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Localization of the Proton Pump of Corn Coleoptile Microsomal Membranes by Density Gradient Centrifugation[1]

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ABSTRACT

Previous studies characterizing an ATP-dependent proton pump in microsomal membrane vesicles of corn coleoptiles led to the conclusion that the proton pump was neither mitochondrial nor plasma membrane in origin (Mettler, Mandala, Taiz 1982 Plant Physiol 70: 1738–1742). To facilitate positive identification of the vesicles, corn coleoptile microsomal membranes were fractionated on linear sucrose and dextran gradients, with ATP-dependent [^{14}C]methylamine uptake as a probe for proton pumping. On sucrose gradients, proton pumping activity exhibited a density of 1.11 grams/cubic centimeter and was coincident with the endoplasmic reticulum (ER). In the presence of high magnesium, the ER shifted to a heavier density, while proton pumping activity showed no density shift. On linear dextran gradients, proton pumping activity peaked at a lighter density than the ER. The proton pump appears to be electrogenic since both [^{14}C]SCN$^-$ uptake and ^{36}Cl$^-$ uptake activities coincided with [^{14}C] methylamine uptake on dextran gradients. On the basis of density and transport properties, we conclude that the proton pumping vesicles are probably derived from the tonoplast. Nigericin-stimulated ATPase activity showed a broad distribution which did not coincide with any one membrane marker.

Primarily on the basis of electrophysiological evidence (24), it is believed that the plant plasma membrane contains an H^+-ATPase which pumps protons electrogenically from the cytoplasm to the cell exterior. According to Mitchell's chemiosmotic coupling theory, the resulting electrochemical proton gradient provides the driving force for the transport of other ions and organic molecules into the cell (16). The plasma membrane ATPase has been implicated in auxin-induced proton extrusion leading to cell elongation in stem and coleoptile tissues (22). However, direct evidence for the existence of a plasma membrane proton pump has been elusive. A comparable primary transport mechanism also appears to operate in tonoplasts. Electrophysiological studies (1) as well as *in vitro* transport experiments with purified vacuoles (12, 13) have indicated that the tonoplast contains an electrogenic H^+-ATPase which pumps protons into the vacuole. In addition to their role in providing the proton motive force for transport, it has been suggested that these pumps may participate in pH regulation of the cytoplasm (19).

Microsomal membrane vesicle preparations from a variety of tissues have recently been shown to possess ATP-dependent proton pumping or electrogenic activity (4, 6, 7, 14, 18, 25, 26, 28). In two cases, the activity was attributed either to plasma membranes (18, 28) or to plasma membrane precursors (6, 7). However, microsomal membrane preparations are enriched in a great diversity of light membrane types, including endoplasmic reticulum, golgi, and tonoplasts. In the preceding paper (15), we reported on the properties of a microsomal H^+ pump isolated from corn coleoptiles. Because of the absence of stimulation by potassium and its insensitivity to vanadate, we suggested that the activity is probably not localized on plasma membranes. In this paper, we show that the proton pump is separable from markers for ER, golgi, mitochondria, and plasma membranes on density gradients. The density of the proton pump is consistent with it being associated with tonoplasts.

MATERIALS AND METHODS

Density Gradient Centrifugation. Corn coleoptiles were harvested and a microsomal pellet was obtained as described previously (15). The pellet was resuspended in 2 ml of buffer (0.25 M sucrose, 1 mM EDTA, 0.5 mM DTT, 5 mM Tris-Mes [pH 7.2] and layered over a 34-ml linear gradient. Sucrose gradients were 15 to 45% (w/w) made in 0.5 mM EDTA, 2.5 mM Tris-Mes (pH 7.2). For the Mg^{2+}-sucrose gradient, 4 mM $MgCl_2$, 1 mM EDTA was present in the homogenization buffer, and 3 mM $MgCl_2$, 1 mM EDTA was included in subsequent buffers, including in the gradient. The dextran gradient consisted of 30 ml of 1 to 12% (w/w) dextran made in 0.25 M sucrose, 2.5 mM Tris-Mes (pH 7.2), with a 4-ml cushion of 40% sucrose. The gradients were centrifuged in a Beckman SW-28 rotor at 80,000*g* for 2 to 3 h. Fractions were 1.2 to 1.5 ml/tube.

