Plant Physiol. (1977) 60, 923-927

Cytochemical Localization of K^+-stimulated Adenosine Triphosphatase Activity in Xylem Parenchyma Cells of Barley Roots[1]

Received for publication December 13, 1976 and in revised form August 9, 1977

ERIKA WINTER-SLUITER,[2] ANDRÉ LÄUCHLI,[2] AND DETLEF KRAMER
Institut für Botanik, Technische Hoschschule Darmstadt, D-6100 Darmstadt, Federal Republic of Germany

ABSTRACT

ATPase activity in xylem parenchyma cells of barley (*Hordeum vulgare* L.) roots was demonstrated cytochemically with a lead precipitation reaction. The methodical parameters of this cytochemical test were optimized for distinction between ATPase-specific and nonspecific precipitates. Optimum conditions were prefixation in 1% glutaraldehyde for 1 hour and incubation for 2 hours in a medium containing 2 mM each of ATP, Ca^{2+}, and Pb^{2+} at pH 7 and 25 C. Problems of cytochemical localizations are discussed.

ATPase activity occurred mainly at the plasmalemma, the endoplasmic reticulum nuclear envelope, and outer mitochondrial membranes of xylem parenchyma cells. The tonoplast of these cells showed only little ATPase activity. High K^+ concentrations stimulated ATPase activity, particularly at the plasmalemma. Diethylstilbestrol prevented the formation of ATPase-specific precipitates. The cytochemical demonstration of a K^+-stimulated ATPase at the plasmalemma of xylem parenchyma cells is discussed in relation to the possible role of this membrane in ion transport to the vessels.

In recent years increasing attention has been paid to the role of ATPases in ion transport through plant cell membranes (10). Positive correlation was observed between ATPase activity and the rate of ion absorption. The ATPase was associated with plasma membranes and stimulated by K^+ and Rb^+. Furthermore, there were similarities between the kinetics of K^+ activation of the enzyme and K^+ absorption by roots. Therefore, it was suggested that ATPases mediate energy transduction to the ion transport systems of plant roots (for general review see ref. 11). These enzymes were activated by Mg^{2+} (3, 16) and/or Ca^{2+} (8, 17), and inhibited by phenolics (2).

There are some reports on the cytochemical demonstration of ATPase activity at the plasmalemma and other membrane systems of tips and young cortical cells of roots, using the lead precipitation technique (5; see 8). Only a few attempts have been made to demonstrate ATPase activity in tissues more complex than root tips and cortical cells (19, 24). Particularly, cytochemical information is needed on ATPase activity in xylem parenchyma cells of roots, because these cells are considered to regulate ion transport into the xylem vessels (12-14, 18, 20). A preliminary report was presented elsewhere (14).

MATERIALS AND METHODS

Plant Material. Seeds of barley (*Hordeum vulgare* L., cv. "Kocherperle") were germinated on 0.2 mM $Ca(NO_3)_2$ for 2 days in the dark and seedlings grown for 3 days in the light (16-hr photoperiod, 10 klux, 20–25 C day and 15 to 18 C night temperature) on a modified Hoagland solution (*cf.* 7), pH 5.3, containing 0.6 mM KNO_3, 0.4 mM $Ca(NO_3)_2$, 0.2 mM NH_4NO_3, 0.1 mM $Mg(NO_3)_2$, 10 μM FeEDTA, and micronutrients at 1/10 concentration of that given by Epstein (7, Table 3-1). This solution contained no Pi and no SO_4^{2-}, which is advantageous in cytochemical demonstration of ATPase because the possibility of unspecific lead precipitates (as phosphate and sulfate) is decreased.

