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Characterization of *In Vitro* Proton Pumping by Microsomal Vesicles Isolated from Corn Coleoptiles[1]

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ABSTRACT

Corn (*Zea mays* L. cv Golden Cross Bantam) coleoptile microsomal vesicles have been isolated which are capable of ATP-driven H^+-transport as measured by [^{14}C]methylamine accumulation and quinacrine fluorescence quenching. Formation of the pH gradient *in vitro* shows a high specificity for ATP•Mg, is temperature-sensitive, exhibits a pH optimum at 7.5, and is inhibited by carbonyl cyanide-*m*-chlorophenylhydrazone. Of the divalent cations tested, Mn^{2+} is almost as effective as Mg^{2+}, while Ca^{2+} is ineffective. Excess divalent cations, particularly Ca^{2+}, reduces the pH gradient. H^+ transport is strongly promoted by anions, especially chloride, while potassium does not affect pump activity. Studies with $^{36}Cl^-$ indicate that ATP-driven H^+ transport into the vesicles is associated with chloride uptake. Both carbonyl cyanide-*m*-chlorophenylhydrazone and the anion transport inhibitor, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene, inhibit methylamine accumulation and $^{36}Cl^-$ uptake. Proton pumping is also blocked by diethyl stilbestrol and *N,N′*-dicyclohexylcarbodiimide, but is insensitive to oligomycin and vanadate. These properties of the pump are inconsistent with either a mitochondrial or plasma membrane origin.

There is increasing recognition of the central role played by electrogenic proton pumping H^+-ATPases in the transport of solutes across the plasma membrane and tonoplast of plant cells (21, 23, 27). A plasma membrane proton pump may also act to acidify the cell wall during auxin-induced cell wall loosening and growth (24). In microbial (11), fungal (26), and animal (14, 15) systems, the use of isolated membrane vesicles capable of carrying out ATP-driven proton transport *in vitro* has been an invaluable aid in characterizing the H^+-ATPase. Recently, progress has been achieved in the development of similar systems in plants. Sze (29) obtained a tobacco microsomal membrane fraction from a dextran step gradient which exhibited ionophore-stimulated ATPase activity, suggesting the presence of sealed vesicles. In the same year, Hager *et al.* (8) demonstrated ATP-driven proton pumping in microsomal vesicles isolated from corn coleoptiles using an indicator dye method. Several laboratories have now demonstrated that similar vesicles from a variety of plant tissues respond to ATP by generating a transmembrane pH and electrical gradient (6, 19, 22, 28, 30). However, in such mixed membrane fractions, the identity of the proton pump has not been unequivocally demonstrated. In this paper, we report on the properties of microsomal vesicles from corn coleoptiles which actively accumulate ^{36}Cl as well as protons. In the following paper (17), the localization of these ATP-dependent transport activities on density gradients in relation to membrane markers is described.

MATERIALS AND METHODS

Plant Material. Corn (*Zea mays* L. cv Golden Cross Bantam) was soaked overnight in aerated tap water, sown in trays with vermiculite, and watered with 0.1 mM $CaCl_2$ solution. The germinating seedlings were maintained in the dark at 20 to 22°C with 2 h of red light daily to inhibit mesocotyl growth.

Microsomal Vesicle Preparation. Coleoptiles from 5- to 7-d-old seedlings were debladed, weighed, and transferred to a chilled mortar. Two ml of buffer (0.25 M sucrose, 2 mM EDTA, 1mM DTT, 50 mM Tris-Mes, pH 7.8) were added per g of coleoptile tissue. The tissue was then ground lightly with a pestle, strained through nylon, and the remaining tissue reground thoroughly with 1 ml buffer/g original tissue. The final homogenate was filtered through nylon and combined with the first homogenate. This was centrifuged for 5 min at 500*g*, and the supernatant recentrifuged for 10 min at 12,000*g*. The supernatant was filtered through cheesecloth and centrifuged for 1 h at 80,000*g* in a Beckman SW28 rotor. The microsomal pellet was resuspended in 2 or 3 ml buffer (0.25 M sucrose, 1 mM EDTA, 0.5 mM DTT, 5 mM Tris-Mes, pH 7.2) and further purified by centrifugation into a dextran step gradient. One ml of homogenate was layered over 2 ml of 8% dextran (bottom) and 2 ml of 1% dextran (top) in transport buffer (0.25 M sucrose, 10 mM KCl, 2.5 mM Tris-Mes, pH 7.0) except in Figure 1 where a 2.5/10% dextran gradient was used. The step gradients were centrifuged for 2 h at 120,000*g* in a Beckman SW-65 rotor. About 0.4 ml of microsomal vesicles were taken from each 1 to 8% interface and diluted with an equal volume of transport buffer.

