Cytochemical Localization of ATPase Activity in Oat Roots Localizes a Plasma Membrane-Associated Soluble Phosphatase, Not the Proton Pump[1]

Donald B. Katz*, Michael R. Sussman, Robert J. Mierzwa, and Ray F. Evert
Departments of Horticulture (D.B.K., M.R.S.) and Botany (R.J.M., R.F.E.), University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

Cytochemical techniques employing lead-precipitation of enzymically released inorganic phosphate have been widely used in attempts to localize the plasma membrane proton pump (H$^+$-ATPase) in electron micrographs. Using *Avena sativa* root tissue we have performed a side-by-side comparison of ATPase activity observed in electron micrographs with that observed in *in vitro* assays using ATPases found in the soluble and plasma membrane fractions of homogenates. Cytochemical analysis of oat roots, which had been fixed in glutaraldehyde in order to preserve subcellular structures, identifies an ATPase located at or near the plasma membrane. However, the substrate specificity and inhibitor sensitivity of the *in situ* localized ATPase appear identical to those of an *in vitro* ATPase activity found in the soluble fraction, and are completely unlike those of the plasma membrane proton pump. Further studies demonstrated that the plasma membrane H$^+$-ATPase is particularly sensitive to inactivation by the fixatives glutaraldehyde and formaldehyde and by lead. In contrast, the predominant soluble ATPase activity in oat root homogenates is less sensitive to fixation and is completely insensitive to lead. Based on these results, we propose a set of criteria for evaluating whether a cytochemically localized ATPase activity is, in fact, due to the plasma membrane proton pump.

Results from biochemical, genetic, and electrophysiological experiments performed over the past decade have demonstrated that there is a proton pump (H$^+$-ATPase) in the plasma membrane of fungi and higher plants (1, 9, 12, 15, 16, 20). This enzyme creates an electrochemical gradient of protons which drives the uptake and accumulation of solutes into the cytoplasm. Microscopic localization of the H$^+$-ATPase would be useful to confirm its role in plasma membrane function as well as to identify differences in its distribution and activity among various cell types.

The most commonly used procedure for cytochemical localization of phosphatases involves incubation of fixed tissue in a medium containing lead and substrate and visualization of lead-phosphate precipitates (25). Numerous investigators have used this approach to localize ATPase activity in plant cells (7, 8, 10, 14, 17), and have observed substantial deposition of precipitate on the plasma membrane. When we recently localized ATPase

activity in corn leaves (RF Evert, RJ Mierzwa, W Eschrich, unpublished data), we likewise observed mainly plasma membrane deposition, but the substrate requirements and inhibitor sensitivities of the cytochemically localized activity were different from those observed with *in vitro* assays of H$^+$-ATPase isolated from higher plants and fungi (3, 24). In addition, although biochemical experiments indicate that the proton pump releases all of its phosphate on the cytoplasmic face (16), much of the cytochemically detected deposition was observed on the extracellular face of the plasma membrane. Taken together, these results led us to question whether the localized ATPase could be the plasma membrane proton pump (H$^+$-ATPase). Rather, our data suggested that some other phosphatase, perhaps loosely associated with the plasma membrane, must be the source of the localized activity.

In order to test this suggestion rigorously, we compared the *in vitro* sensitivities of the H$^+$-ATPase in isolated plasma membranes and of a soluble ATP-hydrolyzing activity in oat root homogenates to the inhibitors and fixatives used in cytochemical studies. We also tested the substrate specificities of these enzymes, for comparison to cytochemical work. Oat roots were used because of the ease of obtaining stable plasma membrane preparations with a high specific activity of ATPase (20). As described below, cytochemical localization of ATPase activity in oat roots gives results qualitatively identical to those obtained in the earlier work on corn leaves. Based on results obtained from the experiments described herein, we suggest a set of criteria for evaluating whether cytochemically localized ATPase activity is in fact due to the activity of the H$^+$-ATPase.

MATERIALS AND METHODS

Plasma Membrane Preparation. Membranes were isolated as described by Surowy and Sussman (20). Briefly, roots were homogenized in 1.15 M sucrose, 25 mM EDTA, 35 mM 2-mercaptoethanol, 250 mM Tris (adjusted to pH 8.5 at room temperature with HCl) 1 mM PMSF.[2] Microsomes were collected by centrifugation of the 8,000g supernatant at 48,000g for 1 h and sedimented through a sucrose density gradient. Plasma membranes were collected at the interface between 33% (w/w) sucrose and 46% (w/w) sucrose and stored at −80°C. Immediately before use, plasma membranes were pelleted at 200,000g and resuspended in buffer containing 10 mM Pipes/Tris or Pipes/NaOH (pH 6.7) and 0.3 M sucrose. In most experiments the buffer also included 100 mM KCl and 1 mM EDTA.

Soluble Phosphatase Preparation. Protein in the 48,000g supernatant from the oat root homogenate was fractionated with ammonium sulfate. All of the phosphatase activity precipitated

[2] Abbreviations: PMSF, phenylmethylsulfonyl fluoride; PCMBS, *p*-chloromercuribenzenesulfonic acid; β-GP, β-glycerol phosphate.

between 40 and 80% saturation. This pellet was resuspended in 10 mM Pipes/Tris (pH 6.7) and stored at $-80°C$ until use.