Cytochemistry of ATPase. Root segments, about 1 mm long from the region 1.5 to 2 cm behind the tip, were prefixed in 1% glutaraldehyde (Anderson-grade purified, see ref. 1) in 50 mM cacodylate buffer (pH 7) at 0 C for 1 hr, and washed for 4 hr in 48 mM tris-maleate buffer (pH 7) at 0 C. The segments were then incubated for localization of ATPase activity using a modified lead phosphate precipitation method originally devised by Wachstein and Meisel (25). The incubations were made for 2 hr at 25 C in a medium containing 2 mM $Ca(NO_3)_2$, 2 mM ATP, 2 mM $Pb(NO_3)_2$ in 48 mM tris-maleate buffer (pH 7) (containing 12 mM K^+). The addition of ATP immediately after $Ca(NO_3)_2$ favors the formation of a Ca-ATP complex in the medium; thus, the danger that soluble calcium phosphate may form in the tissue is minimized. Calcium was included because it activates ATPase in barley roots more than Mg^{2+} (17; *cf.* 20).

Separate samples were incubated under the following conditions: without ATP, without $Pb(NO_3)_2$, at pH 5.0, with 2 mM $Mg(NO_3)_2$ instead of $Ca(NO_3)_2$, at 0 C, with additional 50 mM KNO_3 and with addition of 0.2 mM diethylstilbestrol used as an ATPase inhibitor by Balke and Hodges (2), respectively. Thus, eight assays were made using the standard incubation medium and seven modifications. All samples were washed in 48 mM tris-maleate buffer (pH 7) at 20 C for 1 hr. The segments were postfixed in 1% OsO_4 in 25 mM cacodylate buffer (pH 7.2) at 20 C for 2 hr, dehydrated in a graded ethanol series, followed by propylene oxide, and embedded in Spurr's medium (22). Ultrathin sections were examined without poststaining in a Zeiss EM 9 electron microscope.

RESULTS

Optimum Fixation. In order to combine sufficient preservation of cell structure with maximum enzyme activity, samples were exposed to a range of fixation times and concentrations of glutaraldehyde (0.5 hr, 1 hr, 2 hr; 0.1%, 1%, 2%). Best results were obtained with 1% Anderson-grade glutaraldehyde for 1 hr.

[1] Supported by a research grant from the Deutsche Forschungsgemeinschaft to A. L.

[2] Present address: Botanisches Institut, Tierärztliche Hochschule, D-3000 Hannover 71, Federal Republic of Germany.

Specificity of Precipitates. To test the specificity of the ATPase reaction, control incubations were carried out with the following results. (*a*) Incubation in a medium without ATP and incubation at 0 C with ATP (see Fig. 4) showed only nonspecific precipitates. These can be distinguished electron microscopically from the specific ATPase reaction product by their shape and occurrence. There was no difference in ultrastructure and precipitates between these two assays. (*b*) The lack of specific precipitates after incubation at pH 5 excluded the possibility that the precipitates obtained at pH 7 were due to acid phosphatase activity (*c*) Precipitates were completely absent after incubations without lead, suggesting that the electron density of both specific and nonspecific precipitates is caused by lead. (*d*) Investigations of the chemical components of specific and nonspecific precipitates, and attempts to distinguish them chemically, were made by means of x-ray microanalysis using a Hitachi transmission electron microscope in combination with a Kevex energy dispersive x-ray detector.[3] Precipitates were visible electron microscopically, but they were so small that the amounts of elements present were not sufficient for an accurate analysis.

Site of Specific Precipitates. Specific ATPase precipitates in xylem parenchyma cells of barley roots are mainly found at the plasmalemma (Fig. 1), particularly in the region of the half-bordered pits and along the cisternae of the ER (Figs. 2 and 3), but are also associated with other membrane systems, *i.e.* outer mitochondrial membrane and nuclear envelope (Fig. 1). The tonoplast showed only little ATPase activity (Figs. 2 and 5).

