiments, purified egg PC in CHCl₃ was purchased from Sigma.

Reagents

Histidine free base was prepared from the hydrochloride (Nutritional Biochemicals Co.) by neutralization with NaOH and crystallization. After recrystallization from water it was free of Na by flame test.

Choline free base was prepared from the chloride by ion exchange with a column of Dowex 1 x 8 in the OH⁻ form.

Imidazole, unless from a new bottle, was recrystallized from water to remove brown impurities.

ATP (Sigma) was free of vanadium, according to the manufacturer's analyses. Cations (histidine, imidazole, or K⁺) were substituted for Na as the counterion to ATP by ion exchange with a column of Amberlite CG 120 equilibrated by Na⁺ or the cation to be substituted, followed by adjustment of the pH with the free base. The concentration
of ATP was determined from the 259 nm absorbance, using the extinction coefficient $15.4 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ (Morrell and Bock, 1954, quoted in PL Biochemicals Circular OR-10). Solutions of the Na salt were also standardized for critical experiments.

MgCl$_2$ solutions were made by dilution of a stock solution standardized by the Mohr titration for Cl$^-$ (Fischer and Peters, 1969).

Malachite green oxalate (certified) was from Hartman-Leddon or from Fisher. A stock solution was made by dissolving 420 mg in 20 ml of almost-boiling water, filtering the solution through a pad of Celite analytical filter aid, and rinsing the filter with boiling water. This removes a gray-green impurity which can interfere with the phosphate assay. The combined filtrate was diluted to 200 ml.

Chloroform was redistilled using a fractionating column of glass helices and stored in the dark in brown glass bottles. Ethanol (0.75% v/v) was added to the redistilled material as a preservative. Used chloroform was recycled by distilling it in a rotary evaporator to free it of solids, washing it with water and drying it with Na$_2$SO$_4$, then redistilling as described above.

Polyvinylpyrrolidone (abbreviated PVP; tradename Polyclar AT) was obtained from GAF, Dyestuff and Chemical Division.
It was washed with HCl and H₂O and neutralized with NaOH as described by Loomis (1974) then washed with extraction buffer and stored wet.

Celite analytical filter aid is a product of Johns Manville Inc.

Nigericin sodium was the gift of Dr. Robert J. Hosley of Eli Lilley and Company.

Adenine nucleotides, adenylylimidodiphosphate, Bis-Tris, CCCP, choline chloride, HEPES, imidazole, MES, sodium cholate, sodium dodecylsulfate, bovine gamma globulin, cytochrome C, ferritin, and myoglobin were from Sigma. Diphenylhexatriene, dithiothreitol, polyvinyl alcohol (Mᵣ = 14,000, 100% hydrolyzed), and valinomycin were from Aldrich, Elon was from Eastman Kodak. Aquacide II was from Calbiochem; octyl glucoside was from Calbiochem or from Boehringer-Mannheim. Folin phenol reagent, sodium tripolyphosphate, and Tris (primary standard grade) were from Fisher. Sucrose dilaurate was from ICN. Anhydrous NaClO₄ was from G. Frederick Smith Chemical Co. Sodium borohydride was from Matheson, Coleman, and Bell. Bovine serum albumin was from Pentex division of Miles Laboratories.

Glycine, potassium citrate, potassium formate, sodium citrate, disodium EDTA, EDTA, urea, solvents, and inorganic chemicals not otherwise specified were reagent grade.
Chromatographic media

Sephadex, Sephacryl, and Sepharose gel-chromatography media were obtained from Pharmacia, Inc.

Ion exchange resins were washed alternately with NaOH, H₂O, HCl, and H₂O again until the washes were free of UV absorbing material (A₂₆₀ < .003).

Buffers

The experiments described in this dissertation required the use of several different buffers and buffered salt solutions. These are described fully here and are referred to in the rest of the text by the names given here. The pH was measured at room temperature and in 90 to 95% of the final volume.

Extraction buffer:
- 0.25M sucrose
- 3 mM Na₂EDTA
- 15 mM Tris
adjusted to pH 7.2 with HCl.

Sucrose solutions used for density gradient centrifugation were made like extraction buffer but differed in sucrose concentration, which is specified in the text.

EDTA wash buffer:
- 0.25 M sucrose
- 30 mM Na₂EDTA
- pH 7.2 with Tris
Buffered salt solutions for chromatography:

NaCl, NaClO₄, or Na₂SO₄ at the concentration indicated in the text

1.25 mM MgCl₂

1.25 mM Na₂EDTA

adjusted to pH 8.0 with Tris. The original rationale for including both Mg²⁺ and EDTA was to avoid competition by EDTA with ATP for Mg²⁺ in the assay. Mg²⁺ is less tightly bound than most heavy metal ions by EDTA, so the EDTA would still be effective in binding trace metals.

Glycine buffer:

1.00 M glycine

10 mM NaOH

sucrose, if specified.

The measured pH was 7.8. Sucrose was included in glycine buffer only for density gradient electrophoresis.

1.0 M glycine pH 6.5 contained 10 mM histidine/histidine HCl buffer pH 6.5.

Reconstitution buffers:

4.0% w/v sucrose

40 mM choline chloride

10 mM KOH

0.25 mM K₄EDTA

adjusted to pH 6.5 with HCl. Low-potassium reconstitution buffer substituted choline base for the KOH; high-potassium
reconstitution buffer substituted KCl for the choline chloride. The osmolarity (the sum of the concentrations of all ionic and neutral species) was computed to be 0.222 M, and the ionic strength to be 52 mM.

Magnesium-and-hydrogen-ion buffers:

6.0% w/v sucrose
10 mM Na$_3$ citrate
11 mM KCl
7.0 mM MgSO$_4$
adjusted to pH 6.5 with HCl.

In Na + Mg citrate buffer, NaCl replaced KCl; in K + Mg citrate buffer, K$_3$ citrate replaced Na$_3$ citrate. The pMg was computed to be 3.08 (Perrin and Dempsey, 1974, pp. 96-98), corresponding to 0.83 mM Mg$^2+$. The osmolarity was computed to be 0.245 M, and the ionic strength to be 59 mM.

Histidine/HEPES buffer consisted of 75 mM histidine and 75 mM HEPES. The measured pH was 6.75.

Assay buffers:

Assay buffers were made as stock solutions at the concentrations described below; these were diluted in making the assay mixtures to give the desired final concentration.

Tris/HCl buffer:

25 mM Tris, adjusted to the indicated pH with HCl.
Bis-Tris/HCl buffer:
25 mM Bis-Tris, adjusted to the indicated pH with HCl.

Histidine/histidine HCl buffer:
25 mM solutions of histidine and histidine HCl were combined to make the mixture pH 6.5.

MES/Tris buffer:
40 mM MES, adjusted to pH 6.5 with Tris.

MES/imidazole buffers:
100 mM MES, adjusted to the indicated pH with imidazole.

HEPES/imidazole buffers:
100 mM HEPES, adjusted to the indicated pH with imidazole.

K/HEPES/Cl buffers and K/MES/Cl buffers:
100 mM HEPES or MES
100 mM KOH
adjusted to the indicated pH with HCl. The ionic strength and the total K concentration are both equal to the HEPES or MES concentration, and thus precisely known.

ATPase Preparations

Microsomes
Microsomes were prepared as described by Benson (1977) and by Davis (1979), with minor modifications, as summarized below.
Corn seed (W64A) was weighed, then surface sterilized by soaking for about 4 min in 0.05% NaOCl (dilute bleach) containing a few drops of 2% Tween 80, with occasional stirring. The seed was rinsed, spread on paper towels in plastic crisper boxes, wetted with 60 ml of 0.5 mM KCl, 0.1 mM CaCl₂ per box (35 g seed/box), covered, and incubated in the dark for 3 1/2 days at 28°C.

Primary roots were cut from the seedlings with razor blades and immersed in ice cold deionized water. Shoots were broken off and likewise immersed. The roots or shoots were washed with deionized water, blotted dry, weighed, and cut into 1 cm pieces with a sharp knife. Ice cold extraction buffer (4 ml/g tissue) was added and the roots or shoots were ground and homogenized with a Polytron homogenizer. A 20 box batch of roots (about 130 g) was subjected to two 30s bursts at speed 4, and one 60s burst at speed 6. The blades were cleaned between bursts. Small batches (about 25 g) required only 2 x 15s at speed 4 and 30s at speed 6. Shoots were homogenized similarly, but the second low speed burst was omitted.

Polyclar AT (0.25 g wet weight/g tissue) was mixed into the homogenate. The mixture was filtered through four layers of cheesecloth after 5 min on ice. This removed fibrous material and most of the Polyclar. The root filtrate was centrifuged 25 min at 13,000 x g average
(10,000 rpm in a Sorvall GSA rotor, or 12,000 rpm in a Sorvall SS-34 rotor). The shoot filtrate was first centrifuged 10 min at 1200 g\textsubscript{av} (3000 rpm in the GSA or 4000 rpm in the SS-34), then the supernatant was centrifuged 20 min at 9500 g\textsubscript{av} (8500 rpm in the GSA or 10,000 rpm in the SS-34). The supernatant from roots or from shoots, designated the post-mitochondrial supernatant, was used to make microsomes by ultracentrifugation or by calcium precipitation.

Calcium-precipitated microsomes (Davis, 1979) were prepared by adding enough 3 M CaCl\textsubscript{2} to the post-mitochondrial supernatant, with stirring, to make the mixture 50 mM in Ca. After the mixture had stood on ice 30 min, it was centrifuged 20 min at 13,300 g\textsubscript{av}. The pellets were quickly resuspended in EDTA wash buffer at 1.0 ml/g tissue, homogenized in a glass homogenizer, and recentrifuged at 13,300 g\textsubscript{av} for 20 min. The pellets (calcium-precipitated microsomes) were resuspended in extraction buffer.

Ultracentrifuge microsomes were prepared by layering the post-mitochondrial supernatant over 15 to 20 ml/tube of 20% w/v sucrose in Tris-EDTA buffer, in l 1/2 x 3 7/8 inch polycarbonate centrifuge tubes and centrifuging 80 min at 35,000 rpm in a Beckman 45 Ti rotor at 4°C. The pellets were resuspended in extraction buffer or in reconstitution buffer, with a teflon-and-glass homogenizer.
**NaClO₄ extraction**

Root microsomes made by either procedure were resuspended in extraction buffer at 1.0 ml/14 g roots. An equal volume of 1.00 M NaClO₄ containing 5 mM MgCl₂ and 5 mM Na₂EDTA (Tipton et al., 1975) was added. After the mixture had stood 45 min at 30°C, it was centrifuged 45 min at 40,000 rpm in a Beckman type 40 rotor. The supernatant is the NaClO₄ extract. The pellet was resuspended in extraction buffer for assay.

**Sucrose dilaurate extraction (Davis, 1979)**

Shoot microsomes made by the calcium precipitation procedure were resuspended in extraction buffer at 1.0 ml/7 g shoots. Enough 5% w/v sucrose dilaurate was added to make the mixture 0.5% in sucrose dilaurate, and enough solid KCl to make it 1.0 M in KCl. After the mixture had stood at room temperature for 5 min, it was centrifuged 20 min at 15,000 rpm in the SS-34 rotor (20,000 gₐᵥ). The supernatant is the sucrose dilaurate extract. The pellet was resuspended in extraction buffer for assay.

**Octyl glucoside extraction**

Root or shoot microsomes were resuspended in extraction buffer or in reconstitution buffer at 1.0 ml/7 g tissue. Enough 300 mM octyl glucoside was added to make the
mixture 30 mM in octyl glucoside. The mixture stood on ice 15 to 30 min, then was centrifuged at 50,000 rpm in a Beckman type 50 Ti or type 65 rotor. Extracts made in reconstitution buffer were centrifuged 1 h, but extracts made in extraction buffer required 2 h.

**Hexyl-Sepharose chromatography (Benson, 1977) of extracts**

Hexyl-Sepharose was made as described by Benson (1977), by reaction of cyanogen bromide activated Sepharose 4B with n-hexylamine. The extract was dialyzed against several changes of buffered 0.25 M NaClO₄ (the sucrose dilaurate extract was first dialyzed against buffered 1.0 M NaCl to avoid precipitation of KC1O₄) and applied to a column (1.6 x 25 cm for preparative use) of hexyl-Sepharose equilibrated with buffered 0.25 M NaClO₄. The column was eluted with about 1/2 column volume buffered 0.25 M NaClO₄, then with buffered 2.0 M NaClO₄. As described by Benson (1977), most of the protein and most of the K⁺-independent ATPase activity was eluted by the 0.25 M wash, whereas a peak of K⁺ATPase was eluted by the 2.0 m NaClO₄. The fractions containing this K⁺ATPase were pooled and designated hexyl-Sepharose purified ATPase. This material was used in some experiments without further purification.
Concentration of the ATPase

The pooled active fractions from the hexyl Sepharose column were concentrated by one of two methods. The original procedure was to seal the enzyme solution in a dialysis bag, coat the bag with Aquacide II, and let it stand at 5°C until the Aquacide had absorbed most of the water from the dialysis bag. After the hydrated Aquacide was stripped off, the bag was placed in a beaker of buffered 1.00 M NaCl to dialyze 4 h or more. This procedure was effective in concentrating the enzyme three to five fold, but was slow. Attempts to achieve greater concentrations led to pronounced loss of activity, perhaps by adsorption to the dialysis bag.

To obtain highly concentrated enzyme in good yield, I precipitated the enzyme by making the solution 75% saturated with (NH₄)₂SO₄. Fifty grams of solid (NH₄)₂SO₄ were added to each 100 ml of pooled fractions (dialyzed against buffered 1.00 M NaCl to prevent precipitation of NH₄ClO₄) at 0°C. After the (NH₄)₂SO₄ had dissolved and the solution had stood 1 h, it was centrifuged 20 min at 20,000 rpm (40,000 gav) in the SS-34 rotor. Then the pellet was redissolved in a small volume of buffered 1.00 M NaCl, rubbing the walls of the centrifuge tube with a teflon-ball homogenizer pestle.
Recovery of activity varied between 70% and 100%; the supernatant fluid was devoid of activity.

**Gel filtration**

I used a column of Sephacryl S-200 (2.5 x 8.5 cm) instead of the Sephadex G-100 superfine employed by Benson (1977). The concentrated, hexyl-sepharose-purified ATPase was applied to the column in about 8 ml of buffered 1.00 M NaCl and eluted with that buffer at a flow rate of 0.3 ml/min. The ATPase eluted as a single peak, $K_{av} = 0.50$, 25 ml wide at half height. Calibration of the column with standard proteins confirmed the findings of Benson (1977) and Davis (1979) that the apparent molecular weight of the root and shoot enzymes is $3.0 \times 10^4$. The active fractions were pooled, and are referred to as S200-purified ATPase.