ATPase Assay. Enzyme activities were assayed at 30°C in a reaction mixture containing 10 mM Pipes/Tris (pH 6.7) and, except where noted, containing 5 mM $MgCl_2$, and 100 mM KNO_3. Phosphate released was assayed by the method described in reference (5).

Calculation of [MgATP] and [Pb^{2+}]. The methods of Storer and Cornish-Bowden (19) were used to calculate concentrations of the relevant species in assay solutions containing various total concentrations of $Pb(NO_3)_2$, KNO_3, $MgCl_2$, or $Mg(NO_3)_2$ and ATP. The formation constants for $HATP^{3-}$, $KATP^{3-}$, $MgATP^{2-}$, and $MgHATP^-$ came from Storer and Cornish-Bowden (19), while the constants for $PbOH^+$ and $PbATP^{2-}$ came from Tice (21).

Cytochemistry and Electron Microscopy. Oat roots fixed in cold glutaraldehyde (2.5% v/v) for 90 min were cut into 100 μm thick sections and incubated with a reaction mixture containing 2 mM ATP, ADP or β-GP, 2 mM $Mg(NO_3)_2$, 50 mM KNO_3, 2 mM $Pb(NO_3)_2$, and 50 mM Tris/maleate (pH 6.5) at 35°C for 2.5 h. Some tissue sections were preincubated in buffer containing either 2 mM PCMBS or 0.1 mM NH_4MoO_4 prior to incubation with reaction mixture to which 2 mM PCMBS or 0.1 mM NH_4MoO_4, respectively, had been added. Following incubation, the tissue was rinsed thoroughly with distilled water and then postfixed in 2% osmium tetroxide in 0.05 M cacodylate buffer overnight in a refrigerator. The tissue was dehydrated in a graded acetone series and infiltrated and embedded in Spurr's epoxy resin (18). Thin sections were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome (Sorval) and viewed and photographed unstained with a Hitachi (Tokyo, Japan) HU11C microscope.

RESULTS

In Situ **Cytochemical Localization.** Electron micrographs of glutaraldehyde-fixed oat roots show localization of ATPase activity at the plasma membrane in all cell types of the root; however, localization is most intense in cells of the phloem strands (Fig. 1A). The plasma membrane deposition is seen on both sides of the membrane (Fig. 1B), as well as scattered throughout the cell wall. The ATPase activity was unaffected by PCMBS (Fig. 1C) but was almost entirely eliminated if the tissue sections were treated with 0.1 mM molybdate prior to incubation with substrate (Fig. 1D). A cytochemical activity with the same pattern of localization was observed when either ADP (Fig. 2A) or β-GP (Fig. 2B) was used as the substrate. As with the ATPase activity, these activities were unaffected by PCMBS (data not shown).

Yield of ATPases from Homogenized Oat Roots. A membrane fraction enriched in the plasma membrane proton pump and a soluble fraction enriched in an ATP-hydrolyzing phosphatase were isolated from homogenized oat roots. Ammonium sulfate precipitation of the 48,000g supernatant fraction yielded 0.22 $\pm$ 0.01 units of ATPase activity per g fresh weight of root tissue (mean $\pm$ SE, average of 2 preparations). The average yield of plasma membrane proton pump (H$^+$-ATPase) activity was 0.062 $\pm$ 0.008 units per g fresh weight (mean $\pm$ SE, average of 10 preparations). Therefore, on a fresh weight basis, there is approximately three times as much extractable soluble phosphatase activity as there is plasma membrane proton pump activity.

Substrate Specificity *in Vitro*. As judged by biochemical assays, the plasma membrane H$^+$-ATPase is quite different from soluble ATPase activities in its substrate specificities and divalent cation requirements (Table I). The plasma membrane H$^+$-ATPase showed no activity with substrates other than ATP and displayed no activity in the absence of Mg^{2+}. In contrast, the soluble enzyme was slightly inhibited by divalent cations and readily hydrolyzed ADP and β-GP in addition to ATP. The small amount of mo-

lybdate-insensitive activity in the soluble fraction had a substrate specificity different from the total soluble activity, suggesting it represents a different enzyme or enzymes. Although it could not hydrolyze β-GP, it did hydrolyze ADP.

Effect of Fixatives and Inhibitors *in Vitro*. In order for the lead deposits to be localized by microscopy, the tissue must be fixed prior to incubation with substrate. We tested the sensitivity of soluble and plasma membrane ATPase activities to fixatives by incubating with various concentrations of glutaraldehyde or formaldehyde for 60 min at 0°C. In the case of formaldehyde, we also tested sensitivity by incubating for 30 min at room temperature (22°C). We observed that with either fixative, the plasma membrane activity was more sensitive than the soluble activity (Fig. 3). Fixation of the plasma membrane in 0.5% (v/v) glutaraldehyde left no detectable activity (detection limits were about 1%) while treatment of the soluble fraction under the same conditions left 90% of the activity intact (Fig. 3A). The plasma membrane activity was markedly less sensitive to formaldehyde than to glutaraldehyde (Fig. 3B). After fixation in 5% (v/v) formaldehyde at 0°C, 18% of the H$^+$-ATPase activity remained. By contrast, the soluble activity was more sensitive to formaldehyde than to glutaraldehyde. For both enzymes, the sensitivity to formaldehyde was greatly increased by incubating at room temperature (Fig. 3C).