Effect of Ca^{2+} and K^+ on ATPase Activity. Ca^{2+} and Mg^{2+} were tested for potential activating function. A slight difference in intensity indicated that in the case of barley roots Ca^{2+} activates ATPase preferentially (*cf.* 17). After incubation with additional 50 mM KNO_3 precipitates associated with the plasmalemma of xylem parenchyma cells were estimated from the size of the spots to have increased in intensity more than 3-fold (Fig. 5). At the ER, mitochondrial membranes, and nuclear envelope, no significant and reproducible increase in staining was observed at the high K^+ level.

Effect of Diethylstilbestrol. Incubation in the presence of 0.2 mM diethylstilbestrol prevented completely the formation of specific precipitates (Fig. 6).

DISCUSSION

Reliable localization of ATPases by cytochemical techniques is usually obscured by a number of methodical problems.

A. Enzyme Inactivation by Fixation. There is a need to test each material for its optimum fixation time and concentration (5, 8; *cf.* 21). Furthermore, different fixatives cause different degrees of ATPase inhibition associated with various cellular membrane systems (5). For our purposes glutaraldehyde was chosen as fixative because it is known to inhibit the activity of the inner mitochondrial membrane (5) (*cf.* Figs. 1–3; 5), but to preserve enzyme activity at other membrane systems to a sufficient extent (5, 19, 21).

B. Insufficient Penetration of Incubation Medium into Plant Tissue. Even in small root segments of only 1 mm in length, as used in this study, there is a zone of poor penetration in the middle of the segment. Reasonable enzyme reactions were observed in ultrathin sections from a region between 40 and 80 μm under the cross-sectional surface of the embedded segment.

C. Enzyme Inactivation by Pb^{2+}. High concentrations of Pb^{2+} inhibit enzyme activity in cytochemical phosphatase localizations (21). In our experiments incubations with a Pb^{2+} to ATP ratio of 1:1 showed a much greater extent of specific precipitates than incubations with a ratio of 2:1. A ratio of 2:1 or even higher has been used in previous cytochemical ATPase localizations in plant tissue (5, 8, 9, 19, 24).

D. Production of Nonspecific Precipitates. Nonenzymically produced lead phosphate precipitates in cytochemical reactions can be caused by a spontaneous hydrolysis of ATP (21). This reaction is catalyzed by Pb^{2+} (19, 21), and the Pb^{2+} to ATP ratio again plays an important role. The proportion of Pb^{2+} to ATP of 4.5:1 in the original Wachstein-Meisel medium (25) is considered to produce nonspecific precipitates due to the excess of Pb^{2+} (21); the presence of the tissue enhances the effect of lead (21). This problem also occurs in all modifications of the Wachstein-Meisel medium used previously for plant material (5, 9, 19, 24). In our experiments, relatively few nonspecific precipitates were found at a lead to ATP ratio of 1:1. Nonetheless, they cannot be completely avoided (see ref. 21). Thus, a distinction between specific and nonspecific precipitates becomes necessary.

E. Distinction between Specific and Nonspecific Precipitates. In our experiments nonspecific precipitates can be characterized by their crystalline shape (Fig. 4) and their size (19, 21, 24). In addition, they were not associated particularly with cellular membranes (*cf.* 21, 24), and were neither stimulated by additional K^+ nor inhibited by diethylstilbestrol. Furthermore, they occurred also in incubations without ATP or at 0 C.