Methylamine Uptake. Fractions from sucrose gradients were diluted with 0.5 mM EDTA, 2.5 mM Tris-Mes (pH 7.2), and centrifuged for 1 h in a Beckman 50Ti rotor at 100,000*g*. Pellets were resuspended in transport buffer (0.25 M sucrose, 10 mM KCl, 2.5 mM Tris-Mes (pH 7.0), and the uptake of [^{14}C]methylamine and ^{36}Cl was measured as described previously (15). For dextran gradients, aliquots were used directly from gradient fractions. [^{14}C]KSCN[4] uptake was essentially the same except 5 mM ATP, 2.5 mM $MgSO_4$ was used, KCl was omitted, and the reaction was stopped after a 3-min incubation. [^{14}C]KSCN had a specific activity of 62 mCi/mmol in ethanol and 12.4 μM/assay, 0.77 μCi/ml/assay was used.

Enzymic Assays. NADPH-Cyt *c* reductase, and Cyt *c* oxidase activities were determined spectrophotometrically essentially as described (10). ATP and latent IDP hydrolytic activity was determined by detection of released Pi (5) after a 20 to 30-min incu-

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[4] Abbreviations: KSCN, potassium thiocyanate; NPA, naphthylphthalamic acid; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone.

bation at 37°C in 2.5 mM substrate. Protein was determined by the Lowry procedure (11). α-Mannosidase activity was measured according to Boller and Kende (2). NPA binding was assayed as specific binding according to the procedure of Ray (20).

RESULTS

A 12,000 to 80,000*g* microsomal fraction from a corn coleoptile homogenate was subjected to centrifugation on a sucrose density gradient for 3 h. Figure 1 illustrates the distribution of six marker enzymes. The ER marker, NADPH-Cyt *c* reductase, was located at the light end of the gradient (1.11 g/cc), while Cyt *c* oxidase, the mitochondrial marker, occurred as a sharp band at 1.17 g/cc. Latent IDPase, a golgi marker, peaked at 1.13 g/cc, and the major peaks of the plasma membrane markers, vanadate-inhibited KCl-stimulated ATPase, and NPA specific binding separated from the golgi marker at 1.16 g/cc. A shoulder of vanadate-sensitive ATPase activity, which overlapped the latent IDPase peak, was consistently detected.

In preliminary experiments, it was determined that the crude microsomal KCl-stimulated ATPase activity was enhanced by nigericin, an ionophore which collapses K^+/H^+ transmembrane gradients. Since Sze (27) has proposed that nigericin stimulation of ATPase indicates the presence of sealed membrane vesicles, we examined the location of nigericin-enhanced KCl-stimulated ATPase on the sucrose gradient. The activity had a broad distribution which extends from the ER to the plasma membrane region and was not associated with any particular marker (Fig. 1).