Lead must be present in the cytochemical reaction medium in order to precipitate phosphate and allow visualization of ATPase activity in electron micrographs. Lead forms a soluble complex with ATP with a formation constant of 4.6×10^4 (21). Since the formation constant for the MgATP complex is of the same order of magnitude (3.5×10^4, cited in Ref. 19), the presence of lead reduces the concentration of MgATP, which is the true substrate of the H$^+$-ATPase (2, 3). In order to avoid inhibition of H$^+$-ATPase activity due to this lead-induced reduction in [MgATP], at each level of lead we adjusted the [MgATP] to 5 mM by increasing the total concentration of both Mg^{2+} and ATP. The total concentrations of ATP, Mg^{2+}, lead, and K$^+$ and the calculated concentrations of the relevant species for the *in vitro* study and for the cytochemical work are shown in Table II.

The plasma membrane proton pump (H$^+$-ATPase) is inhibited by low concentrations of lead (Fig. 4). By contrast, the soluble ATPase activity shows no inhibition, even with total [$Pb(NO_3)_2$] at 4.7 mM. The increase in ATPase activity of the soluble fraction seen at higher lead concentrations may be caused by the higher total ATP concentrations in these solutions, suggesting that the soluble activity can hydrolyze PbATP as well as MgATP. The actual species which causes inhibition of the proton pump cannot be determined from this experiment since [PbATP], [Pb^{2+}], and the total lead concentration all increase simultaneously (Table II).

Therefore, we designed a series of four solutions with a calculated [MgATP] of 5.0 $\pm$ 0.2 mM in which either the [PbATP] or the [Pb^{2+}] changed 10-fold with less than a 5% change in the other species (Table III). Inhibition of the plasma membrane ATPase responded to changes in [Pb^{2+}], but did not respond to changes in [PbATP] or total lead, suggesting that Pb^{2+} is the species causing the inhibition.

In previous studies with fractionated membranes and isolated enzyme, the plasma membrane proton pump has been reported to be sensitive to treatment with sulfhydryl reagents (12, 24). We have confirmed these observations in the present study using PCMBS (Fig. 5). In the *in vitro* ATPase assay, this reagent strongly inhibited the plasma membrane proton pump ($K_{0.5}$ less than 0.05 mM) (Fig. 5A). Inhibition of the soluble ATPase activity was much less pronounced, with 50% inhibition occurring at a 40-fold higher inhibitor concentration.

Molybdate has been used in biochemical studies of the plasma membrane ATPase because it inhibits a vanadate-sensitive sol-
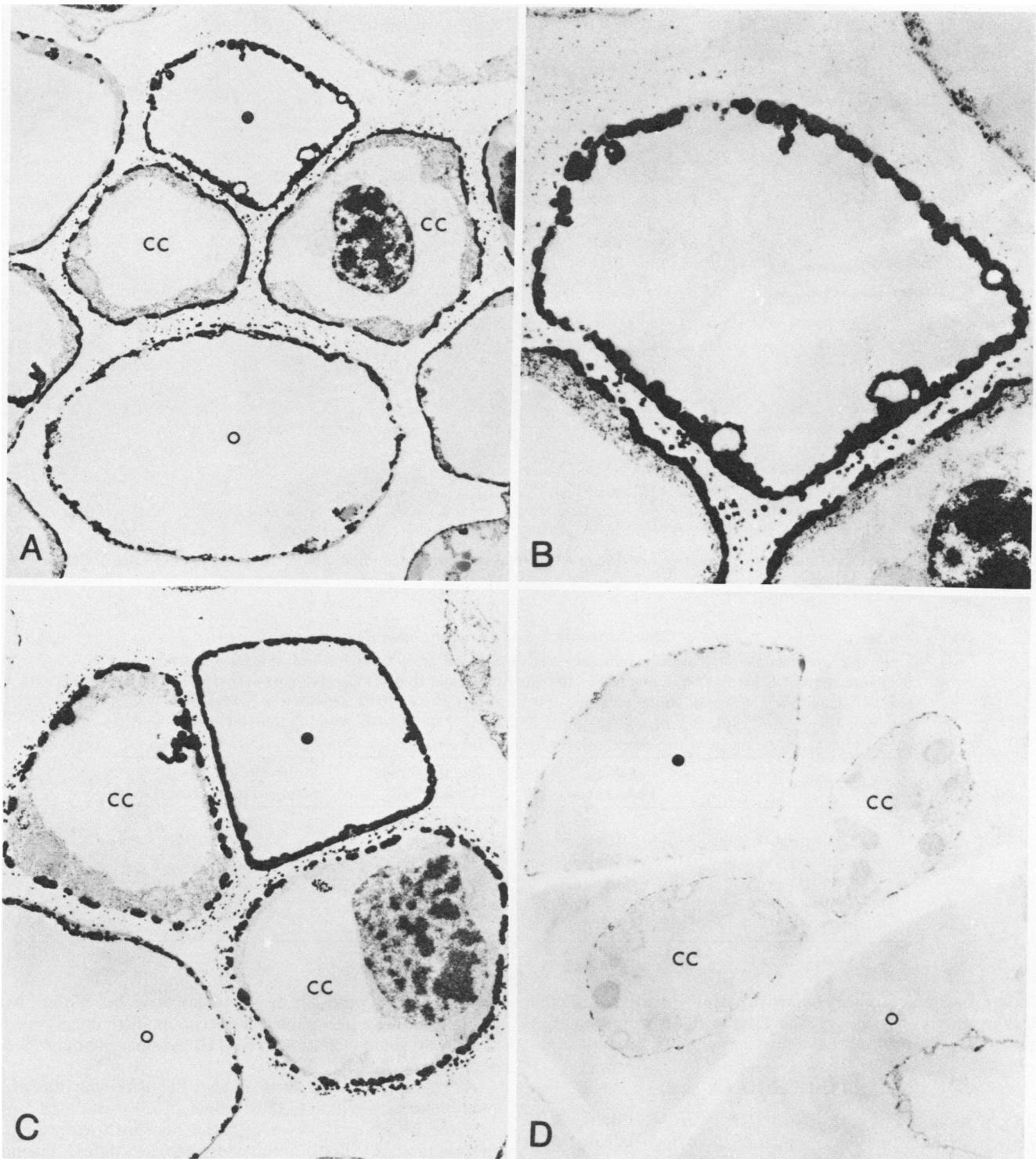
Fig. 1. Cytochemical localization of ATPase activity in oat root phloem. Oat root tissue sections were fixed, incubated with reaction mixture, and prepared for electron microscopy as described in "Materials and Methods." A, Representative section using ATP as substrate, magnified ×6,300; B, closer view of the same section, magnified ×15,450; C, section which was incubated with ATP as substrate in the presence of 2 mM PCMBS, magnified ×6,930; D, section which was preincubated in 0.1 mM NH_4MoO_4 and then incubated in reaction mixture containing 0.1 mM NH_4MoO_4, with ATP as substrate, magnified ×9,960. Cell types are marked as follows: (●) photophloem; (○) metaphloem; cc, companion cells.