**Lipid Vesicles**

Vesicles were made from soy phosphatides or egg phosphatidylcholine by one of three methods, and were characterized by gel filtration in columns of Sepharose 2B and Sepharose 4B. The columns were pretreated with sonicated soy phosphatide vesicles to saturate any lipid-binding sites (Huang, 1969).
Sonication method

The lipid (1 or 2 mg in CHCl₃) was dried with a N₂ stream in the bottom 1 cm of a test tube; 1 or 2 ml of buffer were added and the test tube was filled with N₂ and sealed with several layers of Parafilm. The test tube was shaken intermittently for an hour or more, or vortexed, to disperse the lipid; then it was clamped in place and sonicated 20 min in a Branson B-12 ultrasonic cleaner. For efficient sonication, the bath was filled within 1 cm of the top and the sample was centered in the bath about 2 cm below the surface. The resulting preparation was barely turbid. Gel filtration in Sepharose 2B or 4B gave two peaks, one excluded and one retained by the gel, as is typical of sonicated liposome preparations (Huang, 1969; Szoka and Papahadjopoulos et al., 1980).

Butanol injection method

This method is analogous to the ethanol injection method of Batzri and Korn (1973); ethanol is not a good solvent for soy phosphatides, so butanol was substituted. The volumes of butanol and buffer were chosen so that all the butanol would be soluble in the buffer. Typically, 6 mg lipid was dissolved in 0.10 ml butanol and injected into 1.5 ml buffer while the buffer was gently vortexed (Szoka and Papahadjopoulos, 1980). Vigorous vortexing of the
mixture gave an opalescent emulsion, from which the butanol was removed by Sephadex G-25 chromatography. The resulting vesicles were all excluded from Sepharose 2B.

**Octyl glucoside dialysis method**

The lipid (6 mg in CHCl₃) was dried with a N₂ stream. It was redissolved in 0.40 ml 0.30 M octyl glucoside and diluted with 3.6 ml reconstitution buffer or extraction buffer or combined with 4.0 ml octyl glucoside extract. The mixture was then dialyzed against 800 ml of the same buffer, 12 h at room temperature. When this method was used to make reconstituted vesicles from membrane extracts, the amount of octyl glucoside was reduced to allow for that contained in the extract. Vesicles made by this procedure were excluded from Sepharose 4B.

**Assays**

**Protein**

Protein was determined by the method of Lowry et al. (1953). Interference by sucrose, Tris, EDTA, and other components of the sample was avoided in one of two ways. Either the sample volume was limited to 0.1 ml in a 6 ml assay and all standards and blanks were in the same medium as the sample, or else the samples and standards were precipitated by TCA (5% w/v). In the latter case, the precipitated protein pellets were redissolved in 0.20
ml 1.00 M NaOH and NaOH was omitted from the alkaline copper reagent as described by Lowry et al. (1953); final assay volume was 2.4 ml. Bovine serum albumin was the standard in both versions.

Enzymes

ATPase activity was measured as the rate of inorganic phosphate release from ATP under the specified conditions. Reaction mixtures (1.0 ml) were incubated at 38° for times between 15 min and 1 h in new polystyrene test tubes. The reaction was initiated by adding ATP (or enzyme where indicated) to the otherwise-complete reaction mixture. It was stopped by adding the acid-molybdate reagent used in the phosphate assay, or else by adding 1.0 ml ice cold 0.5 M formic acid and applying the mixture immediately to an anion exchange column, as described below. K⁺ stimulation was computed by subtracting the activity without added KCl from the activity with the indicated KCl concentration.

Phosphate release from ATP by the purified and partly purified K⁺ATPases of roots was found to be linear with time and with the amount of enzyme added at 250 mM and 50 mM KCl. Rapid partial loss of activity at 0 mM KCl was detectable when the purified enzyme was incubated under assay conditions and KCl was added after a time
interval had elapsed. This loss was prevented by inclusion of BSA (0.02 mg/ml) in the assay.

Phosphate was determined similarly, using the assay conditions of Rao and Vaidyanathan (1966).

Phosphate

Phosphate was determined by one of four methods, depending on the amount present and the presence of interfering materials such as phospholipids and detergents in the sample. The relative sensitivity of the four methods is given in Table 1. Routine assays used the method of Fiske and Subbarow (1925), using Elon as the reductant as described by Gomori (1942). When detergent, lipid, or protein was present in sufficient quantity to interfere with other methods, 2% w/v sodium dodecylsulfate was incorporated into the assay as described by Dulley (1975). When small amounts of inorganic phosphate were to be measured, one of two methods using malachite green were used. These are described below.

In version 1 of the malachite green phosphate assay, the reaction mixtures were acidified with 0.2 ml 0.5 M formic acid (H₂C₀₂) per ml, and applied to small ion exchange columns (0.2 ml bed volume Dowex 1 x 8, in the neck of a Pasteur pipet; the body of the pipet served as the eluant reservoir and the bed was supported by a glass
Table 1. Sensitivity of phosphate assays

<table>
<thead>
<tr>
<th>Method</th>
<th>Volume ml</th>
<th>Wave length nm</th>
<th>Absorptivity mol-1 cm-1</th>
<th>Working range nmol P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiske-Subbarow (modified)</td>
<td>5.0</td>
<td>660a</td>
<td>$4.65 \times 10^3$</td>
<td>20-400</td>
</tr>
<tr>
<td>Fiske-Subbarow (with NaDodSO$_4$)</td>
<td>2.0</td>
<td>660</td>
<td>$3.36 \times 10^3$</td>
<td>10-150</td>
</tr>
<tr>
<td>Malachite green version 1</td>
<td>10.0</td>
<td>640</td>
<td>$9.87 \times 10^4$</td>
<td>5-200</td>
</tr>
<tr>
<td>Malachite green version 2</td>
<td>4.0</td>
<td>625</td>
<td>$8.00 \times 10^4$</td>
<td>2-50</td>
</tr>
</tbody>
</table>

$^{a}$ Measured color with a Klett photoelectric colorimeter, using a #66 filter and a 1 cm round cell. 1 Klett unit = 0.002A = 2.18 nmol P.

Wool plug just high enough that bubbles are not trapped in the neck of the pipet). The columns were eluted with 4.5 ml 0.1 M NaHCO$_3$, 0.02 M H$_2$CO$_2$ (modified from Cohn, 1955). The eluate was collected in a plastic test tube which had been marked at the 10.0 ml level with a mechanical guide.

The phosphate reagent used in version 1 was a concentrated version of the reagent of Bastiaanse and Meijers (1968). Equal parts of acid molybdate solution (9.12% w/v ammonium molybdate in 9.6 M HCl) and malachite green solution (0.294% w/v) are combined, stirred 1 h, and
filtered, then 8.4 ml of 2% Sterox solution (Perkin-Elmer) are added to each 100 ml of filtrate. Four milliliters of this reagent were added to each tube of eluate. Each sample was diluted to 10.0 ml; phosphate was measured as $A_{650}$.

Version 2 of the malachite green assay was derived from the method of Altman et al. (1971) and is similar in principle to the method of Muszbek et al. (1977). It avoids interference from acid-hydrolysis of ATP by stopping color development with $H_2SO_4$, which destroys the excess malachite green. The ATPase assay incubation is stopped by addition of 0.70 ml of an acid molybdate reagent (3 parts 0.1 M $Na_2MoO_4$ and 4 parts 2.0 M HCl, mixed the day of use), followed by 0.30 ml of 0.42% malachite green in 1.0% polyvinyl-alcohol (Aldrich, 100% hydrolyzed, $M = 14,000$). The sample is vortexed and set aside for 2 3/4 min; then 2.0 ml of 7.8% v/v $H_2SO_4$ are added and the sample is vortexed again. The color is fully developed within 40 min, and is stable 2 h or more. It is measured at 625 nm.

Version 2 of the malachite green assay differs from the assay of Muszbek et al. (1977) primarily in that trichloracetic acid is not employed and that the amount of malachite green is increased. Substitution of HCl for TCA permits use of a composite acid-molybdate reagent and
simplifies handling of the samples. Increased malachite green extends the linear range of the assay. The chemistry of these methods is discussed by Altman et al. (1971).

Use of polyvinyl alcohol from Sigma led to interference with both this version of the $P_i$ assay and the version of Muszbek et al. (1977). Color development proceeded only above a certain minimum level of $P_i$, suggesting that some contaminant was competing with the malachite green in binding to phosphomolybdate (or with molybdate in reaction with $P_i$). The threshold level of $P_i$ necessary for color formation varied with the PVA concentration. PVA is made by alcoholysis of polyvinyl acetate (Merck and Co., 1976). Gentle hydrolysis of the PVA in base or acid or substitution of PVA (Aldrich) specified as 100% hydrolyzed eliminated the interference, suggesting that the interference was due to residual acetyl groups on the polymer.

**Interferences**  The sensitivity of the methods to $P_i$ is not affected by the presence or absence of KCl (250 mM). Two interferences were noted. Salts of carboxylic acids, including potassium formate and glycine at concentrations above about 0.2 M produce a green color in the assay like that obtained from phosphate. The color increases nonlinearly with concentration. This interference can be eliminated by including extra HCl in the $P_i$ assay to allow for that consumed by the carboxylate. This
requires a new standard curve. For small amounts of carboxylate, comparison with a blank of the same composition is adequate. Phospholipids can reduce the color and cause turbidity when present in high concentration, but 0.2 mg can be tolerated.

Phospholipids

Phospholipid concentration was determined by weighing a dried aliquot of the solution, or by measuring lipid P by the method of Harris and Popat (1954).

The distribution of liposomes in chromatography or centrifugation experiments was monitored as turbidity at 254 nm or 300 nm, or by the fluorescence method of London and Feigenson (1978). The later method entails adding 5 µl of 0.14 mg/ml diphenylhexatriene (DPH) in tetrahydrofuran to 1.0 ml of diluted sample and mixing. The fluorescence is measured after 30 min incubation in the dark. Since the lipids used in these experiments are all above their transition temperatures at room temperature, incubation was at room temperature instead of 38°C. The fluorescence was excited by Hg radiation filtered through a Corning 7-60 filter, and the emitted light was isolated by a Klett 47 filter. Diphenylhexatriene is unstable under UV illumination, and the effect is particularly noticeable in the
liposome assay, presumably because of slow diffusion of the liposomes into and out of the illuminated region of the cell. Therefore, the exciting light was defocussed with a ground glass diffuser; this enhanced the stability of the fluorescence by providing a larger illuminated volume.

**Instruments**

Ultraviolet and visible absorption spectra were measured with a Cary 15 spectrophotometer. A Beckman DU spectrophotometer fitted with a Gilford absorbance-measuring photometer and power supply, or a Perkin-Elmer 552 spectrophotometer was used for single wave length absorbance measurement.

Fluorescence was measured and recorded with a modified Farrand model A fluorometer. Photomultiplier voltage was supplied by a Keithley 242 regulated high voltage supply, through a 10 x 100 kΩ voltage divider. This voltage was varied to control the gain of the photomultiplier, and the fluorometer sensitivity controls were bypassed. The anode current was returned to ground through a 4.5 kΩ resistor and the resulting voltage was measured with a Leeds-Northrup Speedomax W recorder (1 mv full scale, 1 s full-scale response time), so that full scale deflection represents 222 nA. Filters used to select exciting and emitted light are listed in Table 2, along with their spectral parameters.
Table 2. Spectral characteristics of filters

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Designation</th>
<th>Short wavelength cutoff</th>
<th>Transmission optimum</th>
<th>Long wavelength cutoff</th>
<th>Hg line isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corning</td>
<td>2-61</td>
<td>610&lt;sup&gt;a&lt;/sup&gt;</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>7-60</td>
<td>314&lt;sup&gt;b&lt;/sup&gt;</td>
<td>365</td>
<td>388&lt;sup&gt;b&lt;/sup&gt;</td>
<td>365-366</td>
</tr>
<tr>
<td>Klett</td>
<td>42</td>
<td>358&lt;sup&gt;b&lt;/sup&gt;</td>
<td>415</td>
<td>469&lt;sup&gt;b&lt;/sup&gt;</td>
<td>405,436&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>437&lt;sup&gt;b&lt;/sup&gt;</td>
<td>462</td>
<td>492&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>52</td>
<td>474&lt;sup&gt;b&lt;/sup&gt;</td>
<td>518</td>
<td>565&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>542&lt;sup&gt;b&lt;/sup&gt;</td>
<td>559</td>
<td>610&lt;sup&gt;b&lt;/sup&gt;</td>
<td>577-579</td>
</tr>
<tr>
<td>Kodak Wratten</td>
<td>2A</td>
<td>410&lt;sup&gt;a&lt;/sup&gt;</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Bausch &amp; Lomb</td>
<td>Neutral Density 1.2</td>
<td>--</td>
<td>--</td>
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<td>--</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined from the manufacturer's literature as the wavelength of 10% transmittance relative to air.

<sup>b</sup>Measured as the wavelength of 10% transmittance relative to the transmission optimum.

<sup>c</sup>Used with Wratten 2A to isolate 436 nm Hg line.
pH recordings were made with an Orion 601A pH meter and an Orion 91-02 combination electrode. The recorder output of the meter was adjusted to give a 2.00 mV/pH unit signal. The circuit of Figure 1 provided a variable offset voltage, setting the zero position of the recorder. The resulting voltage was applied to the input terminals of a Leeds-Northrup Speedomax recorder (1 mV full scale, 1 s full-scale response time). The test tube containing the sample was purged with N₂ to prevent entry of CO₂. The N₂ stream was delivered through a polyethylene capillary tube attached to the pH electrode. It did not impinge directly on the sample. The sample was stirred magnetically with a 2 x 10 mm spinbar. Samples were routinely 1.0 ml.

Conductivity was measured at 60 Hz with a Yellow Springs model 31 conductivity bridge. Aliquots of column fractions were diluted 100 fold for conductivity measurement.

Refractive index was measured with a Bausch and Lomb Abbe 3L refractometer, at room temperature.

Density Gradient Centrifugation

Gradients

Concave gradients were prepared by layering sucrose solutions (in Tris-EDTA buffer) in cellulose nitrate centrifuge tubes and letting the layers diffuse at 4°C for 12 to 36 h; this method permitted control of the
Figure 1. Recorder zero-adjustment circuit
gradient shape and precise reproducibility of the gradients. To measure and layer the sucrose solutions, I used a Pipetman P-1000 pipeter, with a Pasteur pipet in place of the usual tip. The Pasteur pipet was held tightly by an adapter made by cutting a plastic pipet tip in two. Since the gradient solutions wetted the glass, I pipeted in the reverse mode: that is, the tip was filled by pressing the plunger to the second stop and releasing it slowly, then layering the solution in the bottom of the tube by depressing the plunger to the first stop. The gradient usually consisted of 1.5 ml 5% w/v sucrose in Tris-EDTA buffer and 1.0 ml each of 15%, 20% and 38% solutions. The gradients were centrifuged 18 to 24 h at 50,000 rpm in a Beckman SW-50.1 rotor.