Methylamine Uptake. The uptake of ^{14}C-labeled methylamine was determined by a Millipore filtration technique. Vesicles were incubated in an equal volume of test solution made up in transport buffer. For a single time point, 50 μl vesicles in 100 μl total volume had final concentrations of 2.5 mM ATP·Mg or ADP·Mg (control), 18 μM methylamine (0.77 μCi/ml/assay, 44 mCi/mmol in ethanol), 10 mM KCl, 0.25 M sucrose, 2.5 mM Tris-Mes, pH 7.0. After a 5-min incubation at 30°C, the reaction was stopped by the addition of 3 ml cold transport buffer and filtered immediately on a prewetted 0.45 μm Millipore filter (HATF). The filter was rinsed with an additional 3 ml buffer. The entire stop process took less than 15 s. For a time course, 50 to 100 μl aliquots of the reaction mixture were diluted into 3 ml of buffer, filtered, and rinsed. Radioactivity was counted in a liquid scintillation counter. The maximum levels of ATP-stimulated methylamine uptake during a time course ranged from 0.5 to 1.5 nmol/mg protein. A conversion factor of approximately 3×10^{-4} can be used to convert cpm into nmol/mg protein.

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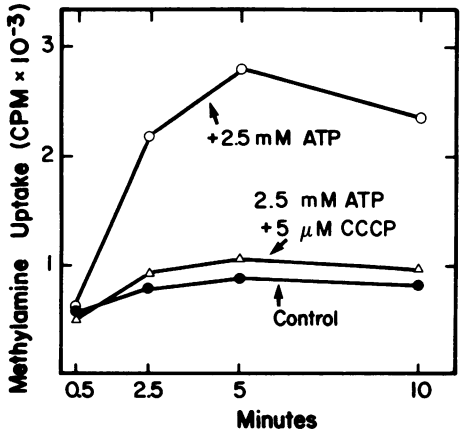

FIG. 1. Time course of [^{14}C]methylamine uptake by microsomal vesicles from corn coleoptiles. Vesicles collected from 2.5/10% dextran interface at pH 7.4 and incubated at 30°C. All treatments had 2.5 mM $MgSO_4$ and 2.5 mM Tris-HCl. Control (−ATP), +ATP (2.5 mM), +CCCP (2.5 mM ATP, 5 μM CCCP).

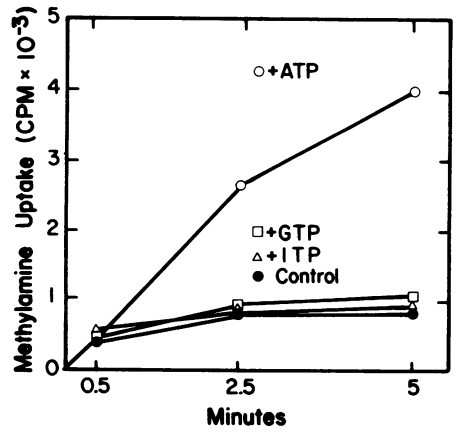

FIG. 2. Substrate specificity of [^{14}C]methylamine uptake. All treatments had 2.5 mM $MgSO_4$, 10 mM KCl. Control (−NTP), nucleotide 5′-phosphates (2.5 mM in Na^+ form).

$^{36}Cl^-$ Uptake. The uptake of $^{36}Cl^-$ (NaCl in H_2O, 0.438 mCi/mmol, 0.5–2.0 μCi/ml/assay) was performed essentially as described for methylamine uptake. Details of the procedure are described in the text.