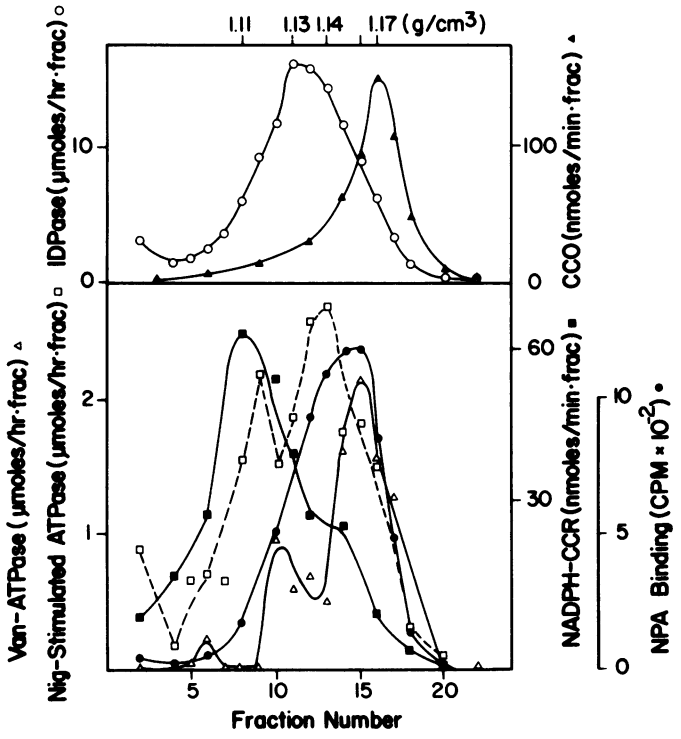

FIG. 1. Fifteen to 45% sucrose gradient of corn coleoptile microsomes centrifuged for 3 h at 80,000*g*. Van-ATPase (△) represents the inhibition of the KCl-stimulation of ATPase activity by 20 μM vanadate. Nig-stimulated ATPase (□) represents the stimulation of the KCl-stimulated ATPase activity by 10 μM nigericin. NADPH-CCR (■) is NADPH-Cyt *c* reductase activity assayed spectrophotometrically. NPA binding (●) is specific cpm calculated as the difference between membranes pelleted with 1×10^{-9} M[^{3}H]NPA and pellets with 1×10^{-5} M NPA in addition to labeled NPA. IDPase (○) is latent activity assayed in the presence of 0.1% digitonin, and CCO (▲) is Cyt *c* oxidase activity. The density of sucrose measured in g/cm^3 is indicated at the top.

An additional marker was also characterized in a separate sucrose gradient: α-mannosidase, a tonoplast marker in yeast (29). NPA binding activity was present in the density range 1.14 to 1.16 g/cc (Fig. 2), similar to the peak of vanadate-sensitive ATPase. The α-mannosidase activity which entered the gradient formed a broad band around 1.13 g/cc. In other plant tissues, it has been found that α-mannosidase is a soluble enzyme (2). The fact that α-mannosidase activity was coincident with the protein peak (Fig. 2) may indicate a nonspecific binding to membranes.

The location of ATP-driven methylamine uptake activity on a sucrose gradient is shown in Figure 3. Proton pumping was restricted to a single peak in the light region of the gradient (~1.11 g/cc) and could not be resolved from the ER marker. Because it seemed likely that the ER was masking the proton pumping membrane fraction, similar gradients were run in the presence of 3 mM MgCl, which shifts the ER to higher densities (17). Figure 4 illustrates the effects of high magnesium on the distribution of various markers. While the densities of several markers have been altered, it is clear that the ER has been shifted away from the proton-pumping fraction which remained in the light region.

A second approach taken to resolve the proton pumping vesicles from the ER membranes was centrifugation on a 1 to 12% linear

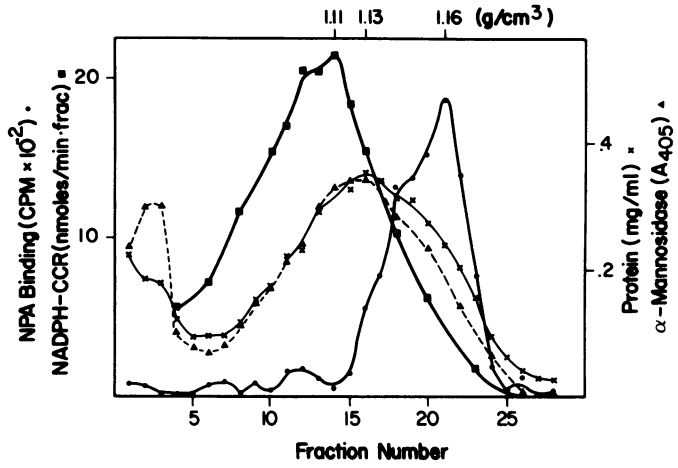

FIG. 2. NPA-specific binding (●), NADPH-Cyt *c* reductase (■), protein (×), and α-mannosidase (▲) activities on a sucrose gradient as in Figure 1.