**Gradient fractionation**

The centrifuge tube containing the gradient was held and pierced with an ISCO 184 gradient fractionator, and a displacing solution was pumped in at 0.28 ml/min with a peristaltic pump. A 0.2 ml aliquot of sucrose-free buffer was layered over each sample before it was fractionated to rinse sucrose from the tubing. The gradient was displaced through a wide-path flow cell and $A_{254}$ was monitored with an ISCO UA2 monitor. Fractions (9 drop) were collected in polyethylene sample collection blocks on a Gilson Micro Fractionator. The sample
collection blocks were sealed with petroleum jelly and Parafilm immediately after the fractions were collected to prevent evaporation; densities were measured by refractometry.

Validation

Figure 2 shows the sucrose profiles of four pairs of gradients prepared, centrifuged, and fractionated by this procedure. The composition of the gradients is specified in Table 3.

<table>
<thead>
<tr>
<th>Pair</th>
<th>Sucrose, % w/w</th>
<th>Volumes, ml</th>
<th>Diffusion time</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5:12:20:38</td>
<td>1.5:1:1:1</td>
<td>12 h</td>
</tr>
<tr>
<td>B</td>
<td>5:15:20:38</td>
<td>1.5:1:1:1</td>
<td>12 h</td>
</tr>
<tr>
<td>C</td>
<td>5:15:20:38</td>
<td>1.5:1:1:1</td>
<td>36 h</td>
</tr>
<tr>
<td>D</td>
<td>5:12:20:38</td>
<td>1.8:1:1:0.7</td>
<td>38 h</td>
</tr>
</tbody>
</table>

The discrepancy between duplicates (other than the initial fractions of pair A) was never greater than half the difference in density between successive fractions, and is usually much less. Thus, the positions of peaks in the gradients should be reproducible to within ±1/2 fraction. (Anomalous points at the top of one gradient
Figure 2. Reproducibility of pairs of sucrose gradients
of pair A are due to omission of the tubing-cleaning overlay in the fractionation of that gradient. Fractions after 19 are the chase solution, not part of the gradient.)

Density Gradient Electrophoresis

The apparatus employed (shown in Figure 3) was of my own design. It consists of a large outer buffer reservoir and a smaller, inner reservoir, each with a platinum loop electrode. The reservoirs are connected by a vertical glass tube, 1.6 x 39 cm, concentric with the reservoirs and projecting down into the outer reservoir. Glass coils immersed in the outer reservoir carry coolant. The inner reservoir is supported by a rubber stopper (not shown) cut to accommodate the reservoir, electrodes, and coolant tubes.

The inner reservoir contains a concentrated buffer less dense than the lighter component of the gradient; the outer reservoir contains a concentrated buffer more dense than the heavier component of the gradient. A linear sucrose gradient (of low ionic strength) is poured in the center tube, on top of the outer reservoir buffer. It is overlayed with inner reservoir buffer to connect it with the inner reservoir, and the level of the outer reservoir buffer is adjusted so that the gradient is in that part of the center tube that projects into the outer reservoir; this promotes effective cooling.
Figure 3. Sucrose gradient electrophoresis apparatus
The sample was layered in the gradient, in the middle or near one end. A density shelf was employed to prevent mixing by a fingering process (Brakke, 1955; Svensson et al., 1957). Electrophoresis was conducted at constant voltage. When the run was completed, the excess inner reservoir buffer was pipeted off and a Pharmacia flow adapter (model A-16) was inserted into the center tube and tightened in place; the gradient was withdrawn through the flow adapter by a peristaltic pump and fractions were collected for analysis.

Membrane Potential and Permeability Measurements

Fluorescence method

Figure 4 shows the dye used in the fluorescence-quenching experiments to measure membrane potentials, and a schematic representation of the equilibria involved in the control and calibration experiments. Figure 5A shows a typical fluorescence trace from control experiments in which K\(^+\)-loaded liposomes (made in reconstitution buffer by dialysis of octyl glucoside) are diluted in low-K\(^+\) reconstitution buffer. Free dye gives the most fluorescence. Addition of liposomes reduces the fluorescence by formation of membrane-bound aggregates of the dye. This is accompanied by a change of color from pink to purple. In agreement with Beck and Sactor (1978), I found that the
Figure 4. Membrane potential measurements with a permeant, fluorescent dye
Figure 5. Typical fluorescence traces: di-0-C₃-(5) with egg PC liposomes

A

0.04 mg PC liposomes each addition

B

0.20 mg PC liposomes

0.5 nmol valinomycin

10% full scale
2 min
greatest fluorescence response to changes of membrane potential is obtained at the lipid:dye ratio which produces the greatest reduction of fluorescence from the free dye (see Figure 5B).

The dye monomer behaves as a permeant cation (Waggoner, 1979). Addition of valinomycin produces a $K^+$ diffusion potential which concentrates the dye inside the vesicle, increasing the formation of nonfluorescent aggregates. This leads to a further drop in the fluorescence (Figure 5B).

After the valinomycin-induced decrease, the fluorescence slowly increases. This might be due to dissipation of the $K^+$ gradient, but the gradient is not dissipated in experiments (without dye) using $H^+$ movements as indicators of the membrane potential. A similar phenomenon has been observed by other investigators (Sims et al., 1974). It occurs with all the liposomes I studied, and may be due to membrane breakdown. Its rate is proportional to the valinomycin concentration, and it can be reduced but not eliminated by use of freshly prepared valinomycin solutions.

The level reached by this increase is somewhat more reproducible than the initial fluorescence level after liposome addition, so the final fluorescence was used as the reference level in measuring fluorescence changes
for sensitivity determinations. Measurements referred to the initial fluorescence level gave similar results.

Figure 6 shows the calibration curve for fluorescence changes using soy liposomes with diO-C$_3$-(5) trapped in the vesicle interior during formation. The sensitivity of this system (1.14%/mv) was the highest obtained. Soy liposomes with dye only on the outside gave 0.5%/mv, and egg phosphatidyl choline liposomes with external dye gave 0.2%/mv. These sensitivities assume ideal Nernstian response of the membrane potential to the K$^+$ gradient, with a slope of 56 mv/decade.

Binding of the dye diO-C$_3$-(5) to charged liposome surfaces is lessened by increased ionic strength and by the presence of divalent cations, increasing the fluorescence. Chelating agents sometimes decreased the fluorescence. In experiments with Mg$^{2+}$ present, Mg$_f$ was held constant with a H$^+$-and-Mg$^{2+}$ buffer such as K$^+$-Mg$^{2+}$-citrate, and ATP was added as a mixture with equimolar MgCl$_2$.

DiO-C$_3$-(5) fluorescence was excited at 578 nm and the emitted light was isolated with a Corning 2-61 filter.

Attempts to measure pH changes inside vesicles by using entrapped carboxyfluorescein as a fluorescent pH indicator (Thomas et al., 1979) were unsuccessful. The dye appeared to respond to changes in the external pH, presumably because it had leaked from the vesicles.
Figure 6. Determination of the sensitivity and linearity of membrane potential measurement.

- Relative change:
  - 49% full scale per decade
  - 64% initial fluorescence per decade
  - $1.14\% \text{mv}^{-1}$
(Szoka et al., 1979). Fluorescence was excited at 436 nm. The exciting light was attenuated by a neutral density (1.2) filter, and the emitted light was isolated with a Klett 56 filter.

**pH method**

Measurements of $H^+$ movements into and out of liposomes permitted estimation of the permeability of the membranes to $K^+$ and $H^+$. In these experiments, the interior of the liposome contained a concentrated buffer, and the external solution was very weakly buffered. The sample volume was 1 ml. $H^+$ movements appeared as changes in the external pH. Exclusion of $CO_2$ was essential; besides causing a pH drift, it appeared to contribute to the $H^+$ permeability of the membranes.

Figure 7 shows a typical experiment, conducted according to Konishi et al. (1979). Soy phosphatide vesicles loaded with $K^+$ and with Tris buffer give a stable pH trace. Addition of valinomycin, a $K^+$ ionophore, led to a slow increase in the pH of the solution. Addition of CCCP, a $H^+$ ionophore, produced a more rapid $H^+$ uptake which quickly leveled off. The initial rate of this uptake is a measure of the membrane potential, so the ratio of the rates before and after CCCP addition is a measure of the permeability to $H^+$. The reciprocal experiment, in
Figure 7. Typical pH trace: determination of the permeability to H⁺ of vesicles made from acetone-washed soy phosphatides
which CCCP is added before valinomycin, provides a measure of the membrane potential with and without valinomycin, and thus of permeability to $K^+$. 

Experiments such as this one established the necessity for washing the soy phosphatides with acetone to reduce the $H^+$ permeability of the vesicle membranes. Deliberate addition of $CO_2$ (by blowing through a Pasteur pipet onto the sample) increased the rate of $H^+$ leakage in the presence of valinomycin and the absence of CCCP. Perhaps $HCO_3^-$ can cross membranes as a valinomycin/$K^+$ complex as has been reported of certain other ions (Marinetti et al., 1978).
RESULTS

ATPase Activities of Membranes and Extracts

Presence of magnesium-requiring and magnesium-independent K^ATPase activities in corn root plasma membrane preparations

Plasma membrane preparations were assayed for ATPase activity under four sets of conditions (Table 4). Stimulation by KCl is evident whether MgCl_2 was present at the same or greater concentration as ATP, or was absent from the assay. Since the K^ATPase of oat root plasmalemmas has been shown conclusively to require Mg^{2+} and Leonard and Hotchkiss found a similar activity in corn root plasma membrane preparations, the activity without MgCl_2 is somewhat surprising.

Table 4. K^ATPase and K^+Mg^{2+}ATPase activities of corn root microsomes and plasma membranes

<table>
<thead>
<tr>
<th></th>
<th>Root Microsomes</th>
<th>Root Plasma Membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>0.30 mM</td>
<td>0.30 mM</td>
</tr>
<tr>
<td>MgCl_2</td>
<td>1.5 mM 0.0 mM</td>
<td>1.5 mM 0.0 mM 1.5 mM 0.0 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>ATPase activity, nmol(/mg h)</td>
<td></td>
</tr>
<tr>
<td>50 mM</td>
<td>3.34 4.37</td>
<td>2.70 2.57 44</td>
</tr>
<tr>
<td>0 mM</td>
<td>2.84 3.99</td>
<td>2.07 2.06 37</td>
</tr>
<tr>
<td>difference</td>
<td>0.52 0.38</td>
<td>0.64 0.51 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>
**Kinetic experiments** I tried several experiments to see if the activities with and without MgCl₂ were assayable separately, and could be resolved and characterized kinetically. Figure 8 shows the K⁺ATPase activity of a plasmalemma preparation as a function of ATP concentration, with and without equimolar MgCl₂. Figure 9 shows the same data plotted against the computed concentrations of ATPᵥ (uncomplexed ATP) and MgATP, and against total ATP, on an expanded scale. These concentrations were computed using 6.37 mM⁻¹ as the formation constant for MgATP (Balke and Hodges, 1975). None of these plots is a simple saturation curve; instead, they suggest at least two distinct activities. At low ATP concentrations (where most of the ATP is ATPᵥ), all of the activity can be accounted for by the activity measured with ATP alone. At and below 0.20 mM ATP, there is more activity without MgCl₂ than with MgCl₂, but at higher concentrations the activity without MgCl₂ is substrate inhibited. This is not seen with MgCl₂, even when the data are plotted against ATPᵥ. Thus, at high MgCl₂ and ATP concentrations a second activity appears to be present which more than makes up for substrate inhibition of the ATPᵥ hydrolyzing activity. This second activity presumably hydrolyses MgATP.
Figure 8. K^+ ATPase activity of corn plasma membranes at several ATP concentrations, with and without equimolar MgCl\textsubscript{2}
Figure 9. Data from Figure 8 plotted against the total ATP concentration and against the computed concentrations of ATP\(_{\text{f}}\) and MgATP.
If this hypothesis is correct, one would expect that if ATP$_f$ were held at a constant concentration, and MgATP were varied, the activity due to hydrolysis of MgATP could be determined by subtraction. Figure 10 shows such an experiment. ATP$_f$ was held at one of two concentrations and MgATP and Mg$_f$ were varied together. Activity without Mg$^{2+}$ was 4.0 nmol/h at 0.05 mM ATP$_f$ and 6.44 nmol/h at 0.20 mM ATP$_f$. This experiment showed substrate inhibition with increasing MgATP, which was not seen in Figures 8 and 9; since substrate inhibition is greater at the lower ATP$_f$ concentration, MgATP may be inhibiting hydrolysis of ATP$_f$. In Figure 9, the concentration of ATP$_f$ increased when MgATP increased, so the increased ATP$_f$ might have prevented inhibition. Alternatively, inhibition might be due to Mg$_f$. The increase in activity with increasing MgATP was greater at the lower ATP$_f$ concentration, suggesting that ATP$_f$ may inhibit the hydrolysis of MgATP. When the reciprocal experiment was performed, holding Mg$_f$ constant and varying MgATP and ATP$_f$ together in constant ratio, the activities with ATP$_f$ and MgATP were not additive: when the activity with ATP alone was subtracted the data at different Mg$_f$ concentrations but the same MgATP concentration did not agree. Detailed kinetic analysis was not warranted by the precision of the data obtainable, considering the
Figure 10. Increment in ATPase activity due to MgATP at fixed ATP$_f$ concentrations of 0.05 mM (o) and 0.20 mM (x).
large number of activities and inhibitions which would have to be taken into account.