Quinacrine Fluorescence Quenching. Fluorescence quenching of the dye quinacrine was performed essentially as described by DuPont *et al.* (6; see 5 for a discussion). Vesicles were prepared as above except the buffers used had sorbitol instead of sucrose, and bis-tris-propane/Mes instead of Tris-Mes. Vesicles (~100 μg protein) were incubated with 10 μM quinacrine and 5 mM ATP·Mg or ADP·Mg. Fluorescence quenching was measured over a period of 30 min.

RESULTS

In vitro proton pumping by microsomal membranes from corn coleoptiles was detected by the accumulation of the weak base methylamine, in the presence and absence of ATP (25). Figure 1 is a time course of ^{14}C-labeled methylamine uptake by the membrane fraction obtained from the dextran interface. The addition of ATP·Mg markedly stimulated methylamine uptake. The ATP·Mg-enhanced uptake was inhibited by the proton ionophore CCCP,[4] indicating that the enhancement is dependent upon a proton gradient across the membrane.

Nucleotide stimulation of methylamine accumulation is highly specific for ATP (Fig. 2). In addition to the compounds shown, ADP, IDP, and AMP were also tested, none of which exhibited significant activity. The dose response to increasing ATP and/or Mg^{2+} is illustrated in Figure 3. Methylamine uptake increased with the ATP·Mg concentration up to 2.5 mM, while higher concentrations were less stimulatory. When the ATP concentration was held constant at 2.5 mM and Mg^{2+} was varied, Mg^{2+} was inhibitory in excess of 2.5 mM. When Mg^{2+} was held constant at 2.5 mM, ATP was inhibitory only at 10 mM. For most subsequent experiments, 2.5 mM ATP·Mg was the concentration used. Kinetic analysis of the ATP·Mg data yielded a K_m of 1.3 mM, but the analysis is complicated by the sharp inhibition of activity above 2.5 mM, and the apparent K_m value may not be accurate. These data are consistent with a proton pump utilizing ATP·Mg as its substrate.

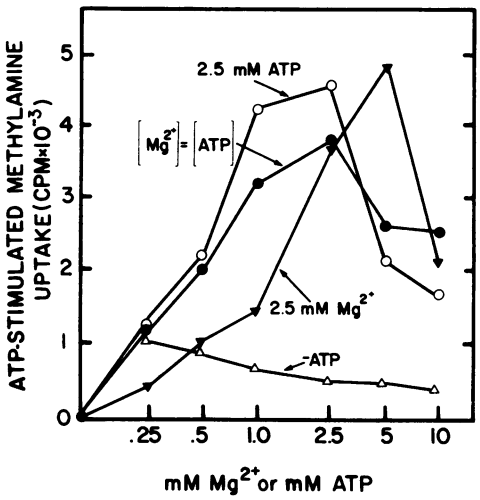

FIG. 3. [^{14}C]Methylamine uptake as a function of ATP and Mg^{2+} concentrations. ATP stimulation calculated after a 5-min incubation by subtracting control values (−ATP). −ATP values plotted for comparison.

The effect of pH on the activity of the proton pump is shown in Figure 4. The membranes were isolated at pH 7.0 and then preincubated for at least 1 h in transport buffer adjusted to the appropriate pH prior to the start of the uptake experiment. This procedure avoided the generation of artificial pH gradients which would obscure the effects of the pump. As indicated in Figure 4, methylamine uptake showed a pH optimum at about 7.5

Inasmuch as the reaction was normally carried out at 30°C, it was of interest to examine the effects of temperature on methylamine uptake (Fig. 5). Pump activity was totally absent at 4°C and increased dramatically with increasing temperature up to 37°C. The Q_{10} between 20 and 30°C was 2.5.

Table I shows the effect of various inhibitors on ATP-driven methylamine uptake. Uptake was inhibited by DES and DCCD with estimated K_i of 40 and 30 μM, respectively. The pump was insensitive to oligomycin. Valinomycin had little effect on the equilibrium value of methylamine uptake but did increase the initial rate of uptake (19). Vanadate, a potent inhibitor of many plasma membrane ATPases (1, 3; M. Jacobs, A. Gepstein, and L. Taiz, unpublished data) did not inhibit even at high concentrations, and consistently caused a slight stimulation of methylamine uptake. Preincubation of membrane vesicles with vanadate for extended periods did not result in any reduction of proton pumping (data not shown).