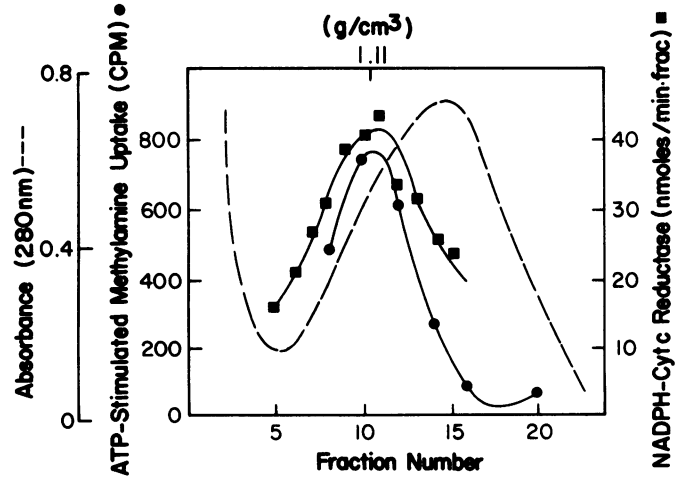

FIG. 3. Absorbance (---), ATP-stimulated methylamine uptake (●), and NADPH-Cyt *c* reductase (■) activites on a sucrose gradient. ATP-stimulated methylamine uptake calculated after a 5-min incubation and subtraction of control (−ATP). Control methylamine uptake ranged from 100 to 300 cpm.

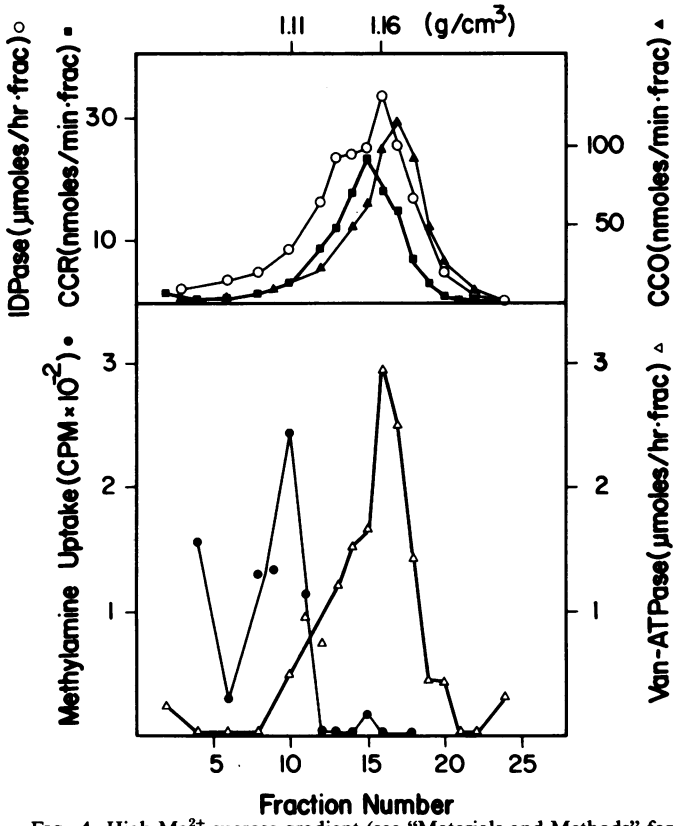

FIG. 4. High Mg^{2+} sucrose gradient (see "Materials and Methods" for details on preparation). Methylamine uptake (●) is the ATP-stimulated component calculated as in Figure 3. Vanadate-inhibition of the KCl-stimulated ATPase (△), latent IDPase (○), NADPH-Cyt *c* reductase (■), and Cyt *c* oxidase (▲).