**Selective inhibition** Since the kinetic results could not readily resolve the two activities, I searched for ways to selectively inhibit one or the other. Iodination with lactoperoxidase and H$_2$O$_2$ (Morrison, 1974) and reaction with DABS (Fraenkel-Conrat, 1957) inhibited about equally with or without Mg$^{2+}$ in the assay. Neither oligomycin (27.5μM) nor ophiobolin A (0.03 mg/ml) inhibited either activity. Diethylstilbestrol (0.1 mM), a known inhibitor of the Mg-requiring oat root K$^+$ATPase, inhibited about 40% of the K$^+$ stimulated ATPase with MgCl$_2$, and about the same fraction of the activity without K$^+$. It did not inhibit without MgCl$_2$. Lower concentrations did not inhibit the K$^+$ stimulation (or else a small inhibition was masked by scatter; results with this inhibitor were typically of poor precision). Reductive alkylation with pyridoxal phosphate and sodium borohydride in HEPES buffer at pH 7.5 almost completely inhibited the activity with excess Mg$^{2+}$ in one experiment, but was ineffective in another. PHMB and DCCD both inhibited the background activity (the activity in the absence of KCl) but did not inhibit the K$^+$ stimulated ATPase activity much.
Figure 11. pH/activity profile of the partly-purified corn root K+ATPase determined in Tris and Bis-Tris buffers in the presence of 50 mM KCl and 67 mM NaClO₄.
ATPase activities of extracts made from root or shoot microsomes

K^+ATPases have been purified to near homogeneity from extracts of microsomes made from corn roots (Benson, 1977) and from corn shoots (Davis, 1979), and have been found to be very similar in their physical and enzymatic properties. Davis found that an ATPase could be purified from shoot microsomes, starting from extracts of the microsomes made with octyl glucoside or with sucrose dilaurate and KCl; it had its greatest activity at pH 7.5, and had no requirement for Mg^{2+}. I found that the root enzyme purified from NaClO₄ extracts according to Benson (1977) was likewise most active near pH 7.5, and had no requirement for Mg^{2+} (Table 5). This result, together with the pH/activity profiles of reconstituted extracts (which will be presented later in Figure 23) prompted the author to examine the pH/activity profiles of the extracts. Table 6 compares the ATPase activity of microsomes and extracts, at pH 6.5, with and without 1.5 mM MgCl₂. Figure 12 (A-C) shows pH/activity profiles of ATPase activities (assayed in the absence of Mg^{2+} with 0 mM, 50 mM, and 250 mM KCl) of root NaClO₄ extract (A) root octyl glucoside extract (B), and shoot octyl glucoside extract (C). The enzyme was incubated in MES-Imidazole buffers (.01M MES in the assay) with 0.3 mM ATP added to start the reaction. The root
Table 5. Activity of purified root ATPase with and without Mg^{2+}

<table>
<thead>
<tr>
<th>K^{+}</th>
<th>ATP</th>
<th>MgCl_{2}</th>
<th>ATPase activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>260 mM</td>
<td>0.15 mM</td>
<td>0.75 mM</td>
<td>60 mM</td>
</tr>
<tr>
<td>ATPase activity, %</td>
<td></td>
<td>260 mM</td>
<td>0.75 mM</td>
</tr>
<tr>
<td>0.75 mM</td>
<td>41</td>
<td>86</td>
<td>46</td>
</tr>
<tr>
<td>0.15 mM</td>
<td>69</td>
<td>88</td>
<td>64</td>
</tr>
<tr>
<td>0.00 mM</td>
<td>100^{a}</td>
<td>97</td>
<td>66</td>
</tr>
<tr>
<td>0.00 mM</td>
<td>97</td>
<td>101</td>
<td>54</td>
</tr>
</tbody>
</table>

with 1.0 mM Na_{2}EDTA

^{a}activity under standard conditions (260 mM K^{+} and saturating ATP) was 3.7 nmol/h, defined as 100%. 

Table 6. ATPase activities and protein content of fractions from extraction of shoot microsomes with 30 mM octyl glucoside, and from extraction of root microsomes with 0.50 M NaClO₄

<table>
<thead>
<tr>
<th>KCl</th>
<th>50 mM</th>
<th>0 mM</th>
<th>difference</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td>MgCl₂</td>
<td>1.5 mM</td>
<td>0 mM</td>
<td>difference</td>
</tr>
<tr>
<td>microsomes</td>
<td>11.0</td>
<td>8.6</td>
<td>2.4</td>
<td>11.6</td>
</tr>
<tr>
<td>pellet</td>
<td>9.3</td>
<td>8.8</td>
<td>0.5</td>
<td>14.8</td>
</tr>
<tr>
<td>extract</td>
<td>8.5</td>
<td>6.8</td>
<td>1.7</td>
<td>12.6</td>
</tr>
<tr>
<td>Root</td>
<td>MgCl₂</td>
<td>1.5 mM</td>
<td>0 mM</td>
<td>difference</td>
</tr>
<tr>
<td>microsomes</td>
<td>2.13</td>
<td>1.70</td>
<td>0.43</td>
<td>2.22</td>
</tr>
<tr>
<td>pellet</td>
<td>1.71</td>
<td>1.58</td>
<td>0.12</td>
<td>2.24</td>
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<tr>
<td>extract</td>
<td>20.6</td>
<td>18.6</td>
<td>2.0</td>
<td>19.3</td>
</tr>
</tbody>
</table>

^a81 g shoots.
^b33 g roots.
Figure 12. $K^+$ATPase activity (A-C) and background ATPase activity (D-F) of corn root NaClO$_4$ extract (A & D), corn root octyl glucoside extract (B & E), and corn shoot octyl glucoside extract (C & F)
NaClO₄ extract shows a pH optimum of 7.5; the octyl glucoside extracts have their greatest activity at pH 6. A NaClO₄ extract of root microsomes assayed in Bis-Tris and Tris chloride buffers with 0.75 mM MgCl₂ and 0.75 mM ATP had a profile similar to that of Figure 12A. Figure 12D-F shows the pH profiles of the same extracts assayed without KCl; these are the background activities which were subtracted from the activities obtained with KCl to get the K⁺-stimulated activities of Figure 12A-C. They are due, at least in part, to acid phosphatase.

Figure 13 shows the elution profiles of ATPase activities of an octyl glucoside extract of shoot microsomes, which I dialyzed against buffered 0.25 M NaClO₄ and fractionated on a small (0.9 x 20 cm) column of hexyl Sepharose. Elution with buffered 0.25 M NaClO₄ removed most of the sample from the column; 2.0 M buffered NaClO₄ then eluted a small peak of bound protein (Figure 13). This latter peak included some K⁺-stimulated ATPase (Figure 14) when assayed at pH 7.5, with and without 250 mM KCl. The earlier fractions, from the elution with buffered 0.25 M NaClO₄ contained a large peak of ATPase which was inhibited by 250 mM KCl under the same conditions. Assays of these earlier fractions at pH 6.0 without Mg²⁺ and pH at 6.5 with 0.75 mM ATP and 0.75 mM MgCl₂ both showed two overlapping peaks of ATPase activity.
Figure 13. Elution profile of shoot octyl glucoside extract fractionated on hexyl Sepharose: $A_{280}$ (x), DPH fluorescence (+), conductivity (o), and glycerophosphatase activity (Δ)
Figure 14. Elution profile of shoot octyl glucoside extract fractionated on hexyl Sepharose: 
K⁺ATPase activity measured at pH 7.5 with 0.3 mM ATP, + 250 mM KCl; at pH 6.0 with 0.3 mM ATP, + 50 mM KCl; and at pH 6.5 with 0.75 ATP and 0.75 mM MgCl₂, ± 50 mM KCl.
Fraction number (40 drops/fraction)

K⁺ stimulated ATPase mmol h⁻¹

- 0.30 mM ATP ± 250 mM KCl
  pH 7.5
- 0.30 mM ATP ± 50 mM KCl
  pH 6.0
- 0.75 mM ATP ± 50 mM KCl
  pH 6.5, ± 0.75 mM MgCl₂
stimulated by 50 mM KCl. The first coincided with a peak of DPH fluorescence (indicative of membrane lipid) and the other with a shoulder in the A_{280} profile. The ATPase of the first peak separated from the DPH fluorescence (which ran at the void volume) when the peak was fractionated by chromatography on Sepharose 4B, indicating that it was not tightly bound to the membranes. The second peak coincided with a peak of glycerophosphatase activity.

A third, smaller peak of K^+-stimulation measured at pH 6.0 or 6.5 was eluted just before the salt front.

The presence of the peak of activity (assayed at pH 7.5, without MgCl_2) which was eluted from the hexyl Sepharose column by buffered 2.0 M NaClO_4 confirms the results of Davis (1979). This activity, though presumably present in the octyl glucoside extracts, is not apparent in the pH profiles of Figure 12. The large peak of KCl-inhibited ATPase activity detected in the earlier fractions in assays at pH 7.5 suggests an explanation: such an activity would mask a smaller amount of K^+-stimulation in assays of the whole extract. The pH 7.5 K^+ATPase activity is seen to be distinct from the bulk of the K^+ATPase assayable at pH 6.0 or 6.5.
Properties of Purified Root $K^+$ATPase

Stability

When the $K^+$ATPase from corn root microsomes is solubilized by extraction of the membranes with NaClO$_4$ solution, and the extract is dialyzed against water or a buffer of low ionic strength, the activity declines rapidly, sometimes vanishing overnight. The presence of salt in the enzyme solution is undesirable in many experiments: it complicates the interpretation of KCl stimulation measurements. To investigate the conditions necessary for the stability of the solubilized ATPase, I dialyzed the partly-purified enzyme (Tipton et al., 1975) against buffered salt solutions of various pH and composition, and stored the dialyzed enzyme in the refrigerator (at about 5°C) or at room temperature. NaCl (0.25 M) and Na$_2$SO$_4$ (0.125 M) were as effective as 0.25 M NaClO$_4$ (all buffered at pH 8.0), giving about 86% recovery after nine days at either temperature. Threefold diluted solutions were less effective, and more enzyme was lost at room temperature than in the cold. Urea, a nonionic chaotropic agent, did not stabilize the enzyme at any concentration, and destroyed the activity at 3.0 M. In experiments with the hexyl-Sepharose-purified $K^+$ATPase, the neutral zwitterion glycine stabilized the activity, but was less effective.
than salts. In 0.25 M glycine, about half (54%) of the K⁺-stimulated ATPase was lost in three days, compared with no loss in 0.25 M NaClO₄ and 68% loss in 5 mM Tris/HCl pH 8.0 (the glycine and the NaClO₄ were dissolved in 5 mM Tris/HCl pH 8.0). In NaClO₄ or glycine solutions, stability increased with pH up to pH 8.7. In 1.0 M glycine pH 7.5, activity persisted for several days with negligible loss. This is sufficient to permit experimentation in the absence of inorganic salts.

Early experiments showed that lipids extracted from corn root microsomes by the procedure of Bligh and Dyer (1959), then dried and sonicated in buffer, stimulated the NaClO₄-extracted root K⁺-ATPase, as did sonicated soy phosphatides. Moreover, even in unsuccessful reconstitution experiments in which the ATPase did not seem to be associated with lipid vesicles, the presence of phospholipids frequently led to greater recovery of activity when purified ATPase from roots or shoots was subjected to conditions of low ionic strength. Octyl glucoside at 7 mM and at 20 mM stimulates the purified root ATPase two fold. Davis (1979) found that detergents such as octyl glucoside and sucrose dilaurate stimulate the purified shoot ATPase. These results indicate that the purified ATPases interact with lipids and detergents; this was investigated further.
to find suitable conditions for reconstituting the association of the purified ATPases with membranes.

Table 7 shows the results of an experiment in which shoot ATPase prepared by chromatography on hexyl-Sepharose was dialyzed against reconstitution buffer. The enzyme (0.3 mg/ml in buffered 1.0 M NaCl) was mixed with sonicated soy phosphatides, octyl glucoside, both octyl glucoside and soy phosphatides, or reconstitution buffer alone before dialysis.

Table 7. Effects of phospholipids and detergents on stability of the ATPase at low ionic strength

<table>
<thead>
<tr>
<th>Hours since start of dialysis</th>
<th>42</th>
<th>66</th>
<th>80</th>
<th>162</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffered NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Reconstitution buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>83</td>
<td>49</td>
<td>41</td>
<td>31</td>
</tr>
<tr>
<td>octyl glucoside</td>
<td>42</td>
<td>46</td>
<td>nd</td>
<td>19</td>
</tr>
<tr>
<td>soy phosphatides</td>
<td>&gt;100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>250</td>
<td>249</td>
<td>187</td>
</tr>
<tr>
<td>soy phosphatides + octyl glucoside</td>
<td>101</td>
<td>69</td>
<td>65</td>
<td>48</td>
</tr>
</tbody>
</table>

<sup>a</sup>Control activity was 29 μmol mg<sup>−1</sup> hr<sup>−1</sup>.

<sup>b</sup>Overshot.
Final concentrations were: protein, 0.09 mg/ml; lipid, 7.1 mg/ml; and octyl glucoside (before dialysis) 32 mM. After dialysis and storage at 5°C for the times indicated, the preparations were assayed at pH 7.5, with and without 0.25 M KCl, using 0.30 mM ATP as substrate. Octyl glucoside destabilized the activity, while soy phosphatides stabilized and stimulated it, giving more activity than a control kept in buffered 1.0 M NaCl. Octyl glucoside and soy phosphatides together gave partial stabilization: there was more activity than after storage in extraction buffer alone, but still a substantial loss.

**Stimulation by phospholipids**

To see how much of the increased activity of ATPase stored with soy phosphatides was due to stimulation by lipid carried into the assay with the enzyme, I assayed the samples stored in extraction buffer and in buffered NaCl with added lipid, adding the same amount (0.07 mg/assay) as was in the assays of enzyme stored with lipid. As Table 8 shows, both samples were stimulated about three fold by the added phosphatides. If the lipid in the dialyzed mixture stimulates to the same relative extent, the enzyme dialyzed and stored with lipid lost about 16% of its activity relative to enzyme stored in 1.0 M NaCl. Experiments not shown here established that gramacidin
Table 8. Stimulation of ATPase activity by soy phosphatides

<table>
<thead>
<tr>
<th>Storage medium</th>
<th>Phospholipids (in assay)</th>
<th>250 mM KCl</th>
<th>0 mM KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 M NaCl pH 8</td>
<td>absent</td>
<td>57.3</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>added</td>
<td>99.1</td>
<td>6.1</td>
</tr>
<tr>
<td>Reconstitution buffer</td>
<td>absent</td>
<td>15.1</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>added</td>
<td>39.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Reconstitution buffer with detergent</td>
<td>absent</td>
<td>9.2</td>
<td>0.05</td>
</tr>
<tr>
<td>present during dialysis</td>
<td>added</td>
<td>17.3</td>
<td>2.0</td>
</tr>
</tbody>
</table>

did not affect the activity of the enzyme dialyzed with lipid, that the enzyme dialyzed with octyl glucoside was stimulated 1.7 fold by added phosphatides, and that added phosphatides caused no further stimulation of the enzyme dialyzed with lipid.