uble phosphatase that sometimes contaminates plasma membranes during isolation (4, 24). It has been less commonly used in cytochemical studies in part because of its insolubility in the presence of lead (14). In the present study we observed that the isolated plasma membrane H+-ATPase was indeed much less

sensitive to molybdate than the soluble activity (Fig. 5B). The membrane activity showed no inhibition at concentrations of molybdate as high as 0.1 mM while the soluble activity was 80% inhibited at a 10-fold lower concentration. The response to molybdate seems to distinguish two activities within the soluble
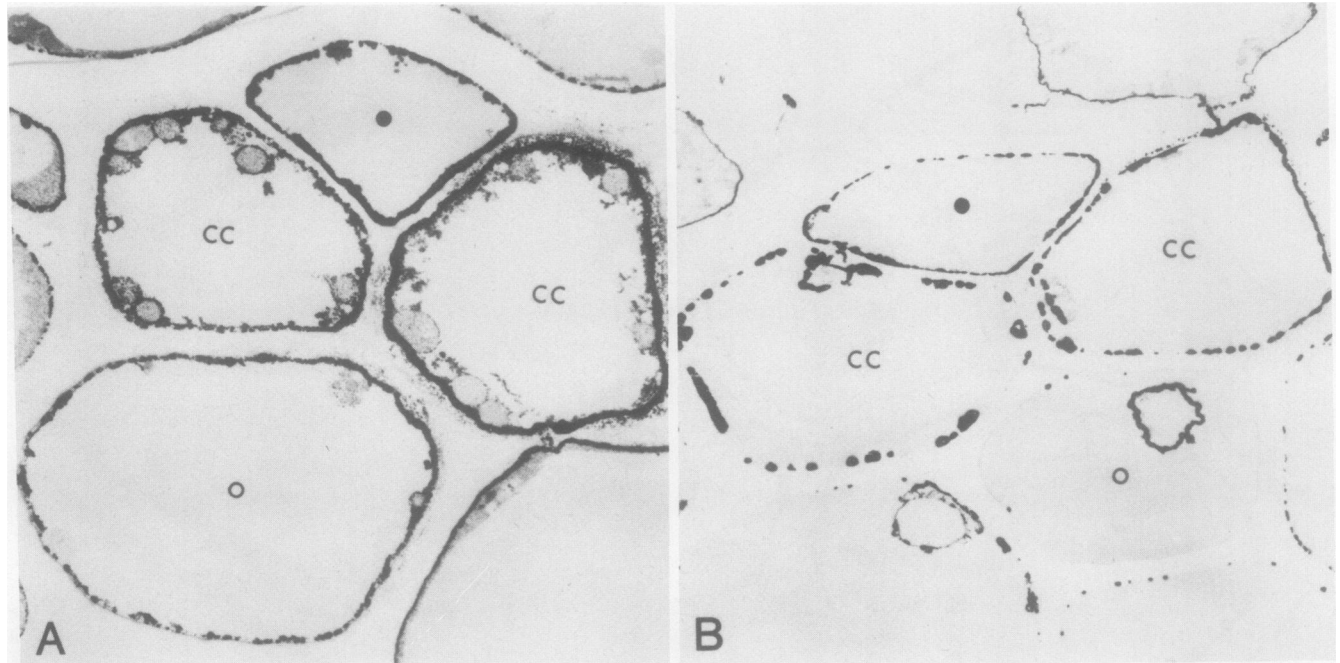
FIG. 2. Cytochemical localization with alternative substrates. A, ADP as substrate, magnified ×6,050; B, β-GP as substrate, magnified ×5,490. Cell types are marked as in Figure 1.