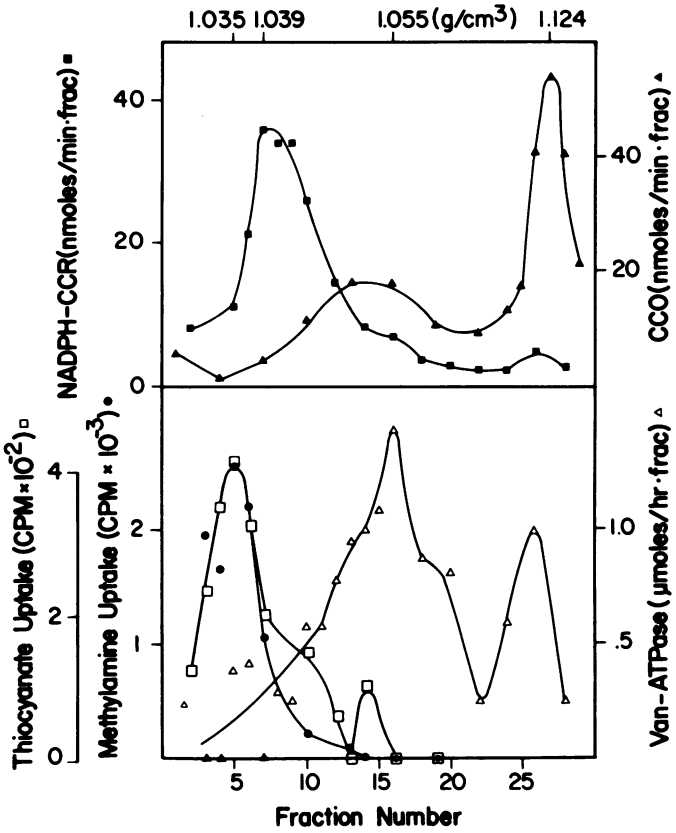

FIG. 5. One to 12% dextran gradient with a 40% sucrose cushion after a 2-h centrifugation of corn coleoptile microsomes at 80,000*g*. Thiocyanate uptake (□) is the ATP-stimulated component calculated by subtracting control values (−ATP) after a 3-min incubation. Methylamine uptake (●), vanadate-inhibition of the KCl-stimulated ATPase (△), NADPH-Cyt *c* reductase (■), and Cyt *c* oxidase (▲).

dextran gradient. A cushion of 40% sucrose trapped any activity which sedimented to the bottom. The dextran gradient clearly separated ATP-stimulated methylamine uptake from the ER and mitochondrial markers, and the vanadate-inhibited ATPase (Fig. 5).

Inasmuch as there have been recent reports of microsomal preparations containing ATP-dependent electrogenic pumps (15, 18, 25, 26, 28), the uptake of thiocyanate ($[^{14}C]SCN^-$) was also monitored in fractions from the dextran gradient. ATP-driven SCN^- uptake was detected on the dextran gradient, and the peak of activity coincided exactly with methylamine uptake (Fig. 5). This is strong evidence that the same population of vesicles involved in proton pumping contains an electrogenic (inside positive) ATPase, and that it is, in fact, an electrogenic proton pump. A second, much smaller peak of SCN^- uptake was detected in the heavier region of the gradient.

Vesicles isolated from the dextran step gradient accumulated $^{36}Cl^-$ in the presence of ATP (15), so we localized this activity on the linear dextran gradients (Fig. 6). The peak of ATP-driven chloride uptake coincided with that of methylamine uptake, similar to the thiocyanate uptake results. On the basis of density, it seems that all of the electrogenic proton transport activities we have measured reside on the same light membrane fraction.