Stimulation by salts

The finding that glycine preserves the ATPase activity without stimulating it made possible a set of experiments to assess the contributions of specific anion stimulation, specific cation stimulation and of ionic strength to the observed stimulation of the ATPase activity by salts.
I dialyzed purified root ATPase against 1.0 M glycine, pH 6.5 or 7.8, and maintained 0.20 M glycine in the assay mixtures. Preliminary experiments showed that histidine did not stimulate, so it was used as the buffer and the counterion to ATP in the experiments at pH 6.5. When I incubated ATPase purified by hexyl Sepharose chromatography with 0.75 mM ATP and 0.75 mM MgCl₂ at pH 6.5, the activity without KCl was 20% to 25% of that with 50 mM KCl. The activity was not due to contaminating cations: pooled aliquots of assay mixtures without added KCl contained 1.1 x 10⁻⁵ M K and 2.2 x 10⁻⁵ M Na, as determined by plasma emission spectrometry. The activity without KCl varied between different batches of enzyme, however, so some of it may have been due to contaminating enzymes. Sephacryl-S200-purified ATPase from roots and from shoots retained a trace of activity (about 5% of the 50 mM KCl activity) at pH 7.5 with imidazole (also nonstimulating, see below) as the sole cation. Figure 15 compared stimulation by KCl and K₂SO₄ of hexyl-Sepharose-purified root ATPase at pH 6.5. Figure 16 compares stimulation by KCl, KHCO₃ (potassium formate), K₂SO₄, LiCl, and Li₂SO₄ of Sephacryl-S200-purified ATPase at pH 7.8. The buffer was 10 mM HEPES containing 10 mM K⁺ or Li⁺ and 0.02 mg/ml BSA. At pH 6.5, stimulation by KCl increases over the whole range of concentrations.
Figure 15. Activity of the root $K^+\text{ATPase}$ at several $K^+$ concentrations, with $SO_4^{2-} (\Delta)$ or $Cl^- (o)$ as the counterion.
Figure 16. Activity of the root K\textsuperscript{+}ATPase at pH 7.5 as a function of K\textsuperscript{+} or Li\textsuperscript{+} concentration, with S\textsubscript{0}\textsuperscript{2-}, Cl\textsuperscript{-}, or H\textsubscript{CO}\textsubscript{3}\textsuperscript{-} (formate) as the counterion
tested. \(K_2SO_4\) stimulates more than KCl from 25 mM to 125 mM. Above that level, activity with \(K_2SO_4\) levels off. Both curves at pH 6.5 are concave upward at low salt concentrations, suggesting cooperativity. At pH 7.8, all the salt stimulation curves were sigmoid (Figure 16). As at pH 6.5, sulfate salts stimulated more effectively than chloride salts at low concentrations, but less effectively at higher concentrations; the leveling off was more abrupt at the higher pH. The curve with KHC\(O_2\) was similar to that with KCl. The curves obtained with lithium salts were qualitatively similar to those with potassium, but the activities were lower and the concentrations needed to obtain half-maximal activity were higher.

These data confirm that at least part of the KCl stimulation is due to specific activation of the ATPase by K\(^+\), and that the enzyme does not discriminate between monovalent anions of different size and shape. The effect of \(SO_4^{2-}\) appears to be two-fold: enhanced activity at low concentrations gives way to sharply reduced activity at higher concentrations. The activity is more readily saturated with salt at pH 7.8 than at pH 6.5.

Figure 17 is a Hill plot of the data from three experiments in which KCl was varied: the two from Figure 16 and a third set at 50 mM and below. To compute the monovalent cation concentration at each point, I
Figure 17. A Hill plot of $K^{+}$ATPase activity at various KCl concentrations, combining data from three experiments.
added 15 mM (10 mM K from the buffer, 3 mM K from the ATP, and 2 mM Na from the glycine buffer) to the amount of KCl added to the reaction mixture. The activity at 0 mM monovalent cation concentration was extrapolated from the activity with no added KCl, using the average increase between 0 mM and 10 mM KCl added to estimate the slope. Maximal activity was estimated from a double-reciprocal plot of the data at high KCl. Since the estimated activity without monovalent cations was subtracted from the total activity, and these estimates were based on the data at 15 mM total monovalent cation concentration, this point is common to all three sets of data. The resulting plot can be represented well by two straight lines: below 90 mM, the slope is 2.9; above 90 mM the slope is 1.1.

If a single straight line is drawn through the points, its slope is 2.2. The points from the different experiments do not agree well in absolute activity, but each set is a good fit to lines of the given slopes intersecting at 90 mM total cation concentration.

Figure 18 shows the effect of increased concentration of imidazole/HCl buffer (pH 7.5) on the activity of hexyl-Sepharose-purified ATPase from roots. With no other monovalent cations present, imidazole does not stimulate the ATPase activity. With 50 mM KCl present, however, total activity increased by 1/3 and K⁺ stimulation
Figure 18. Activity of the root $K^+$ ATPase in imidazole/HCl buffers of various concentrations, with and without KCl.
increased by 1/2 when imidazole was increased from 5 mM to 50 mM. This result suggests that increased ionic strength enhances stimulation by $K^+$. 

**Aggregation**

Preliminary gel filtration experiments suggested that the purified root and shoot ATPases aggregated under some conditions at low ionic strength. I investigated this aggregation by dialyzing aliquots of S200 root ATPase against buffered salt solutions of various compositions and then chromatographing them on a column of Sepharose 4B in the same solution. Figure 19 shows the elution profile this author obtained from a column run in 1.0 M glycine with 10 mM NaOH, pH 7.8. ATPase activity was assayed at pH 7.8 (HEPES buffer) with 0.75 mM ATP, 0.75 mM MgCl$_2$, and 0.25 M KCl. Data from the other elution profiles are summarized in Table 9. These profiles usually consisted of one or more peaks corresponding in position to peaks in Figure 19. Peak 1 eluted at the void volume, peak 2 eluted at an intermediate position, and peak 3 eluted at the position expected for monomeric $K^+$ ATPase. The remaining two peaks represent enzyme which bound to the gel and was retained on the column past the salt volume (that volume of buffer necessary to elute salts and other small molecules which are not excluded at all from the gel;
Figure 19. Elution profile of purified root K^+ATPase fractionated on Sepharose 4B in 1.0 M glycine pH 7.8, showing the locations of the peaks listed in Table 8.
Table 9. Summary of elution profiles from aggregation experiments

<table>
<thead>
<tr>
<th>Buffer</th>
<th>NaCl</th>
<th>Peak</th>
<th>( K_{av} )</th>
<th>Area mol/hr</th>
<th>% total recovered activity</th>
<th>Est. % overall recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.40</td>
<td>3</td>
<td>1.00</td>
<td>5.60</td>
<td>100</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>3</td>
<td>0.98</td>
<td>0.80</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>-</td>
<td>2.68</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>-</td>
<td>0.96</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>-</td>
<td>4.44</td>
<td>100</td>
<td></td>
<td>74</td>
</tr>
<tr>
<td>1.25 mM EDTA, 1.25 mM NaCl, pH 8.0 with Tris</td>
<td>0.10</td>
<td>3</td>
<td>0.85</td>
<td>0.15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>-</td>
<td>0.03</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>-</td>
<td>3.66</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>-</td>
<td>3.84</td>
<td>100</td>
<td></td>
<td>71</td>
</tr>
<tr>
<td>1.00 M Glycine, 10 mM NaOH pH = 7.8</td>
<td>0.40</td>
<td>3</td>
<td>1.08</td>
<td>5.00</td>
<td>100.0</td>
<td>nd</td>
</tr>
<tr>
<td>0.10</td>
<td>1</td>
<td>-0.06</td>
<td>0.039</td>
<td>4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.69</td>
<td>0.011</td>
<td>1.3</td>
<td></td>
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<td></td>
<td>3</td>
<td>1.06</td>
<td>0.067</td>
<td>8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>-</td>
<td>0.000</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>-</td>
<td>0.70</td>
<td>86.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>-</td>
<td>0.81</td>
<td>100.</td>
<td></td>
<td>73</td>
</tr>
<tr>
<td>1.00 M Glycine, 10 mM NaOH pH = 7.8</td>
<td>0.050</td>
<td>1</td>
<td>0.02</td>
<td>0.014</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>0.26</td>
<td>0.022</td>
<td>3</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>0.76</td>
<td>0.29</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>-</td>
<td>0.017</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>-</td>
<td>0.53</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>-</td>
<td>0.87</td>
<td>100</td>
<td></td>
<td>73</td>
</tr>
<tr>
<td>none</td>
<td>1</td>
<td>0.00</td>
<td>0.15</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.61</td>
<td>2.26</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.83</td>
<td>0.23</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>-</td>
<td>0.56</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>-</td>
<td>4.90</td>
<td>61</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>-</td>
<td>8.10</td>
<td>100</td>
<td></td>
<td>127</td>
</tr>
</tbody>
</table>

Shoot ATPase

| Extraction buffer | 1  | +0.07 | <0.06 | <8  |
|                  | 3  | 0.91  | 0.26  | 40  |
|                  | B2 | -     | 0.38  | 60  |
| total            | -  | 0.65  | 100   | nd  |

Reconstitution buffer

| B2 | 0.17 | 100 | nd |

nd = not determined
measured in my experiments as the elution position of inorganic phosphate). The peak designated Bound 1 is considered to include all enzyme retained past the salt volume and eluted with the original buffer; Bound 2 is the peak of enzyme eluted by washing the column with buffered 1M NaCl.

In buffered 0.40 M NaCl, all the enzyme eluted as monomer. Progressive reductions of the NaCl concentration led to binding of more enzyme to the column, but produced no evidence of aggregation. Binding to the column limited the range of salt concentrations tested.

With 1.0 M glycine present at pH 7.8, 0.4 M NaCl again produced the monomer peak. Aggregates were detected in glycine buffer when NaCl was 100 mM, 50 mM or absent. The presence of 1.0 M glycine led to less binding to the column than was seen with the other buffers. In both sets of experiments, recovery of the enzyme activity applied was 70% or greater.

Similar experiments were performed with purified shoot ATPase in reconstitution buffer and extraction buffer, in preparation for reconstitution experiments. There was little or no aggregation but considerable binding to the column. Recovery of activity was poor.
Adsorption to gels

As noted above, the root and shoot ATPases bind to Sepharose 4B at low ionic strength. Binding is not limited to Sepharose: I have observed the root enzyme to bind to Sephadex G15, Bio-Gel P2, and Sepharose 2B, and the purified shoot ATPase to bind to Sephadex G100 and Sephacryl S200. M. H. Davis and C. L. Tipton (personal communication) observed the shoot enzyme to bind to Sepharose 2B and 4B, and to Bio-Gel A1.5 M. In all these cases, the enzyme bound to the gel at an ionic strength of about 0.1 or less, and was released from the gel as a sharp peak upon washing with buffered 1.0 M NaCl. When the conductivity was monitored, the enzyme began to elute in the first fractions having increased conductivity.

Electrophoretic mobility

In sucrose gradient electrophoresis (in 1.0 M glycine buffer, pH 7.8), the purified root ATPase migrated toward the anode (the positive electrode). Its mobility was about $6 \times 10^{-5} \text{cm}^2\text{V}^{-1}\text{s}^{-1}$ (uncorrected, at 4°C in a 5% to 25% w/v sucrose gradient). Migration was also toward the anode at pH 6.8. Hence the enzyme is anionic at neutral pH, and the binding to the gels (which tend to carry a weak negative charge) is not due to simple electrostatic effects.
Reconstitution of Membrane-bound ATPase

If the $K^+$-ATPase could be incorporated into the membrane of a lipid vesicle, it could then be tested for ability to transport ions across the membrane in the presence of ATP. Ion translocation would result in the appearance of a concentration gradient or an electrical potential across the membrane. I first sought procedures whereby the ATPase could be stably incorporated into an artificial lipid membrane.

Reconstitution from extract

When octyl glucoside extracts of shoot microsomes are combined with soy phosphatidès (solubilized in octyl glucoside solution) and the mixture is dialyzed against extraction buffer (or reconstitution buffer), the clear mixture becomes turbid as the lipids form membrane vesicles. Such vesicles are excluded from Sepharose 4B. The elution profile of Figure 20 shows that $K^+$-ATPase (measured at pH 6.5 as stimulation of activity by 50 mM KCl, using 0.3 mM ATP as substrate) is associated with the lipid peak, and enriched in that peak relative to unstimulated ATPase and protein. A similar profile was obtained from a sucrose dilaurate extract of CaCl$_2$ precipitated microsomes, likewise reconstituted with lipids solubilized in octyl glucoside. Extraction buffer was the eluant in both
Figure 20. Elution profile of shoot octyl glucoside extract, reconstituted with soy phosphatides and fractionated on Sepharose 4B
Sepharose 4B

- $K^+\text{ATPase}$
- Unstimulated ATPase
- Liposomes

ATPase, nmol Pi/hour

Conductivity, mho, 1:100 dilution

Fraction number
experiments. Liposomes were detected by DPH fluorescence. Figure 21 shows profiles obtained from concave sucrose density gradients centrifuged 22.5 hours. The samples were an aliquot of the same preparation applied to the column of Figure 20, and aliquots of fractions 13 and 40 from the column. Column fraction 13 gave a single peak at low density (Figure 21B); fraction 40 gave a single peak at high density (Figure 21C). The original reconstituted extract gave a profile approximating the sum of the profiles from fractions 13 and 40 (Figure 21A). As in the gel filtration experiment, K\textsuperscript{+}ATPase was enriched in the liposome peak. Root extract, reconstituted by the same procedure, gave a similar pattern (Figure 22A). Figure 22b shows a liposome sample run as a control, and the sucrose profile of these gradients. Figure 22B shows the pH profiles of a reconstituted shoot extract preparation, made by the above procedure and isolated as the V\textsubscript{o} fraction from Sepharose 4B chromatography. Activity was measured in MES/imidazole and HEPES/imidazole buffers with 0.3 mM ATP as substrate.

Reconstitution of purified ATPases

Several procedures were employed in attempts to reconstitute the purified ATPases. In one set of experiments, I dialyzed concentrated shoot
Figure 21. Sucrose density gradient profiles of reconstituted shoot extract and fractions from Sepharose 4B chromatography
Figure 22. Sucrose density gradient profiles of reconstituted root ATPase, and of control liposomes made from soy phosphatides alone.
Figure 23. pH profile of background ATPase activity and K$^+$ATPase activity of reconstituted shoot extract
ATPase (purified by chromatography on hexyl Sepharose) against reconstitution buffer, then combined it with lipids solubilized in octyl glucoside and dialyzed the mixture against reconstitution buffer at room temperature. This method closely resembles the one used to reconstitute the ATPase of octyl glucoside extracts. The results depended on the lipid used. After such treatment, the ATPase could be separated from egg phosphatidylcholine vesicles by gel filtration or density gradient centrifugation. When soy phosphatides were used, the enzyme appeared to be associated with the lipid in density gradients, but recovery of activity was low.