Table I. *Substrate Specificity and Divalent Cation Requirements*

Soluble phosphatase or plasma membranes were incubated at 30°C with 5 mM substrate, either 5 mM divalent cation or 1 mM EDTA, 5 mM NaN_3, 100 mM KNO_3, and 10 mM Pipes/Tris (pH 6.7). The molybdate resistant activity was measured in the presence of 0.1 mM NH_4MoO_4; 100% activity was 2.6 units ml^{-1} (total soluble), 3.5 units ml^{-1} (plasma membrane) and 0.43 units ml^{-1} (molybdate-resistant soluble). The % activity shown represents the mean of two replicates ± the standard error.

Substrate + Cation	Soluble Phosphatase	Plasma Membrane H^+-ATPase	Molybdate-Resistant Soluble Phosphatase
		% activity	
ATP + Mg^{2+}	100	100	100
ATP + Ca^{2+}	121 ± 1.2	0	ND^a
ATP + EDTA	151 ± 3.9	0	ND
ADP + Mg^{2+}	119 ± 0.7	0	28 ± 2.3
β-GP + Mg^{2+}	37 ± 2.4	0	0

[a] No data.

fraction, one which is very sensitive to molybdate and another which is nearly insensitive. The latter activity only comprises about 10 to 20% of the total activity using ATP as substrate.

DISCUSSION

In oat roots, as in corn leaves (RF Evert, RJ Mierzwa, W Eschrich, unpublished data), much of the cytochemically localized ATPase activity is found near the plasma membrane. Nevertheless, comparison of the substrate requirements and inhibitor sensitivities of the cytochemical activity to those observed *in vitro* for the H^+-ATPase demonstrate that this cytochemical activity cannot be due to the proton pump.

The molybdate-sensitive soluble ATPase activity we measured *in vitro* is probably the source of the ATPase activity observed cytochemically. Both activities are resistant to fixatives and lead, sensitive to molybdate and insensitive to PCMBS, and both hydrolyze ADP and β-glycerol phosphate in addition to ATP. It is possible that *in situ* this activity is loosely associated with membranes and is stripped from these structures by the high ionic strength (250 mM) and low divalent cation concentration

(25 mM EDTA) present in our homogenization buffer. A molybdate-sensitive phosphatase does contaminate membrane fractions from some plant tissues (4; MR Sussman, unpublished observations).

Alternatively, association of lead deposits with the plasma membrane may be due to short-range diffusion of the phosphate released by cytoplasmic or cell wall phosphatases prior to its precipitation by lead (6). If the phosphate does diffuse, the unique surface properties of the plasma membrane, which allow its isolation in two-phase polymer systems (11) may account for the special affinity of that membrane for the reaction product.

The proton pump will be difficult to localize successfully by cytochemical methods. Since lead is essential to the Wachstein-Meisel technique, its inclusion in the reaction mixture cannot be avoided, despite its inhibitory effect on the H^+-ATPase (Fig. 4). Reducing the lead concentration would reduce the inhibition of the proton pump, but may not give satisfactory localization due to increased diffusion of phosphate before precipitation, causing a change in the observed distribution of reaction product (13).

The extreme sensitivity of the proton pump to glutaraldehyde

A
GLUTARALDEHYDE 0° C 60 min

B
FORMALDEHYDE 0° C 60 min

C
FORMALDEHYDE 22° C 30 min

ATPase ACTIVITY (%)

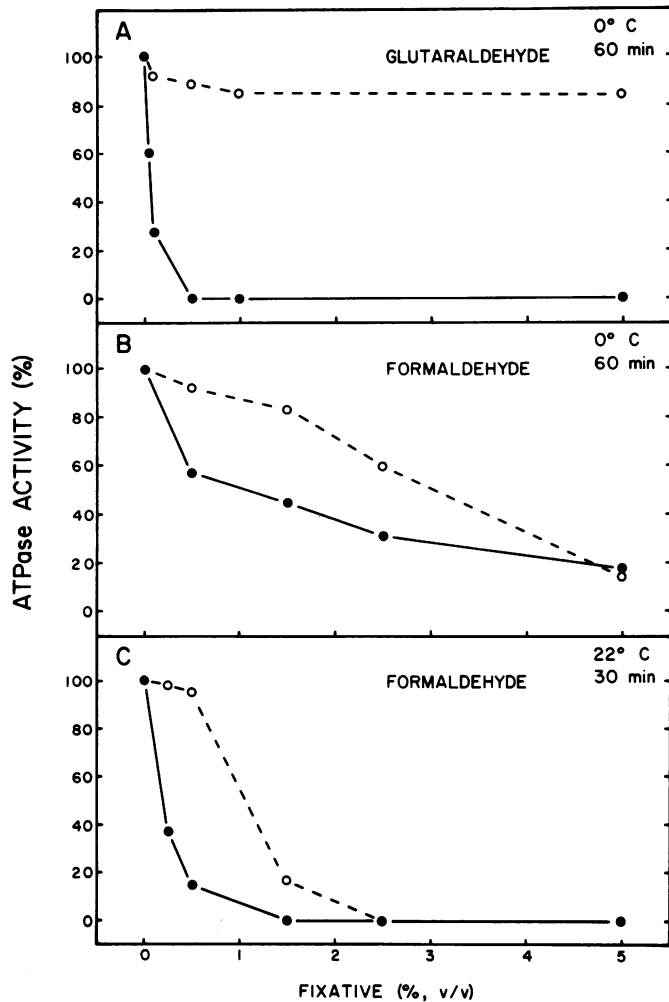FIXATIVE (%, v/v)