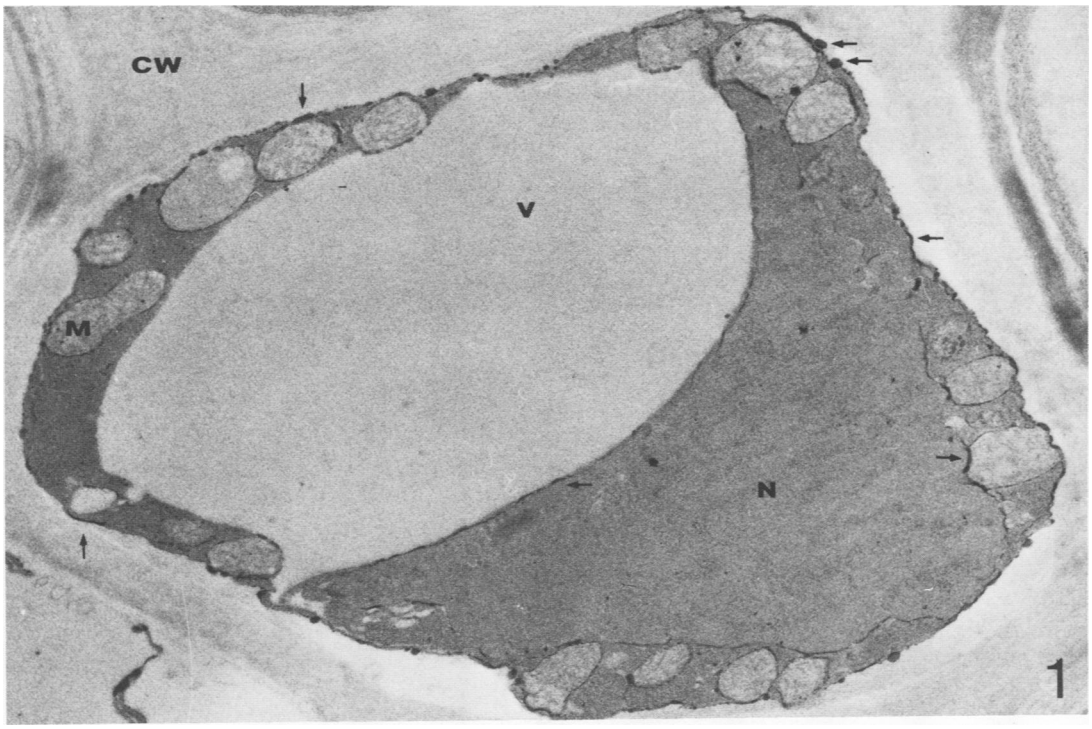
F. Migration of Pi and Dislocation Artifacts. The concentration of Pb^{2+} can be kept low in order to prevent the two sources of artifact due to Pb^{2+} described under C and D, but then the amount of Pb^{2+} may not be sufficient to trap all of the Pi produced. The excess Pi may migrate from the enzyme sites and result in dislocation artifacts (21, 24). In the present study we do not consider nonspecific deposits (D) to be caused by enzymically produced Pi that has moved away from the location of the ATPase.

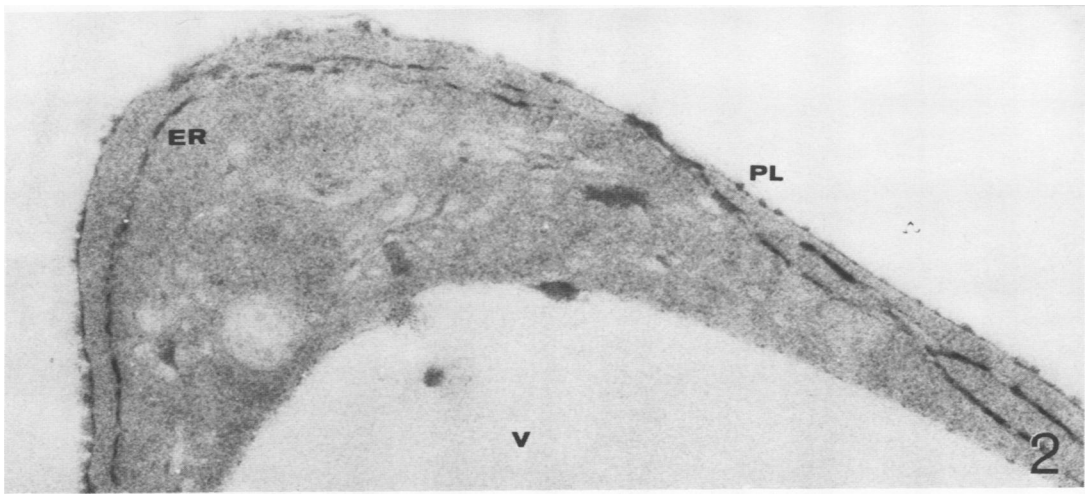
It has been recently considered that the regulating step of ion release from the stelar tissue into the lumen of the xylem vessels is located at the plasmalemma of the xylem parenchyma cells (7, 12, 14, 20). Recent reports on structure and transport-function of xylem parenchyma cells (13, 14) support this hypothesis. However, there are also other views on the location and mechanism of ion transport to the vessels (see ref. 18 for review).