I also tried the freeze-thaw-sonication method of Kasahara and Hinkle (1977) which Davis (1979) reported led to reconstitution of the shoot ATPase. The author dialyzed S200-purified shoot ATPase against extraction buffer containing 20 mM KCl, then combined it with soy phosphatides sonicated in the same buffer. The author let the mixture stand 10 min at room temperature, then rapidly froze it in a dry-ice/ethanol bath. When the dark area in the center of the frozen mixture had disappeared and it had popped away from the walls of the test tube, it was set in a rack on the bench to thaw and warm to room temperature, then sonicated at 45s to break up any large aggregates.
I treated four samples as described above, stopping at a different point in the procedure with each, and applied them to concave sucrose density gradients. After centrifuging the gradients 24 h and fractionating them, the author obtained the activity and lipid profiles of Figure 24. Dialyzed enzyme alone was applied to gradient A; there was marked loss of activity but a peak was detectable in fractions 15 and 16 (at about 21% sucrose w/w). Enzyme and lipid mixed and fractionated without further treatment (gradient B) gave better recovery of activity; the peak was again centered on fractions 15 and 16. The liposomes banded at about 13% sucrose w/w. Enzyme and lipid, mixed, frozen, and thawed, gave identical profiles with or without sonication. The ATPase peak was now centered on tube 14 (17% sucrose w/w), overlapping but resolved from the lipid peak (gradients C and D). Thus, the position of the ATPase band is somewhat altered by the freezing and thawing in the presence of soy phosphatides, but it still does not coincide with the lipid peak.

Two 1 ml portions of a similar preparation were centrifuged 22 h in shallower preparative gradients (Figure 25). The ATPase-containing fractions were pooled and recentrifuged 22.5 h in standard concave gradients. Fractionation and assay showed the ATPase peak at
Figure 24. Interaction of the shoot $K^+$ ATPase with lipids in a freeze-thaw-sonication experiment. Sucrose gradient profiles: A, ATPase alone; B, ATPase mixed with liposomes; C, ATPase + liposomes, frozen and thawed; D, ATPase + liposomes, frozen, thawed, and sonicated
Figure 25. Interaction of the ATPase with lipids in a freeze-thaw-sonication experiment:
A. Preparative sucrose density gradient centrifugation of a larger sample of lipid-treated ATPase
B. Re-fractionation of the lipid-treated ATPase on an analytical sucrose density gradient
fractions 15-16 (21% sucrose w/w) with no accompanying peak of DPH fluorescence (Figure 25B). The pooled ATPase fractions retained good activity for a month at low ionic strength in the refrigerator. In this and other experiments, soy lipids seem to preserve the ATPase activity, even though the enzyme is not tightly associated with the bulk of the lipid. The ATPase may bind a small amount of lipid tightly. If the acyl chains did not form an apolar fluid domain, similar to the interior of a lipid bilayer, they would not be detected by DPH fluorescence enhancement. Alternatively, the enzyme may associate reversibly with the liposomes in the freeze-thaw procedure. The activity might have been preserved at low ionic strength by an amount of lipid too small to be detected in the second gradient. Some association of the enzyme with lipid is indicated by the alteration of its position in the gradient by freezing and thawing with lipid present.

Brief sonication (10 min) of root ATPase with soy liposomes in 1.00 M glycine pH 7.8, followed by chromatography on Sepharose 2B and assay of fractions gave a profile which showed that some of the ATPase was associated with the lipid peak at the void volume. Recovery of activity was much better with lipid than without. Longer sonication, with or without lipid, tended to destroy the activity.
When root ATPase was incubated at room temperature with soy liposomes made by the butanol injection method in 0.10 M buffered NaCl or in 1.00 M glycine pH 7.8, 5 to 20% of the activity was eluted from Sepharose 2B with the lipid peak (at the void volume). A similar result was obtained by sucrose gradient electrophoresis when sonicated soy liposomes (4.0 mg in 0.4 ml 1.00 M glycine pH 7.8) and root enzyme (in 0.6 ml buffered 1.00 M NaCl) were combined and dialyzed against 1.00 M glycine pH 7.8. Most of the enzyme ran with its usual mobility although as a broader peak, but a small peak of activity accompanied the liposomes at a mobility of $11 \times 10^{-5}$ cm$^2$V$^{-1}$s$^{-1}$ (Figure 26).

**Salt dialysis** More extensive reconstitution was achieved by combining 0.12 mg shoot ATPase (purified by hexyl-Sepharose chromatography) in 0.4 ml of buffered 1.00 M NaCl with 10 mg soy lipids (sonicated) in 1.0 ml of reconstitution buffer, and dialyzing the mixture overnight against reconstitution buffer at 5°C. Figure 27 shows the Sepharose 4B elution profile of such preparation. Peaks of ATPase accompany both peaks of liposomes. No free ATPase was observed, but about 30% bound to the column and was eluted by buffered 1.00 M NaCl. Figure 28 shows the Sepharose 4B elution profile of a similar preparation, made with liposomes which were
Figure 26. Sucrose gradient electrophoresis of the root ATPase after incubation with soy phosphatide liposomes.
Figure 27. Elution profile of partly purified shoot K^+ ATPase reconstituted by salt dialysis and fractionated on Sepharose 4B
Figure 28. Sepharose 4B elution profile of shoot ATPase reconstituted by salt dialysis with soy phosphatide liposomes having a larger average size than those of Figure 27.
not so thoroughly sonicated. Most of the lipid, and most of the enzyme which did not bind to the column, were eluted at the void volume; a small peak of lipid and enzyme was retained \((K_{av} = 0.35)\). A larger fraction of the ATPase bound to the gel in this experiment than in the preceding one. In both experiments, the ratio of ATPase to lipid (DPH fluorescence) was greater in the void volume peak than in the retained peak. Since the distribution of ATPase was changed by a change in the elution profile of the liposomes, it is clear that binding to the lipid rather than aggregation is responsible for its exclusion from the gel.

The preparation used for Figure 28 was also fractionated by centrifugation in a concave sucrose density gradient for 24 h. The resulting ATPase and lipid profiles are shown in Figure 29. ATPase again accompanied the liposomes. The peak of activity was offset about 1/2 fraction down the gradient from the peak of liposomes. This may result from increased density of those vesicles associated with protein. Lower levels of activity were present down to the bottom of the gradient, confirming that not all the enzyme was associated with the liposomes.

Properties of the reconstituted ATPase

**pH profile** To prepare a larger batch of reconstituted ATPase for further study, the author sonicated 68 mg of
Figure 29. Sucrose gradient profile of shoot ATPase reconstituted by salt dialysis
soy phosphatides in 1.0 ml reconstitution buffer. I combined the lipids with concentrated shoot ATPase (0.30 mg protein in 1 ml buffered 1.00 M NaCl), dialyzed the mixture against reconstitution buffer, and fractionated it on Sepharose 4B.

I pooled and concentrated the fractions from both liposome peaks to 2 ml by ultrafiltration using an Amicon PM30 membrane. Figure 30B shows the pH/activity profile of that preparation, assayed in MES-imidazole and HEPES-imidazole buffers with 0 mM, 50 mM, or 250 mM KCl present, along with 0.30 mM ATP as substrate. Figure 30A shows the activity of the original ATPase (prepared by chromatography on hexyl-Sepharose) which was used to make the reconstituted preparation. The curves representing stimulation by 250 mM KCl are similar. The pH optimum for K⁺ stimulation of the reconstituted shoot ATPase shifts from 7.5 to 8.0 when the KCl concentration is reduced from 250 mM to 50 mM, but the pH optimum for K⁺ stimulation of the original ATPase remains at 7.4. There is much less unstimulated activity in the reconstituted preparation than in the original concentrated ATPase. A similar reduction in background activity can be obtained by simply adding sonicated soy phosphatides to hexyl-Sepharose-purified ATPase (Table 8). Addition of soy phosphatides to ATPase purified further by Sephadryl S200
Figure 30. pH/activity profile of partly purified shoot K⁺ATPase before (A) and after (B) reconstitution by salt dialysis with soy phosphatide liposomes, showing stimulation by 50 mM (+) and 250 mM (x) KCl, and activity without KCl (o)
chromatography does not reduce the background activity, which is already much less than that of the ATPase from the hexyl-Sepharose chromatography step.

**Resolubilization** The similarity of the pH profiles of stimulation by 250 mM KCl of the hydrophobic and reconstituted hydrophobic ATPases suggested that the enzyme might be resolubilized in the assay. To test this possibility, I applied the remaining concentrated reconstituted ATPase to the Sepharose 4B column, which was equilibrated and eluted with 0.25 M KCl in 0.010 M K Hepes, pH 7.5.

This medium simulates the composition of the assay mix. Figure 31 shows the elution profile. A substantial peak of free ATPase shows that this medium can indeed dissociate the enzyme from liposomes. Some liposome-bound ATPase was also present.

When shoot octyl glucoside extract reconstituted with soy phosphatides by the octyl glucoside dialysis method and purified by Sepharose 4B chromatography in reconstitution buffer was rechromatographed in 0.25 M KCl, 10 mM K HEPES pH 7.5, K⁺-stimulated ATPase (assayed at pH 6.0 as stimulation of hydrolysis of 0.3 mM ATP by 50 mM KCl) was recovered at the void volume. No K⁺-stimulated ATPase was found in any later fraction, free or adsorbed, in assays at pH 6.0 ± 50 mM KCl or in assays at pH 7.5 ± 250 mM KCl, indicating
Figure 31. Sepharose 4B elution profile of reconstituted shoot K⁺ATPase in 0.25 M KCl, 10 mM HEPES, pH 7.5
that the reconstitution of ATPase activity in these preparations is stable to salt, in contrast to the reconstitution of the purified ATPase.

Transport and Permeability Measurements

**Reconstituted extracts**

I tested several reconstituted preparations for cation permeability and for ATP driven cation transport, using either the external pH (glass electrode) or the membrane potential (carbocyanine fluorescence) method, or both. Shoot membrane extracts reconstituted with soy phosphatides by the octyl glucoside dialysis method were generally permeable to protons and potassium ions: vesicles loaded with KCl and transferred by Sephadex G25 chromatography to weakly buffered media of low KCl concentration did not change the external pH on addition of the ionophores CCCP and valinomycin or of either alone. Control vesicles without protein took up protons under these conditions, giving pH increases several hundredths of a unit. Shoot extracts reconstituted with soy phosphatides (1.5 mg/ml extract) in reconstitution buffer in the presence of di-O-C3-(5) (.43 mg/mg lipid), freed of excess dye by Sephadex G25 chromatography and diluted into low-potassium reconstitution buffer did not generate a demonstrable membrane potential upon addition of valinomycin, although control liposomes made
without extracts did. Similar results were obtained by reconstituting in the absence of dye and adding it to the assay. Addition of EDTA to the reconstitution buffers did not alter the leakiness, and reconstituted root extracts were also leaky. Accordingly, one would not expect ATP-driven cation transport to be detectable: any membrane potential or pH gradient would spontaneously dissipate. Nevertheless, I tested these preparations for development of a membrane potential in response to ATP, with or without MgCl₂. No potential was observed, whether or not CCCP was added to convert pH gradients to membrane potentials.

When egg phosphatidylcholine was substituted for the soy lipids and the lipid:protein ratio was doubled, tight vesicles sometimes resulted. Such vesicles, diluted into low KCl reconstitution buffer or into Na⁺-Mg²⁺-citrate buffer containing di-O-C₃-(5), responded to valinomycin by generating a membrane potential which was visible as a decrease in fluorescence lasting several minutes. MgATP rapidly reversed this decrease. The vesicles seemed to have some permeability to K⁺ without added ionophores: when they were diluted to low KCl buffer, the initial fluorescence declined by several per cent in the first minute. Dilution in reconstitution buffer (so there was no K⁺ gradient) gave no such decline. MgATP also reversed this initial decline; subsequent addition of
valinomycin gave a normal fluorescence decrease. These responses are shown in the recorder tracings of Figure 32.

Such results are suggestive of a reconstituted ion pump delivering $H^+$, $K^+$, or some other cation into the liposomes, although the observed effect is small. The fluorescence changes shown are the most pronounced that I obtained. Two other preparations also showed reversal of the valinomycin effect by ATP, but I have subsequently been unable to obtain tight vesicles from reconstituted extracts. Such changes are not observed in egg phosphatidylcholine vesicles, or in 90% egg phosphatidylcholine/10% soy phosphatide vesicles, or (as noted below) in preparations of purified ATPase reconstituted with 90% egg phosphatidyl choline and 10% soy phosphatides.

Reconstituted purified ATPase

Purified shoot ATPase, combined with sonicated soy phosphatides, frozen, thawed, and briefly sonicated, then put into low KCl buffer by Sephadex G25 chromatography, was tested for vesicle permeability and cation transport by the glass electrode method. The vesicles were tight: valinomycin alone gave a slow pH rise, which was greatly accelerated by CCCP. Addition of ATP to such preparations caused a slow pH decrease, with or without MgCl$_2$. Since the pH decrease was not reduced by ionophores, it was not
Figure 32. di-0-C₃-(5) fluorescence trace from a preparation of shoot extract reconstituted with egg PC: effect of MgATP and valinomycin
due to $H^+$ release by the vesicles, but rather to protons generated by ATP hydrolysis.

Reconstitution of concentrated ATPase from the hexyl-Sepharose chromatography step with soy phosphatides or with a mixture of 10% soy phosphatides and 90% egg phosphatidyl choline by the octyl glucoside dialysis method gave tight vesicles when they were kept at room temperature. Permeability was measured by the carbocyanine fluorescence method. No ATP-dependent membrane potentials were detected with or without ionophores or MgCl$_2$. A small ATP-dependent fluorescence decrease (which would ordinarily indicate a potential negative inside the vesicles) was detected in several experiments with soy phosphatides, but adenylylimidodiphosphate, pyrophosphate, tripolyphosphate, and EDTA all produced similar effects, and the decrease was reversed by MgCl$_2$. Such decreases are likely due to chelation of membrane-bound divalent cations, with an accompanying change in the surface potential leading to increased dye binding. A similar but smaller decrease occurred in control soy phosphatide vesicles made without enzyme.

Reconstitution of concentrated, hexyl-Sepharose-purified ATPase from shoots by the salt dialysis method likewise gave vesicles (this time with good recovery of
ATPase) which were impermeable to $H^+$ and $K^+$ but did not respond to ATP.
DISCUSSION

ATPase Activities of Corn Membranes

Mg-dependent and Mg-independent activities of plasma membranes

Since the $K^+\text{ATPase}$ of oat root membranes has been shown conclusively to require $Mg^{2+}$ or some other divalent cation for activity (Balke and Hodges, 1975), and a similar enzyme with apparent requirement for $Mg^{2+}$ has been found in corn (Leonard and Hotchkiss, 1976), the finding (Davis, 1979, and this dissertation) that the isolated ATPases from corn roots and shoots did not require $Mg^{2+}$ was surprising.