Pb(NO₃)₂ (mM)

ATPase ACTIVITY (%)

FIG. 3. Inhibition *in vitro* by fixatives. Plasma membrane (●) or soluble phosphatase (○) were preincubated with varying concentrations of glutaraldehyde or formaldehyde in 10 mM Pipes/NaOH (pH 6.7) for the times and at the temperatures shown in the figure. The reaction of enzymes with fixatives was stopped by 100-fold dilution into ATPase reaction mixture and the residual ATPase activity was assayed as described in "Materials and Methods." Controls were preincubated for the same time and temperature in the absence of fixative, and then assayed at the same diluted fixative concentrations, to correct for any additional inhibition occurring during the assay. Residual NH_4SO_4 in the phosphatase preparation was shown to have no protective effect.
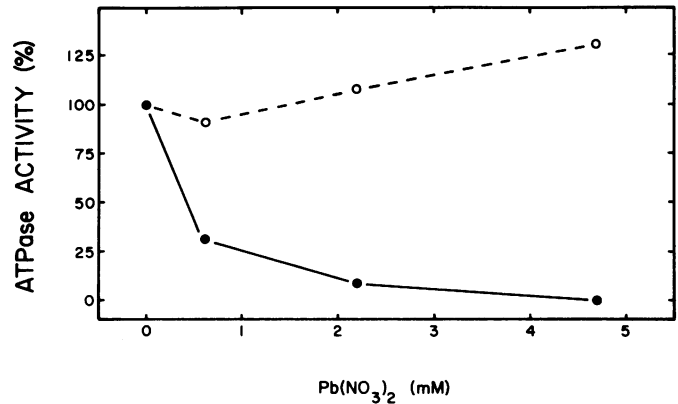
FIG. 4. Inhibition *in vitro* by lead. Plasma membranes (●) or soluble phosphatase (○) were incubated for 10 or 20 min, respectively, at 30°C, with 100 mM KNO_3, 10 mM Pipes/Tris (pH 6.7), and varying concentrations of $Pb(NO_3)_2$, ATP, and $MgCl_2$ to provide the total concentrations shown in Table II. Phosphate released during the incubation was measured as described. Solutions were made fresh to avoid loss of lead from the solutions due to precipitation by phosphate released by nonenzymic hydrolysis of ATP catalyzed by lead (13). Controls were included to enable correction for this nonenzymic hydrolysis. Residual NH_4SO_4 in the phosphatase preparation was shown to have no protective effect. Lead had no effect on the phosphate assay.

(Fig. 3A) would seem to preclude the localization of its activity in glutaraldehyde-fixed tissue. Although the proton pump is more tolerant of fixation by cold formaldehyde (Fig. 3B), good fixation may be difficult using cold formaldehyde alone (RF Evert, personal communication). Pesacreta *et al.* (14) have reported adequate structural preservation after fixation of beet leaves in 3% formaldehyde at room temperature. However, 30 min in 1.5% formaldehyde at room temperature was enough to leave no detectable H⁺-ATPase activity in our *in vitro* studies (Fig. 3C). Thus the 'signal' generated cytochemically by the proton pump will necessarily be diminished, both by lead and by the fixation conditions.

Additional problems arise due to 'noise' generated by other plant ATPases. Plants contain a large number of ATP-hydrolyzing enzymes, some of which have no known specific inhibitors. In oat roots, there is three times as much soluble ATP-hydrolyzing activity as there is plasma membrane H⁺-ATPase. This activity creates a background which must be reduced in order to see the signal arising from the pump.

Molybdate is a useful reagent with which to reduce this background. The cytochemical ATPase activity in oat roots is com-

Table II. *Concentrations of Relevant Species in Solutions Containing Pb^{2+}, Mg^{2+}, and ATP*

All concentrations are calculated using the methods in Storer and Cornish-Bowden (19) with the K_f for Pb^{2+}-containing species from Tice (21). The first four columns are the four lead concentrations used in Figure 3. The last column gives the concentrations for the cytochemical incubation mixtures used in this paper.