The activity of the proton pump was strongly promoted by KCl

[4] Abbreviations: CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; DES, diethylstilbestrol; DCCD, *N,N′*-dicyclohexylcarbodiimide; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; FCCP, carbonyl cyanide-*p*-trifluoromethoxy-phenylhydrazone.

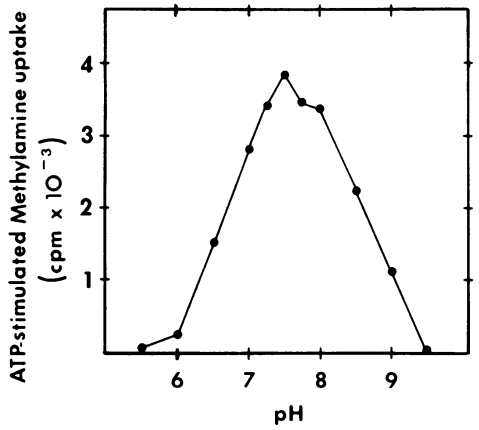

FIG. 4. [^{14}C]methylamine uptake as a function of pH. Vesicles collected from 1/8% dextran interface made in pH 7.2 transport buffer. They were brought to the appropriate pH with an equal volume of transport buffer containing 10 mM Tris-Mes and were allowed to equilibrate for 1 h. The data represents the average of three experiments. Control (−ATP) values ranged from 300 to 500 cpm.

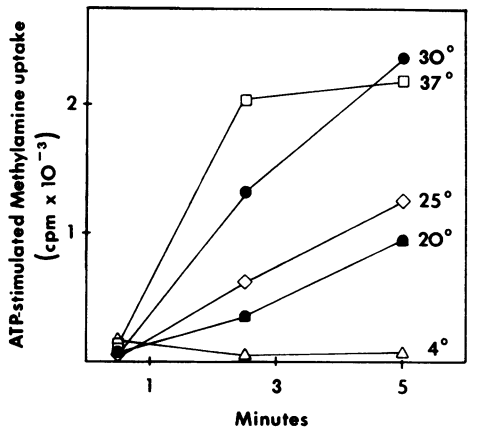

FIG. 5. Time course of [^{14}C]methylamine uptake as a function of temperature. Ice-cold vesicles and test solutions were mixed to start the reaction and incubated in a water bath at the appropriate temperature. Control values (2.5 mM ADP, Mg) ranged from 100 to 600 cpm.

up to 10 mM (Fig. 6). An examination of the effects of various monovalent salts (Table II) demonstrated that methylamine accumulation was more sensitive to anions than to cations, and that chloride was the most stimulatory. The organic acids malate, citrate, and succinate were unable to significantly stimulate methylamine uptake. Potassium had no detectable effect on the pump, since potassium-Mes and potassium sulfate supported less than 10% of the activity with KCl. Of the divalent cations, Mg^{2+} and Mn^{2+} were equally effective, and both were inhibitory when present in excess of the ATP concentration (Table II). Calcium exhibited very low activity alone and was strongly inhibitory when added with Mg^{2+}.

The stimulation of H^+-pumping by chloride could be due to the uptake of Cl^- into the vesicles, which would neutralize an inside positive membrane potential produced by an electrogenic pump. This possibility was directly tested by measuring the ATP-promoted accumulation of $^{36}Cl^-$ by the vesicles. For comparison, methylamine uptake was determined in separate experiments on the same vesicle preparation. $^{36}Cl^-$ uptake was enhanced by ATP·Mg to about the same extent as methylamine uptake (Fig. 7). Importantly, $^{36}Cl^-$ uptake was abolished by the proton ionophore CCCP, demonstrating that chloride accumulation was driven by the proton gradient. DIDS, an amino reagent which blocks anion

Table I. *Effect of Inhibitors on ATP-Stimulated Methylamine Uptake by Microsomal Vesicles from Corn Coleoptiles*

Vesicles were incubated for 5 min at 30°C in 0.25 M sucrose, 2.5 mM Tris-HCl (pH 7.0), 18 μM [^{14}C]methylamine, 2.5 mM $MgSO_4$, and 2.5 mM ATP (except where indicated).