DISCUSSION

ATP-driven proton pumping or electrogenic activity has now been detected in the microsomal membranes of a number of different plant tissues (4, 6, 7, 14, 18, 25, 26, 28), but the subcellular origin of this activity remains unresolved. Hager and Helmle (7) speculated that the microsomal vesicles involved may actually be native vesicles derived either from the golgi and destined for the plasma membrane or from the ER in the form of 'prevacuoles.' Sze and Churchill (28) and Rasi-Caldogno *et al.* (18) proposed a plasma membrane origin for the electrogenic pump. In the preceding paper (15), we used the accumulation of $[^{14}C]$methylamine in microsomal membranes from corn coleoptiles to show that proton accumulation was insensitive to vanadate and oligomycin, stimulated by chloride, and had a pH optimum of 7.5. These results are strong, albeit indirect evidence against the involvement of plasma membrane or mitochondrial vesicles in generating the pH gradient. In this paper, we employed density gradient fractionation to show that the peak of proton pumping is distinct from markers for the ER, golgi, mitochondria, and plasma membranes.

Centrifugation of corn coleoptile membranes in a sucrose gradient preserved the proton transport capacity of the vesicles and facilitated their localization. Proton pumping activity was clearly separated from the golgi, mitochondrial, and plasma membrane markers. Vanadate inhibition of the KCl-stimulated ATPase was used as a plasma membrane marker since the major peak coincided well with the NPA binding peak. There was consistently, however, a smaller peak or shoulder of vanadate-inhibited ATPase activity found possibly associated with the golgi. Ray *et al.* (21) reported a weak ATPase as well as phosphatase activity associated with the golgi in peas, which is supported by the finding that a second peak of vanadate-sensitive ATPase is coincident with glucan synthetase I and latent IDPase activity in peas (M. Jacobs, A. Gepstein, and L. Taiz, unpublished results). Occasionally, there was also some activity in the light region of the gradients as in Figure 5, but because these peaks were not found consistently, we treated them as scatter and drew the line accordingly. There is

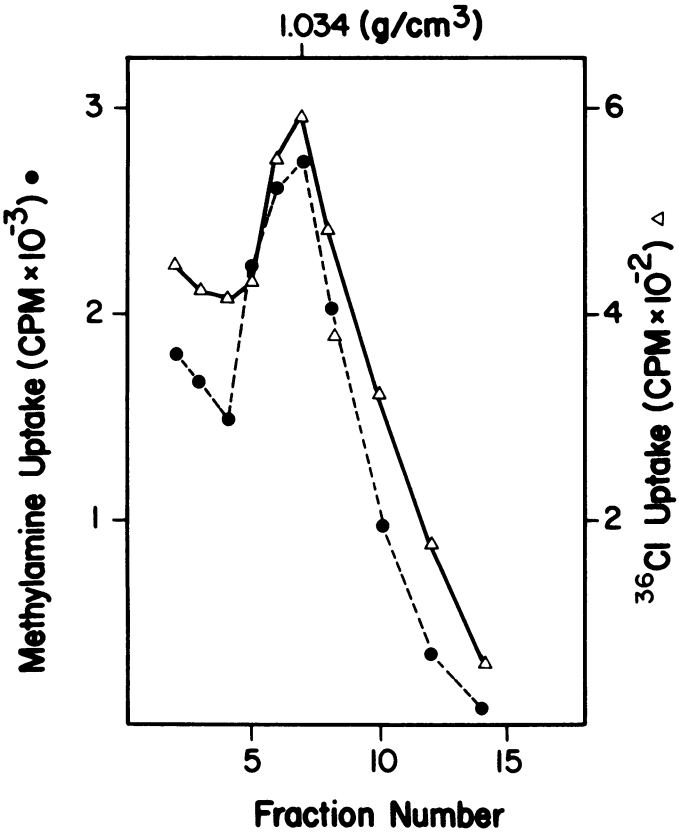

FIG. 6. Methylamine uptake (●) and ^{36}Cl uptake (△) on a dextran gradient prepared as described in Figure 5 and in "Materials and Methods." Both activities represent the ATP-stimulated components.

still the possibility that vanadate is not a specific inhibitor of the plasma membrane ATPase even when expressed as inhibition of the KCl-stimulated component. Vanadate can also inhibit phosphatase activity (23).