Species	Concentration				
	In vitro				Cytochemical
	mM				
Total $Pb(NO_3)_2$	0.0	0.62	2.2	4.7	2.0
Total Mg^{2+}	5.7	6.3	7.0	6.9	2.0
Total ATP	6.0	6.0	7.0	9.0	2.0
Total KNO_3	100	100	100	100	50
[Pb^{2+}]	0.0	0.10	0.50	1.0	0.92
[PbATP]	0.0	0.51	1.7	3.6	1.0
[MgATP]	5.0	5.0	5.0	5.0	0.90

Table III. *Determination of the Lead Species Causing Inhibition of Plasma Membrane H^+-ATPase*

Plasma membranes were incubated at 30°C in reaction mixtures containing the indicated total amounts of Pb^2, Mg^{2+}, and ATP, contributed by $Pb(NO_3)_2$, $Mg(NO_3)_2$, and KATP, and also containing 100 mM KNO_3 and 10 mM Pipes/Tris (pH 6.7). After 30 min the phosphate released was assayed. The percent activity values shown are the mean of three replicates, ± the standard error. The concentrations of the solution species were calculated as described in Table II.

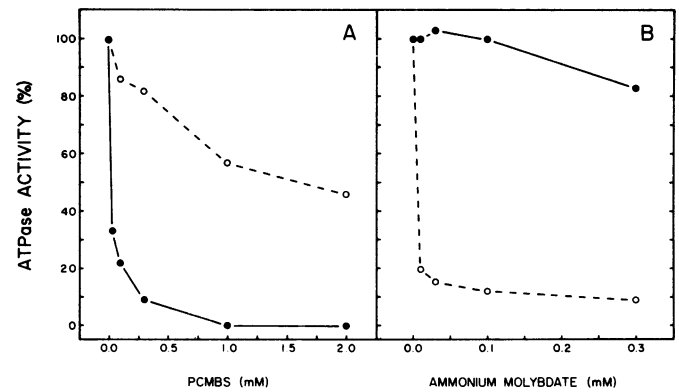| Species	Concentration			
	mM			
Total $Pb(NO_3)_2$	0	2	1.5	0.6
Total $Mg(NO_3)_2$	10	7	5	25
Total ATP	5	7	10	5
[Pb^{2+}]	0	0.42	0.038	0.43
[PbATP]	0	1.54	1.46	0.14
[MgATP]	4.84	5.10	4.83	4.83
ATPase Activity				
	%			
	100 ± 0.7	34 ± 0.3	50 ± 0.7	33 ± 0.7

FIG. 5. *In vitro* inhibition by specific inhibitors. ATPase activity of plasma membrane (●) and soluble phosphatase (○) was measured with varying concentrations of PCMBS (A) or NH_4MoO_4 (B) added to the reaction mixture. Molybdate had no effect on the phosphate assay up to 0.3 mM, but could not be tested at higher concentrations due to inconsistent effects on the assay blanks.

pletely eliminated by 0.1 mM molybdate (Fig. 1D). In contrast, there is no significant inhibition by molybdate of the biochemically assayed plasma membrane H^+-ATPase at concentrations below 0.3 mM, while the soluble activity is inhibited by 90% at concentrations below 0.1 mM (Fig. 5B). However, molybdate alone is not a sufficient distinguishing criterion since 10% of the soluble ATPase activity was nearly insensitive to this reagent.

Earlier investigators have similarly compared the characteristics of *in vitro* activities with those localized *in situ* (*e.g.* Ref. 8); however, much of that work was done before the introduction of adequate procedures for isolating H^+-ATPase uncontaminated by other phosphatase activities. Our review of the literature suggests that the lack of established criteria for evaluating whether cytochemical lead deposits are due to the proton pump have led other investigators to conclude that they have localized the pump when their data does not support that conclusion. The *in vitro* work described herein, as well as the characterization of purified and partially purified plasma membrane H^+-ATPase in fungi and higher plants by other investigators (3, 24) suggest such a set of criteria, as follows:

1. Deposition should be specific for ATP as substrate. ADP and β-GP should produce no deposition.

2. Reaction product should have a specific requirement for Mg^{2+} as divalent cation. Since endogenous Mg^{2+} may be present, either deposition should be increased by Mg^{2+} but not by Ca^{2+}, or activity should show no requirement for divalent cations (suggesting sufficient Mg^{2+} is present endogenously). Preincubation of fixed sections with EDTA prior to incubation with ATP and lead may allow a Mg^{2+} requirement to be shown.

3. The cytochemical activity should be sensitive to covalent sulfhydryl reagents such as PCMBS and *N*-ethylmaleimide. This is not a specific requirement since some apyrases (membrane-associated enzymes which hydrolyze both ADP and ATP, see *e.g.* Ref. 23) are also sensitive (22).

4. Reaction product due to the proton pump should be insensitive to 0.1 mM molybdate. Again this is not a specific requirement since some apyrases are also insensitive (24).

5. Since phosphate is released into the cytoplasm by the proton pump during hydrolysis of ATP (16), reaction product should be observed primarily or entirely on the cytoplasmic side of the plasma membrane.