Treatment	Methylamine Uptake	ATP Stimulation
	cpm	*% of control*
No ATP	448	0
+ATP (control)	1,938	100
−$MgSO_4$	492	3
+DES (100 μM)	753	20
+DCCD (100 μM)	695	16
+Oligomycin (10 μg/ml)	1,899	97
+Vanadate (20 μM)	2,051	107
+Vanadate (100 μM)	2,176	115

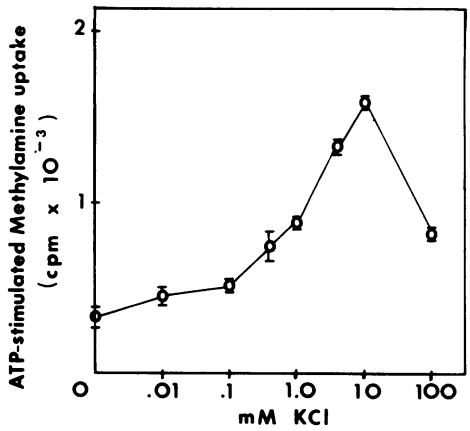

FIG. 6. [^{14}C]Methylamine uptake as a function of KCl concentration. Vesicles were isolated in the absence of KCl and incubated for 5 min at 30°C with the appropriate concentrations. Control values (−ATP), ~600 cpm. Bars indicate SE. (n = 2).

Table II. *Effect of Various Ions on ATP-Stimulated Methylamine Uptake*

Data, which was compiled from several experiments, was determined as in Table I, except the buffer was Tris-Mes (pH 7.0) and had 10 mM mono- and 2.5 mM divalent salt (except where indicated).

Monovalent Salts[a]	ATP Stimulation	Divalent Salts[b]	ATP Stimulation
	% of control		*% of control*
No monovalent	10	No divalent	3
KCl (control)	100	$MgSO_4$ (control)	100
NaCl	102	$MgCl_2$	109
NaBr	87	$MnSO_4$	98
KI	43	$CoCl_2$	60
KNO_3	21	$ZnSO_4$	22
KSCN	20	$CaSO_4$	9
K-Mes	9	$MgSO_4$ (5 mM)	75
K_2SO_4	3	$MgSO_4$ + $MnSO_4$	52
Tris-Malate	18	$MgSO_4$ + $CaSO_4$	7
Tris-Citrate	9		
Tris-Succinate	14		

[a] Divalent salt was $MgSO_4$ (2.5 mM).

[b] Monovalent salt was KCl (10 mM).

transport in red blood cell membranes (2), inhibited methylamine uptake as well as chloride accumulation (Fig. 7). In contrast, vanadate, which does not inhibit proton pumping, had no effect