On sucrose gradients, proton pumping coincided with the ER. In the presence of high magnesium, however, the ER shifted toward the heavy end of the gradient, leaving the proton transport vesicles at the light end. The inclusion of magnesium thus provides a means of unmasking certain fractions for which definitive markers are lacking and also suggests a method for the isolation of these vesicles. The results of the high magnesium sucrose gradient were supported by the distribution pattern obtained by centrifugation in a linear dextran gradient. Here again, H^+ transport activity accumulated at a lighter region of the gradient relative to the ER. Thus, it seems clear that the active vesicles do not represent fragments of ER.

That the proton pump is electrogenic was confirmed by the ability of the vesicles to accumulate thiocyanate in the presence of ATP. Since the vesicles can also take up $^{36}Cl^-$, chloride probably moves into the vesicles in response to the inside-positive electrical potential. This result is in agreement with the results of CCCP inhibition of $^{36}Cl^-$ uptake reported in the preceding paper (15). All three of the probes localized a population of vesicles active in transporting protons in the low-density region of the gradients. Only thiocyanate showed an additional, much smaller peak of activity in the heavier region of the gradient. This activity could be associated with the plasma membrane or another membrane fraction such as the golgi, since it is not inhibited by vanadate (S. Mandala and L. Taiz, unpublished results). The lack of any detectable methylamine uptake in this region suggests that cations other than protons are responsible for this electrogenic gradient.

Nigericin is reported to act specifically and electroneutrally to collapse H^+/K^+ transmembrane gradients. Nigericin-stimulated ATPase, however, had a broad distribution across most of the gradient including the peaks of ER, golgi and plasma membrane, whereas proton pumping was located in only one area. There are two possible explanations for the broad distribution of nigericin stimulation: either nigericin is exerting nonspecific stimulatory effects on general ATPase activity or some of the nigericin stimulation is due to the activity of a K^+-transporting ATPase. The inability to detect methylamine uptake over much of the distribution of nigericin-stimulation suggests that caution should be exercised when interpreting the response of membrane ATPases to nigericin.

For several reasons, we infer that the proton pumping vesicles are derived from the tonoplast. (a) The activity bands at a density of 1.11 g/cc on sucrose gradients. This is the same density as that reported for radioactively-labeled tonoplasts obtained from purified vacuoles from tobacco (3). (b) Many of the properties of the proton pump are similar to the properties of intact vacuoles and tonoplast vesicles isolated from *Hevea brasiliensis*, *e.g.* a high specificity for ATP, dependence on anions, and insensitivity to vanadate (12, 13). (c) It has been reported that a tonoplast-associated ATPase isolated from beet roots is stimulated by chloride, rather than potassium, and is insensitive to vanadate (30). Although anion-stimulated ATPase activity has been reported to be present in some plasma membrane preparations (*e.g.* 8), the plasma membrane ATPase of corn roots does appear to be potassium-stimulated (9). In oat roots, the potassium-stimulated ATPase was distributed mainly in the pellet of the dextran step gradient, while the interface vesicles were enriched in a chloride-stimulated activity (26). The weight of the evidence thus supports the interpretation that the proton pumping vesicles of corn coleoptile microsomes are derived from the tonoplast.

Less is known about the tonoplast ATPase than the plasma membrane ATPase, in part because the absence of a reliable tonoplast marker (17) has hindered isolation and study of this membrane. If ATP-driven methylamine uptake is, as we have indicated, a tonoplast function, it may prove to be a useful marker for tonoplasts as well as an indicator of an important physiological function.

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