Our results further suggest three conditions which should favor the cytochemical observation of plasma membrane H^+-ATPase

activity: (a) formaldehyde, rather than glutaraldehyde, should be used at 0°C for fixation; (b) the free lead concentration should be kept low, or other phosphate-precipitating agents employed; and (c) molybdate (0.1 mM) should be used to reduce background deposition from soluble phosphatase activity. With these conditions, and the criteria suggested above, it may be possible to design a protocol for unambiguously localizing the plasma membrane proton pump in plant tissue.

LITERATURE CITED

1. ANTHON GE, RM SPANSWICK 1986 Purification and properties of the H^+-translocating ATPase from the plasma membrane of tomato roots. Plant Physiol 81: 1080–1085
2. BENNETT AB, SD O'NEILL, M EILMANN, RM SPANSWICK 1985 H^+-ATPase activity from storage tissue of *Beta vulgaris*. Plant Physiol 78: 495–499
3. BOWMAN BJ, F BLASCO, CW SLAYMAN 1981 Purification and characterization of the plasma-membrane ATPase of *Neurospora crassa*. J Biol Chem 256: 12343–12349
4. BRISKIN DB, WR THORNLEY 1985 Plasma membrane ATPase of sugarbeet. Phytochemistry 24: 2792–2802
5. DRYER RL, AR TAMMES, RI ROUGH 1957 The determination of phosphorus and phosphatase with *N*-phenyl-*p*-phenylenediamine. J Biol Chem 225: 177–183
6. FIRTH JA 1978 Cytochemical approaches to the localization of specific adenosine triphosphatases. Histochem J 10: 253–269
7. FRANCESCHI VR, WJ LUCAS 1982 The relationship of the charasome to chloride uptake in *Chara corallina*: physiological and histochemical investigations. Planta 154: 525–537
8. GILDER J, J CRONSHAW 1974 A biochemical and cytochemical study of adenosine triphosphatase activity in the phloem of *Nicotiana tabacum*. J Cell Biol 60: 221–235
9. HAGER K, S MANDALA, J DAVENPORT, D SPEICHER, E BENZ JR, CW SLAYMAN 1986 Amino acid sequence of the plasma membrane ATPase of *Neurospora crassa*: deduction from genomic and cDNA sequences. Proc Natl Acad Sci USA 83: 7693–7697
10. HALL JL, AJ KINNEY, A DYMOTT, JR THORPE, DA BRUMMELL 1982 Localization and properties of ATPase activity in pea stems and wheat coleoptiles. Histochem J 14: 323–331
11. LARSSON C 1983 Partition in aqueous polymer two-phase systems. *In* Isolation of Membranes and Organelles from Plant Cells. JL Hall, AL Moore, eds, Academic Press, London, pp 278–309
12. KATZ DB, MR SUSSMAN 1987 Inhibition and labelling of the plant plasma membrane H^+-ATPase with *N*-ethylmaleimide. Plant Physiol 83: 977–981
13. MOSES HL, AS ROSENTHAL 1968 Pitfalls in the use of lead ion for histochemical localization of nucleoside phosphatases. J Histochem Cytochem 16: 530–539
14. PESACRETA TC, AB BENNETT, WJ LUCAS 1986 Spectrophotometric and cytochemical analysis of phosphatase activity in *Beta vulgaris* L. J Histochem Cytochem 34: 327–338

15. SERRANO R 1985 Plasma Membrane ATPase of Plants and Fungi. CRC Press, Boca Raton, FL

16. SERRANO R, MC KIELLAND-BRANDT, GR FINK 1986 Yeast plasma membrane ATPase is essential for growth and has homology with $(Na^+ + K^+)$, K^+- and Ca^{2+}-ATPases. Nature 319: 689-693

17. SOSSOUNTZOV L, Y HABRICOT 1985 Ultracytochemical localization and characterization of membrane-bound ATPases in lateral buds from intact and decapitated plants of an aquatic fern, *Marsilea drummodii* A. Br Protoplasma 127: 180-191

18. SPURR AR 1969 A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26: 31-43

19. STORER AR, A CORNISH-BOWDEN 1976 Concentration of $MgATP^{2-}$ and other ions in solution. Biochem J 159: 1-5

20. SUROWY TK, MR SUSSMAN 1986 Immunological cross-reactivity and inhibitor sensitivities of the plasma membrane H^+-ATPase from plants and fungi. Biochem Biophys Acta 848: 24-34

21. TICE LW 1969 Lead-adenosine triphosphate complexes in adenosine triphosphatase histochemistry. J Histochem Cytochem 17: 85-94

22. TOGNOLI L, E MARRÈ 1981 Purification and characterization of a divalent-cation activated ATP-ADPase from pea stem microsomes. Biochem Biophys Acta 642: 1-4

23. VARA F, R SERRANO 1981 Purification and characterization of a membrane bound ATP diphosphohydrolase from *Cicer arietinum* (chick-pea) roots. Biochem J 197: 637-643

24. VARA F, R SERRANO 1982 Partial purification and properties of the proton-translocating ATPase of plant plasma membranes. J Biol Chem 257: 12826-12830

25. WACHSTEIN M, E MEISEL 1957 Histochemistry of hepatic phosphatase at a physiologic pH. With special reference to the demonstration of bile canaliculi. Am J Clin Pathol 17: 13-23