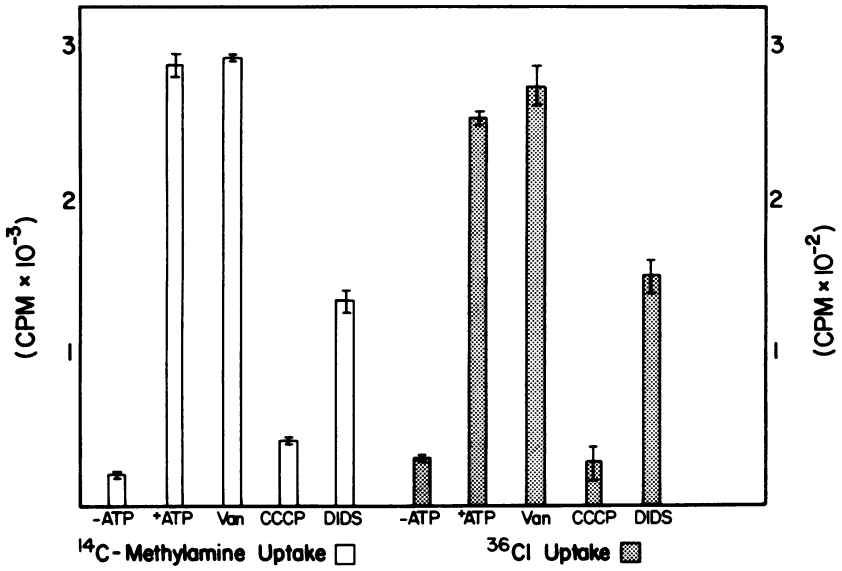

FIG. 7. Effect of inhibitors on [^{14}C]methylamine uptake and $^{36}Cl^-$ uptake. [^{14}C]Methylamine uptake studies done in the presence of 10 mM KCl; $^{36}Cl^-$ uptake experiments were carried out without any additional KCl (final chloride concentration, ~1.5 mM). −ATP (2.5 mM ADP, $MgSO_4$), all others in the presence of 2.5 mM ATP, $MgSO_4$. Vanadate (Van, 100 μM), CCCP (5 μM), DIDS (10 μM). Data, compiled from several experiments, represents the average ± SE of four experiments.

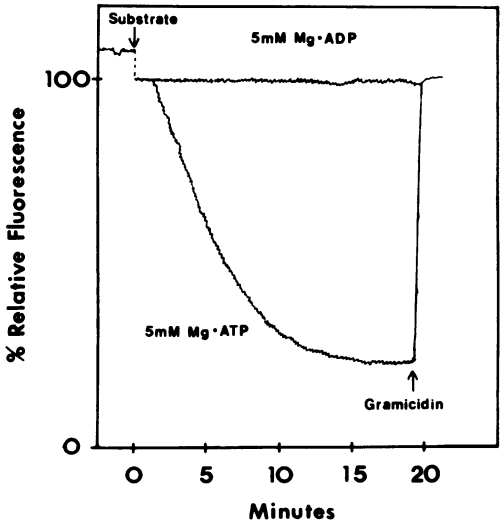

FIG. 8. Time course of quinacrine fluorescence quenching by microsomal vesicles from corn coleoptiles. Dextran interface vesicles (~100 μg protein) were incubated in a 1-ml mixture of 10 μM quinacrine, 0.25 M sorbitol, 10 mM KCl, 2.5 mM bis-tris propane/Mes (pH 7.0) at 25°C. Five mM ADP, $MgSO_4$ or 5 mM ATP, $MgSO_4$ was added to start the reaction. Three μM gramicidin was added after 20 min. The excitation wavelength was 420 nm and the emission wavelength was 495 nm.

on $^{36}Cl^-$ uptake.

A potential complication inherent in the use of methylamine is the possible transport of the charged species on an ammonium ion carrier. Although the inhibition of methylamine uptake by proton ionophores argues against this possibility, proton pumping was also determined by the fluorescence quenching method using the bulkier diamine compound quinacrine. A typical quench curve obtained upon the addition of ATP·Mg is shown in Figure 8. The reaction kinetics are similar to those of methylamine uptake, and ADP·Mg shows no activity. Gramicidin, which nonspecifically collapses the proton gradient, induced a rapid increase in fluorescence due to the leakage of quinacrine from the vesicles. In other experiments, proton pumping as measured by the fluorescence quenching technique was inhibited by the proton ionophore FCCP, but was insensitive to vanadate (data not shown). The quinacrine and methylamine results thus are in good agreement.

DISCUSSION

The use of a dextran step gradient provides a convenient method for isolating membrane vesicles with proton transport capability. We have characterized the proton pumping activity of microsomal vesicles from corn coleoptiles using the method of [^{14}C]methylamine accumulation. Parallel studies using the technique of fluorescence quenching of the diamine quinacrine yielded similar results, suggesting that methylamine is a suitable probe for transmembrane pH gradients.

As reported previously (19), the inside-acid proton gradient generated by corn coleoptile membranes is strictly dependent on ATP and divalent cations. Of the divalent cations tried, Mg^{2+} and Mn^{2+} were the most effective while others showed varying amounts of stimulation. Ca^{2+} appeared to be inhibitory, which may be due to a H^+/Ca^{2+} antiport mechanism as suggested by Hager and Hermsdorf (10).