The enzymes were originally isolated on the basis of assays which included 0.75 mM MgCl$_2$ and 0.75 mM ATP in the reaction mixture, conditions established to give maximum activity of the ATPase in the NaClO$_4$ extract of corn root microsomes. This mixture contains 0.28 mM ATP$_f$ at pH 6.6, assuming a pH-adjusted $K_d$ of 6.5 mM$^{-1}$ (Davis, 1979). This is adequate for full activity of the purified ATPases, as the $K_m$ for ATP is about 0.03 mM (Davis, 1979; Tipton, Karl and Topping, unpublished results).

The pH optima (pH 7.5 to 7.8) of the purified enzymes are at least a full pH unit higher than the pH optimum of the $K^+Mg^{2+}\text{ATPase}$ studied by Leonard and Hotchkiss (1976), but are also one pH unit higher than the optimum for ATP$_f$ hydrolysis by shoot microsomes. This raises the question
of whether the pH optimum shifted during solubilization and purification, or whether the enzyme assayed on the microsomes was distinct from the enzyme isolated. Hydrolysis of ATP_f by shoot microsomes is inhibited by high concentrations of substrate, so it seemed plausible that addition of 3 mM MgCl_2 to 3 mM ATP (which would reduce the ATP_f concentration to 0.64 mM) might give an apparent Mg^{2+} stimulation by releasing the substrate inhibition. Davis (1979) believed that the ATP_f hydrolyzing activity of shoot microsomes represented the only K^+-stimulated ATPase present.

I investigated the K^+Mg^{2+}ATPase and K^+ATPase activities of root plasma membranes to see if they were due to distinct enzymes. To avoid the problem of ATP_f in equilibrium with MgATP, the author used excess MgCl_2 and kept the ATP concentration low (0.30 mM) in routine assays; 1.2 mM Mg_f would be sufficient to hold ATP_f below 0.035 mM at pH 6.5, so the activity hydrolyzing free ATP would be considerably reduced (Davis found a K_m of 0.15 mM for ATP with shoot plasma membranes; I was unable to determine a Km for root plasma membranes because of pronounced substrate inhibition but my results show that hydrolysis of ATP_f would be quite low under these conditions).
The kinetic data are consistent with the presence of at least two ATPases, one hydrolyzing ATP with a relatively low Km, and the other hydrolyzing MgATP with a higher Km (similar to the values of about 1 mM reported for the K\(^{+}\)Mg\(^{2+}\)ATPase; Balke and Hodges, 1975; Leonard and Hotchkiss, 1976; DuPont et al., 1981). The marked substrate inhibition precluded detailed analysis. At the minimum, it would have been necessary to consider K\(^{+}\)-dependent and K\(^{+}\)-independent hydrolysis of ATP and of MgATP, along with substrate inhibition by ATP and by MgATP or Mg\(_{f}\) or both. Since the K\(^{+}\)-stimulation is determined by difference, this would have required extremely precise data.

Although experiments with other inhibitors were not very informative, DES proved to be selective for inhibition of hydrolysis of MgATP, both with and without KCl. Since it has now been shown (Balke and Hodges, 1976; DuPont et al., 1981) that a substantial part of the MgATPase activity measured without KCl is due to the K\(^{+}\)Mg\(^{2+}\)ATPase (which is inhibited by DES), this probably represents inhibition of a single enzyme. This K\(^{+}\)Mg\(^{2+}\)ATPase is presumably the enzyme studied by these investigators and by Leonard and Hotchkiss (1976). Although Davis (1979) found that the purified shoot K\(^{+}\)ATPase was inhibited by DES, he found little or no DES inhibition of ATP\(_{f}\) hydrolysis by the membrane-bound activity. Inhibition would probably
have been more pronounced in my experiments if the ATP concentration had been higher, since 0.3 mM is below the Km of the K⁺Mg²⁺ATPase (Leonard and Hotchkiss, 1976).

**Does the pH optimum change during purification?**

The pH profile of the K⁺ATPase activity of NaClO₄ extract indicated that the enzyme form purified by Benson (1977) was present, and that no major shift in its pH optimum accompanied its further purification. The octyl glucoside extracts, however, have low pH optima (about pH 6) for stimulation by 50 mM KCl, and 250 mM KCl was inhibitory. The presence of an ATPase with a higher pH optimum was not apparent, even though Davis had purified the shoot K⁺ ATPase from octyl glucoside extracts made by the same procedure (as well as from other detergent extracts). This might be taken for evidence of a change in the pH optimum of the K⁺ATPase during purification, but the presence of ATPase activity inhibited by KCl at pH 7.5 hinders interpretation. KCl-inhibited and KCl-stimulated activities will subtract from each other in an assay based on the difference in ATPase activity with and without KCl. Fractionation of the octyl glucoside extract by hexyl Sepharose chromatography separated the pH 7.5 activity from the species with activity at lower pH. The marked inhibition of the unretained ATPase activity by 250 mM KCl at pH 7.5 is sufficient to account for the absence
of the pH 7.5 K⁺ATPase in the pH profile of the whole extract. The activity of the pH 7.5 K⁺ATPase may be low because of its instability in octyl-glucoside-containing buffers.

It remains possible that hexyl Sepharose or NaClO₄ breaks up a complex composed of the K⁺ATPase and one or more other proteins in the octyl glucoside extract with alteration of the properties of the ATPase. However, the gentler treatment of dialysis of the extract against buffered 0.10 M NaCl and fractionation by chromatography on Sephacryl S200 gives a peak of K⁺ATPase at the expected position (Kav 0.50) for the purified K⁺ATPase. At present there is no reason to believe that the purified K⁺ATPase undergoes any major change in its properties during solubilization and purification. Simple reassociation with phospholipids raises rather than lowers the pH optimum for stimulation by 50 mM KCl, an effect which could be expected on simple electrostatic grounds, since the phospholipid vesicles bear a negative surface charge (the local pH will be lower than the bulk pH). Further resolution of the question of assaying the pH 7.5 ATPase in the presence of interfering activities will require use of a specific inhibitor. Antibody to the purified ATPase might be a suitable inhibitor. Preparation of such antibodies is planned (S. G. Carter, personal communication).
Relationship to other ATPases

The solubilization and partial purification of the corn root K⁺Mg²⁺ATPase (DuPont and Leonard, 1980; DuPont et al., 1981) has made it clear that that enzyme is distinct from the purified pH 7.5 K⁺ATPase. The K⁺Mg²⁺ATPase retains its requirement for Mg²⁺ or another divalent cation, it has a high Km for ATP, and its pH optimum is about 6.5, but it is stimulated only about 20% by KCl. The preparation contains no polypeptide of M = 30,000, but is enriched in a polypeptide of M = 100,000. It shows numerous other similarities to the plasma membrane ATPases of yeast and Neurospora, including high activity with cobalt ATP.

As mentioned earlier, I found that the purified corn root K⁺ATPase of Benson (1977) does not require Mg²⁺; the activity is not due to traces of magnesium in the reagents, since EDTA is not inhibitory. None of the well-characterized transport ATPases have this property.

Myosin, however, has an ATPase activity which is activated by potassium in the absence of Mg²⁺ (Offer, 1964; Seidel, 1969). The corn K⁺ATPases differ in molecular weight from myosin, from all mammalian transport ATPases, and from the catalytic (β) subunits of F₁ ATPases.
Properties of the Purified ATPase

Stimulation by salts

The finding that glycine could stabilize the ATPase activity in the absence of salts let me study the salt stimulation over a wide range of salt concentrations, with only one species of monovalent cation present. Like Benson (1977), I found that the activity increased sigmoidally with increasing salt concentration, but in my experiments activity did not level off until higher salt concentrations were reached. The marked difference in stimulation between lithium and potassium salts demonstrated cation specificity, while the similarity of stimulation by potassium chloride and potassium formate argues against anion specificity.

KCl gives twenty-fold or greater stimulation of highly purified K^+ATPase and of partly purified K^+ATPase in the presence of phospholipids, when no other stimulating ions are present. It is possible that the requirement for monovalent cations is absolute; the small background activity could represent a contaminant. If the requirement for K^+ or some other monovalent cation is indeed absolute, then perhaps K^+ substitutes for Mg^{2+} in the catalytic mechanism. A K^+ATP complex can be detected in solution (K_f = 10, Martel and Smith, 1977), and its formation
could be favored by the conformation and environment of ATP in active site of the enzyme.

Ionic strength contributes to the stimulation as well, since increased concentrations of imidazole-HCl buffer increase the activity when KCl is present but not when it is absent. Ionic strength effects may account for the appearance of cooperativity and the biphasic Hill plot. The effect of ionic strength might be related to the aggregation of the enzyme; although the aggregation is reversible, it is not known whether the aggregates themselves are active. The ionic strength effect is probably not on substrate binding. The Km's of the root and the shoot enzyme are well below the concentration of ATP in the assay.

**Aggregation**

Aggregation is typical of membrane enzymes in the absence of detergent, but the ionic strength dependence of the aggregation of the root and shoot K⁺ATPases (the aggregation of the shoot enzyme resembles that of the root enzyme (Davis, 1979; D. W. Karl and M. H. Davis unpublished data) suggests that electrostatic forces control the aggregation. Hydrophobic forces may also contribute, of course. The enzyme has a net negative charge at the pH of the aggregation experiments, but it might be a dipole—that is, it
might have a region of positive charge which could contribute to aggregation or to binding to negatively charged surfaces. As noted above, binding to soy lipid vesicles is at least partly reversed by increased KCl concentration.

Aggregates formed in the presence of 50 or 100 mM NaCl are excluded from Sepharose 4B, presumably indicating molecular weights of several million. The principal species in 1.0 M glycine is an aggregate of intermediate size, perhaps a dodecamer of the ATPase. This species is unique to the low-ionic-strength glycine buffer, and the presence of 50 mM NaCl eliminates it with formation of larger aggregates. The large aggregates might conceivably be polymers of the intermediate-sized aggregates which are destabilized by long-range electrostatic forces at low ionic strength.

Such a two-fold effect of ionic strength is possible because electrostatic interactions in salt solutions are effective (to a first approximation) only within the effective radius of the ionic cloud surrounding each charge. This radius is known as the Debye length; assuming univalent electrolytes, the Debye length is 0.5 nm at 400 mM, 1.2 nm at 60 mM, and 3.0 nm at 10 mM (interpolated from values in Moore, 1972). Reduction of the ionic strength to 10 mM would permit mutual repulsion
by negative charges about 3 nm apart (a distance comparable to the diameter of the ATPase monomer if it is a globular molecule) which would be effectively screened from each other at 60 mM. At 400 mM even short-range interactions acting over distances of a few angstroms would be screened appreciably.

Interaction with lipids

The K⁺ATPase appears to be intermediate in its properties between intrinsic proteins and extrinsic proteins. It is released from the membranes much more efficiently by the chaotropic salt NaClO₄ than by NaCl (Benson, 1977). (Chaotropic salts are so named because they are believed to break up water structure; they increase the water solubility of hydrophobic solutes, presumably by disrupting hydrophobic interactions; Jencks, 1969). It interacts with phospholipids and detergents, both of which stimulate its activity, but it is stable without them and will stay in solution as a monomer at high ionic strength.

There are several lines of evidence of interaction of the enzyme with phospholipids. Soy phosphatides and lipid extracts from corn membranes stimulate ATPase activity. Phospholipids tend to stabilize the activity at low ionic strength. Freezing and thawing with phospholipids alters the sedimentation properties of the ATPase,
probably because of a decrease in its buoyant density
due to binding of phospholipids, either as molecules or
as vesicles. The presence of phospholipid vesicles
alters the electrophoretic mobility of the enzyme, and
some of the activity migrates with the vesicles. Under
certain conditions the ATPase binds firmly to vesicles
to give a reconstituted preparation in good yield.

Although octyl glucoside stimulates ATPase activity,
it destabilizes the enzyme rather than stabilizing it as
phospholipids do. Phospholipids added with octyl
glucoside protect the activity.

Results such as these have been attributed to
delipidation (Warren et al., 1974) so they may indicate
that the \( \text{K}^+ \text{ATPase} \) contains tightly bound phospholipid.

DuPont and Leonard (1980) criticized the report of
Benson and Tipton (1978) of the isolation of root \( \text{K}^+ \text{ATPase} \)
(along with other reports of solubilization of plant ATPases)
because the enzyme is resistant to chaotropic salts and to
EDTA, which they assert would remove bound lipids. They
imply that bound phospholipids are essential to the
stability and function of all intrinsic membrane proteins,
and that only intrinsic proteins can be involved in ion
transport. These criticisms appear to be based on a mis-
derstanding of the properties of membrane proteins.
It is well-documented that $F_1$ ATPases are involved in $H^+$ transport, and they behave as extrinsic proteins. Furthermore, not all intrinsic proteins require lipid for stability or for activity (for example cytochrome b5) and many can be reversibly delipidated and relipidated with restoration of activity (Hobbs and Albers, 1980; Shamoo and Tivol, 1980). Moreover, EDTA and NaClO$_4$ are incapable of dissociating intrinsic proteins from phospholipids; this requires detergents or organic solvents (Tanford, 1973). Although NaClO$_4$ disrupts hydrophobic interactions, it is not sufficient to solubilize a phospholipid molecule. Octyl glucoside, on the other hand, is capable of delipidating intrinsic proteins (Stubbs and Litman, 1978). The possibility of bound phospholipid being associated with the ATPase is deserving of further investigation.

Reconstituted $K^+$ATPases

Reconstitution of the purified ATPase

Some association of the purified ATPases with phospholipids, was clearly indicated by the stabilization and stimulation of the enzyme activity by added lipid. The results of the freeze-thaw-sonication experiments supported this conclusion, but they were equivocal as to whether the association was loose binding to vesicles, tight binding to a small number of vesicles, or binding of individual
phospholipid molecules to the enzyme. In other experiments, either recovery of activity was low, or only a small portion of the activity was associated with the vesicles when they were separated from the free enzyme.

In the stability experiments, stimulation and stabilization were especially good when a mixture of ATPase and phospholipid vesicles was dialyzed against a buffer of lower ionic strength. Such preparations prove to consist in large part of ATPase firmly associated with membrane vesicles. The distribution of the ATPase in gel chromatography is controlled by the distribution of phospholipid, which rules out the possibility that the results reflect simple aggregation of the ATPase. Aggregates of ATPase are not found in this buffer when lipid is omitted.

This tight association of ATPase with artificial membrane vesicles did not lead to any ion transport activity. Neither did it shift the pH optimum for ATPase activity to lower values; at 50 mM KCl, the pH optimum was instead shifted to pH 8.0. At 250 mM KCl, the pH/activity curve was similar to the pH/K⁺ stimulation curve of the K⁺ stimulation of free enzyme.

This similarity is probably due to reversal by KCl of the binding of the enzyme to the phospholipid membrane, since 250 mM KCl at pH 7.5 and 4°C was sufficient to resolubilize most of the ATPase. Except for the temperature
and the absence of substrate, these are the conditions of the assay.

**Reconstitution of extracts**

It could be that reconstitution of the purified ATPase did not lead to reconstitution of ion transport because an essential subunit was left behind in the purification. As noted earlier, the $F_1$ ATPases of bacteria, chloroplasts, and mitochondria behave as extrinsic proteins but are involved in ion pumping. $H^+$ translocation driven by these enzymes is mediated by intrinsic protein components of the membranes, to which the $F_1$ ATPases bind. These membrane components include a proteolipid with ionophoric properties. The $K^+$ ATPases of corn might transport ions in a similar fashion by interaction with intrinsic proteins, in spite of the difference in size and other properties between the $K^+$ ATPases and $F_1$ ATPases. The ATPase could bind to this subunit in such a way as to cap an ion channel (Figure 33).

If the ATPase is involved in ion transport, another argument speaks for such a structure: its molecular weight (30,000) is rather low for a transport protein. If it were cylindrical and its partial specific volume were $0.78 \text{ g/cm}^3$, its dimensions could be $3.15 \text{ nm} \ (\text{diameter})$ and $5 \text{ nm} \ (\text{height})$. These are sufficient to span the bilayer and provide an ion channel, but if an ATP-hydrolyzing site
Figure 33. Possible structure of an ion-translocating ATPase complex
were present and coupled to the ion channel, the protein would be an unprecedented wonder of compactness. Bacteriorhodopsin and mitochondrial adenine nucleotide translocator are in this size range, but neither couples a hydrolytic chemical reaction to transport. The well-known transport ATPases are at least three times as large (Hobbs and Albers, 1980). Moreover, any protein buried so deeply in the membrane would presumably be an intrinsic protein. The K+ATPases of corn are not intrinsic proteins although they resemble them in some respects.

If a complex of the K+ATPase with an intrinsic membrane-spanning subunit existed in the membrane, it would not be solubilized intact by chaotropic salts, which would instead tend to break the complex up. Such disruption of the F1-F0 complex by chaotropic agents occurs with submitochondrial particles (Kagawa, 1974). Detergents such as octyl glucoside might solubilize the complex intact, although there is no evidence they do. Alternatively, the dissociated components of the complex might reassociate during reconstitution with phospholipids. Because of these considerations. I reconstituted membrane vesicles from crude detergent extracts and tested them for ATPase activity and for ion transport activity.

Mg-dependent and Mg-independent K+ATPase activities were found to be associated with membranes reconstituted
by addition of phospholipids to the octyl glucoside extracts and removal of the octyl glucoside by dialysis. 

Background ATPase (the activity present in the absence of KCl) was considerably reduced.

The K⁺ATPase of reconstituted extracts differs from the reconstituted purified ATPase. The pH optimum is low (although a smaller peak of activity near pH 7.5 exists). Over most of the pH range, activity is the same with 50 mM KCl or 250 mM KCl. No resolubilization of activity by KCl could be detected.

The vesicles usually appeared permeable to K⁺, although I obtained vesicles capable of sustaining a valinomycin/KCl diffusion potential in a few experiments. These appeared to have a MgATP-dependent uptake of positive charge. In no case, however, did I find an ATP-dependent membrane potential without Mg²⁺, even when having added ionophores to convert concentration gradients of potassium or of protons to membrane potentials.

The problem of vesicle leakiness

The leakiness of the reconstituted extract vesicles could be due to any of several causes. Specific transport proteins might have been reconstituted. If the suggestion of Hanson (1978) that passive uniports and antiports of complementary specificity exist in different plant tissues
so that both uptake and secretion can be driven by the proton-motive-force (Figure 34) are correct, then recon-
stitution of an extract made from membranes of several tissues could lead to short-circuit paths which uncouple transport from the membrane potential. Such transporters would probably be proteins, and might be subject to denaturation by heat. The leakiness was not abolished, however, by heating in a boiling water bath. Another possible origin for the leakiness is enzymatic degradation of the lipids. Such degradation is a problem when plant membranes are prepared (Kates, 1972; Scherer and Morre, 1978; Gibrat and Rossignol, 1977; Galliard, 1974) and the enzymes responsible might be extracted along with the ATPase. Several breakdown products of phospholipids could alter the permeability or integrity of the membrane vesicles. Free fatty acids, especially the polyunsaturated fatty acids found in plants, can act as $H^+$ ionophores (Racker, 1975) and might also carry other cations. Lysolecithin, free fatty acids, and neutral lipid breakdown products might also make the membrane fragile and easily ruptured; lysolecithin and fatty acid salts have detergent action (Tanford, 1973), and neutral lipids are believed to facilitate membrane breakage (Yatvin et al., 1980). Degradation of phospholipids in a detergent extract of corn membranes was observed in experiments (not shown)
Figure 34. A chemiosmotic model (Hanson, 1978) of translocation of salts into the interior of a root, showing translocators of complementary specificity operating in different tissues.
wherein the $^{31}$P NMR spectrum of the extract was observed for several hours.

Although I obtained no evidence that the K$^+$ATPases are involved in ion transport, the permeability of the reconstituted extract vesicles in most of the experiments means that I cannot entirely rule it out. Even the vesicles which maintained a detectable valinomycin-induced K$^+$ diffusion potential must have been somewhat leaky, since the fluorescence change was small compared with controls; hence the experiments were insensitive. Any further attempts to measure transport in reconstituted corn membranes must await development of tight reconstituted membrane vesicles.

Sze's results (1980a, 1980b) with native membranes from tobacco protoplasts are interesting. She succeeded in selecting tight vesicles by centrifugation of crude membranes from tobacco callus in a dextran/sucrose step gradient. Tight vesicles banded atop the dextran layer while a pellet of unsealed vesicles formed at the bottom of the tube. The sealed vesicles show ionophore-stimulation of the K$^+$Mg$^{2+}$ATPase. Such methods may make it possible to characterize ATPase-driven transport in native membranes without solubilization and reconstitution—at least as a first step. Such separation methods might be useful with reconstituted vesicles. The method relies on permeability
of unsealed vesicles to dextran, however, and would not be effective if the vesicles were selectively leaky to small molecules or ions. In many of my experiments, vesicles which effectively trapped the dye di-0-C$_3$-(5) appeared to be permeable to K$^+$. 

Investigation of the biological role of the K$^+$ATPase would be aided by knowledge of its location in the cell. Localization of plant membrane components has until recently been ambiguous when accomplished by cell fractionation (Quail, 1979), but the experiments of Perlin and Spanswick (1980) make it very likely that K$^+$Mg$^{2+}$ATPase is a reliable marker for the plasma membrane. There are still no good markers for the tonoplast, but it can be prepared from isolated vacuoles. Part of the difficulty in localizing membrane activities had been the apparent density heterogeneity of the plasma membrane fraction; plasma membrane markers appear spread out over a wide range of densities in continuous sucrose gradients (see, for example, Leonard and VanDerWoude, 1976), and various presumed plasma membrane markers are distributed differently (Hendricks, 1978). Sze's procedure for isolating tight vesicles might help in isolating narrow, homogeneous bands if the broad bands are caused by osmotic effects, since vesicles selected for tightness could be subfractionated on sucrose gradients. Isolation of a subpopulation of
membranes might permit detection of transport activities which are masked in the mixture. Purified membrane fractions might have less lipid-degrading activity and so be more suitable for reconstitution experiments. Starting with homogeneous membrane preparations might also avoid uncoupling of active transport in the reconstituted system by passive transporters originating in other membranes.

Physiological Role of the $K^+\text{ATPase}$

If the $K^+\text{ATPase}$ is not involved in transmembrane transport, it presumably has some other role in membrane function. Although its aggregation behavior suggests that its role could be cytoskeletal, it does not resemble any of the well-known cytoskeletal proteins. It does share with myosin the ability to hydrolyze $\text{ATP}_f$ in the presence of $K^+$. It would be interesting to know whether it can interact with microfilaments or microtubules, especially when it is bound to lipid vesicles or aggregated, and whether its aggregates are filamentous. If the aggregates formed in dilute NaCl solutions are filamentous, their exclusion from Sepharose 4B would be accounted for.

If, on the other hand, it is involved in ion transport, a possible role would be in the accumulation of salts in the vacuole. It is activated cooperatively by KCl over
a range of concentrations that includes the cytoplasmic $K^+$ concentration. Hence it would be strongly activated by any increase in cytoplasmic $K^+$ and could provide the energy for storage of $K^+$ salts in the vacuole. It could conceivably be responsible for the second component of the cell potential of corn cortical cells observed by Cheeseman et al. (1980), which was stimulated by high $K^+$ concentrations and was not inhibited by inhibition of the $K^{+}\text{Mg}^{2+}\text{ATPase}$. Location on the tonoplast would not rule this out since the cell potential is measured with an electrode inserted into the vacuole. Leigh and Walker (1980) have found that an ATPase of beet vacuoles has a high pH optimum, about pH 8.0, although it appears to differ from the corn $K^+$ ATPase in its substrate specificity. Davis (1979) found that most of the $K^+\text{ATPase}$ of shoot microsomes was located on membranes less dense than the plasma membrane.

Is There a Passive $K^+$ Translocator in Corn Plasma Membranes?

In order for an ATPase on the tonoplast to drive uptake of an ion from the external solution (outside the plasmalemma), a translocator for that ion must be present in the plasmalemma. In the experiments of Cheeseman et al. (1980), inhibition of the $K^{+}\text{Mg}^{2+}\text{ATPase}$ did not block $K^+$ uptake in the system 2 concentration range. As noted
before, $K^+$ appears to be taken up passively in this range. These considerations suggest the existence of a passive $K^+$ translocator in the plasma membrane, active in the system 2 concentration range. This is reminiscent of the Tori-Laties hypothesis (Tori and Laties, 1966; Luttge and Higinbotham, 1979).

Such a translocator would have to be regulated by the extracellular $K^+$ concentration, since otherwise $K^+$ could not be taken up actively from solutions of low $K^+$ concentration. Uptake in the system 1 concentration range is active (Cheeseman et al., 1980) and is highly specific for $K^+$, even in the presence of excess $Na^+$ (Rains and Epstein, 1967). The external regulatory site would have to be specific for $K^+$, but the specificity of the transport site would reflect the broad specificity of system 2 cation uptake.

**Ca$^{2+}$ and membrane permeability**

If regulation of the passive cation translocator by $K^+$ were also dependent on Ca$^{2+}$, the requirement of plants for Ca$^{2+}$ to maintain the specificity of cation uptake from dilute solutions (Epstein, 1972) would be explained. In the absence of Ca$^{2+}$, the passive cation transporter would operate, and its broad specificity would allow Na$^+$ to enter in response to the membrane potential or in
exchange for $K^+$. Slayman (1965) found that the plasma membrane of *Neurospora* appeared to be permeable to $K^+$ and $Na^+$ in the absence of $Ca^{2+}$, since either cation reduced the membrane potential when present in the bathing solution. $Ca^{2+}$ increased the membrane potential and greatly reduced its sensitivity to $K^+$ and $Na^+$, suggesting that it reduced the passive permeability of the membrane to these ions.

**Kinetics of the $K^+, Mg^{2+}$ATPase**

The existence of a passive cation translocator in the same membrane as the $K^+Mg^{2+}$ATPase could explain several aspects of the relationship of the ATPase to ion transport. The ATPase activity is highly correlated with the rate of $K^+$ uptake into intact root tissue as a function of KCl concentration, but it has always been unclear why $K^+$ on the same (intracellular) side of the membrane as the enzyme should stimulate, since $K^+$ is taken up from the extracellular solution. The extracellular side of the membrane is exposed to the interior of the plasma membrane vesicles, at least of those with the ATPase exposed for assay. The vesicle interior may be rapidly depleted of $K^+$ by ATP-driven transport during the assay; this could reduce the activity. If a passive $K^+$ carrier delivered $K^+$ into the vesicle interior, it would stimulate the ATPase by dissipating the $K^+$ gradient. The stimulation would be
due to the recycling of $K^+$ into the vesicles and would have the concentration dependence of the $K^+$ carrier, so that an ATPase active in system 1 $K^+$ transport would show system 2 kinetics. $Ca^{2+}$ is not usually included in assays of the $K^+Mg^{2+}$ATPase, nor was it present when Sze and Hodges (1976) measured the permeability of oat root plasma membranes to cations. DuPont et al. (1981) found that the solubilized and partly purified $K^+Mg^{2+}$ATPase was only slightly stimulated by $K^+$, and this stimulation was not correlated with the concentration dependence of ion uptake. This change in properties would be explained if the $K^+$ stimulation of the ATPase in the membrane were controlled by some other component of the membrane, such as a passive $K^+$ translocator as described above.

The presence of a cation translocator could account for the leakiness of the vesicles I reconstituted from corn membrane extracts. $Ca^{2+}$ was never present in these preparations since it is an activator of lipases (Galliard, 1974; Kates, 1972). Such a translocator could presumably be isolated, reincorporated into a membrane, and characterized, if an assay could be found to detect it. An assay based on induction of cation permeability in reconstituted membrane vesicles would be direct and fairly simple, but other sources of cation permeability such as lipid degradation could interfere.
The translocator's affinity for monovalent cations would be too low to permit detection of $^{86}\text{Rb}^+$ or $^{42}\text{K}^+$ binding, except in very concentrated solutions of the translocator. If the hypothesis of $\text{Ca}^{2+}$ regulation were correct, $^{45}\text{Ca}^{2+}$ binding might be detectable. Perhaps the best solution would be an inhibitor or activator of the $\text{K}^+$ stimulation which does not affect the $\text{Mg}^{2+}$ ATPase activity or the solubilized $\text{K}^+\text{Mg}^{2+}$ ATPase. The cation translocator would be a likely site of action for such an effector.
BIBLIOGRAPHY


Cassagne, C., and Lassire, R. (1979), *Biochimie* 61, VII-XI.


Cheeseman, J. M., and Hanson, J. B. (1979b), *Plant Physiol.* 64, 842-845.


Leonard, R. T., and VanDerWoude, W. J. (1976), Plant Physiol. 57, 105-114.

Lin, W., and Hanson, J. B. (1974), Plant Physiol. 54, 250-256.

Lin, W., and Hanson, J. B. (1976), Plant Physiol. 58, 276-282.


Rogers, T. B., and Lazdunski, M. (1979), *Biochemistry* 18, 135-140.


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