ATP-driven proton pumping is temperature-sensitive and shows a pH optimum around 7.5. Our preliminary report of a plateau of activity between pH 7.0 and 8.5 (19) can now be explained by incomplete buffering in the alkaline range in the earlier study. A pH optimum of 7.5 is consistent with the action of a plasma membrane or tonoplast Mg^{2+}-ATPase, which typically has a pH optimum in this range (12, 16).

Although different methods of membrane isolation and ΔpH measurement were used, our results with corn coleoptiles are similar to those obtained by Hager and coworkers (8–10). Proton pumping is insensitive to monovalent cations, but is strongly promoted by chloride and to some extent by bromide. The lack of stimulation by K^+ is significant because it is an indication that these are not vesicles from the plasma membrane for which a K^+-stimulated Mg^{2+}-ATPase in considered to be a marker (12). The dependence of H^+ transport on chloride has several possible explanations: (a) the proton pump may operate as an electroneutral H^+/Cl^- symporter; (b) chloride may relieve electrical back pressure on an electrogenic pump by diffusing into the vesicle in response to an inside-positive membrane potential generated by a H^+-pumping ATPase; (c) chloride may directly stimulate the H^+-ATPase. As will be shown in the following paper (17), it is

probable that the proton pump we have measured is electrogenic, since ATP-driven thiocyanate (SCN^-) uptake is detectable in the same population of vesicles isolated from density gradients, in agreement with the findings of other workers using similar membrane preparations obtained from a variety of tissues (18, 22, 26, 28, 30). We have also found that ATP stimulates $^{36}Cl^-$ uptake by the vesicles. The fact that $^{36}Cl^-$ uptake and methylamine accumulation are inhibited in parallel by the proton ionophore CCCP and the anion transport inhibitor DIDS is consistent with the idea that movements of H^+ and Cl^- are coupled in our vesicle preparation. Together, these observations argue in favor of the passive diffusion of chloride in response to an inside-positive membrane potential. Cl^- may have an additional stimulatory affect on the ATPase since Walker and Leigh (31), working with beet root vacuoles, found a chloride stimulation of an ATPase which was insensitive to 0.01% Triton X-100, and DuPont *et al.* (7) observed chloride stimulation of ATPase activity in corn root microsomal membranes in the presence of FCCP.

Organic anions may also be transported into microsomal vesicles. Marin *et al.* (18) have demonstrated ATP-driven citrate uptake by lutoid vesicles of *Hevea brasiliensis*. Citrate uptake was closely correlated with acidification of the vesicle interior. However, our system differs in this respect since we have shown that under the conditions used, malate, citrate, and succinate are unable to support significant methylamine accumulation (Table II) and do not compete with $^{36}Cl^-$ uptake (data not shown).

The lack of inhibition by oligomycin and the pH optimum of 7.5 make it unlikely that mitochondrial membranes are responsible for the proton pumping activity. However, sensitivity to DES and DCCD is consistent with the involvement of an H^+-ATPase. Vanadate, a specific inhibitor of the plasma membrane proton pump ATPase of *Neurospora* (1), has recently been shown to block both proton extrusion. (4, 13) and the plasma membrane ATPase (7, 20; M. Jacobs, A. Gepstein, and L. Taiz, unpublished data) of plant tissues. It is therefore significant that we observed no inhibition of proton pumping by vanadate even at high concentrations (100 μM). Hager and Helmle (9), also working with corn coleoptiles, obtained similar results. However, it should be noted that there have been reports of vanadate-sensitive H^+ transport in vesicles (28, 30). The reason for the discrepancy is unclear at present. Assuming that vanadate is a specific probe for the plasma membrane ATPase, our membrane preparation appears to be enriched in vesicles of nonplasma membrane origin. It is of interest that the tonoplast ATPase of beet roots shows features which are very similar to the activity we have described, including anion-enhancement, slightly alkaline pH optimum, and insensitivity to vanadate (31). Studies aimed at localizing this activity are described in the following paper.

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