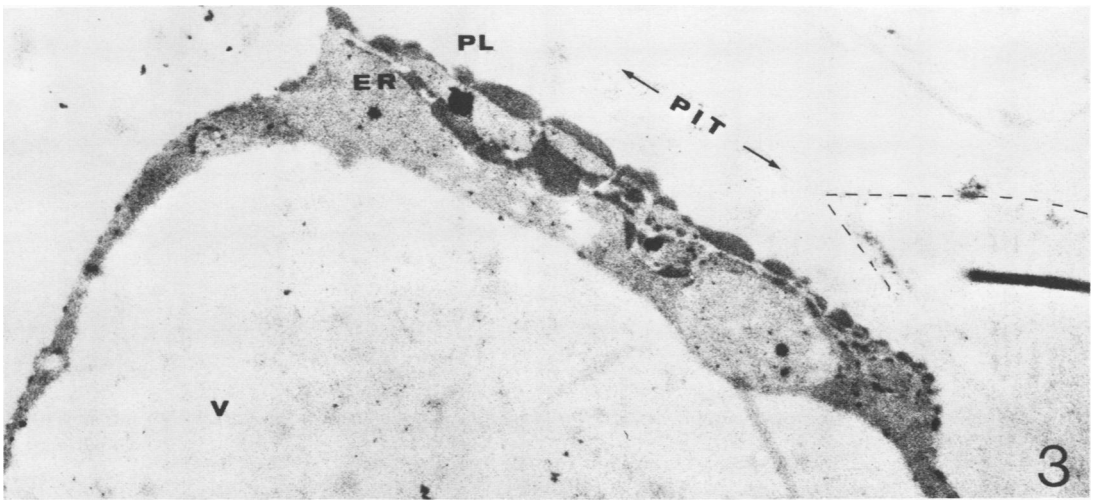
Our cytochemical demonstration of ATPases in xylem parenchyma cells provides a further indication for their participation in ion transport through the root, particularly because no other stelar cells contained high ATPase activity, apart from phloem parenchyma cells and sieve tubes. A function of the xylem parenchyma cells in ion secretion appears supported by the extremely high intensity of the ATPase precipitates at the plasmalemma in the region of the half-bordered pits (Fig. 3). Intracellular ATPase-dependent transport processes are also indicated by our results, *i.e.* xylem parenchyma cells show ATPase activity at the ER (Fig. 2). The ATPase activity at the membranes of the ER is consistent with the hypothesis that the ER forms an integral part of the symplasmic pathway in roots (12, 23).

The involvement of the ER in symplasmic transport was deduced from experiments on Cl^- transport, hence a study on the effect of Cl^- on ATPase activity at the ER would be of interest. Hodges (11) suggested the presence of an anion-sensitive ATPase at the tonoplast of oat roots. Our cytochemical study indicated that, under the experimental conditions used, the tonoplast showed much less ATPase activity than the ER membranes. High K^+ concentrations considerably enhance the extent of precipitates at the plasmalemma of xylem parenchyma cells (Fig. 5), indicating a K^+ stimulation of plasmalemma-bound ATPases. This cytochemically obtained result corroborates the existence of a K^+-stimulated ATPase in oat roots.

[3] Department of Inorganic Chemistry, University of Berne, Switzerland.





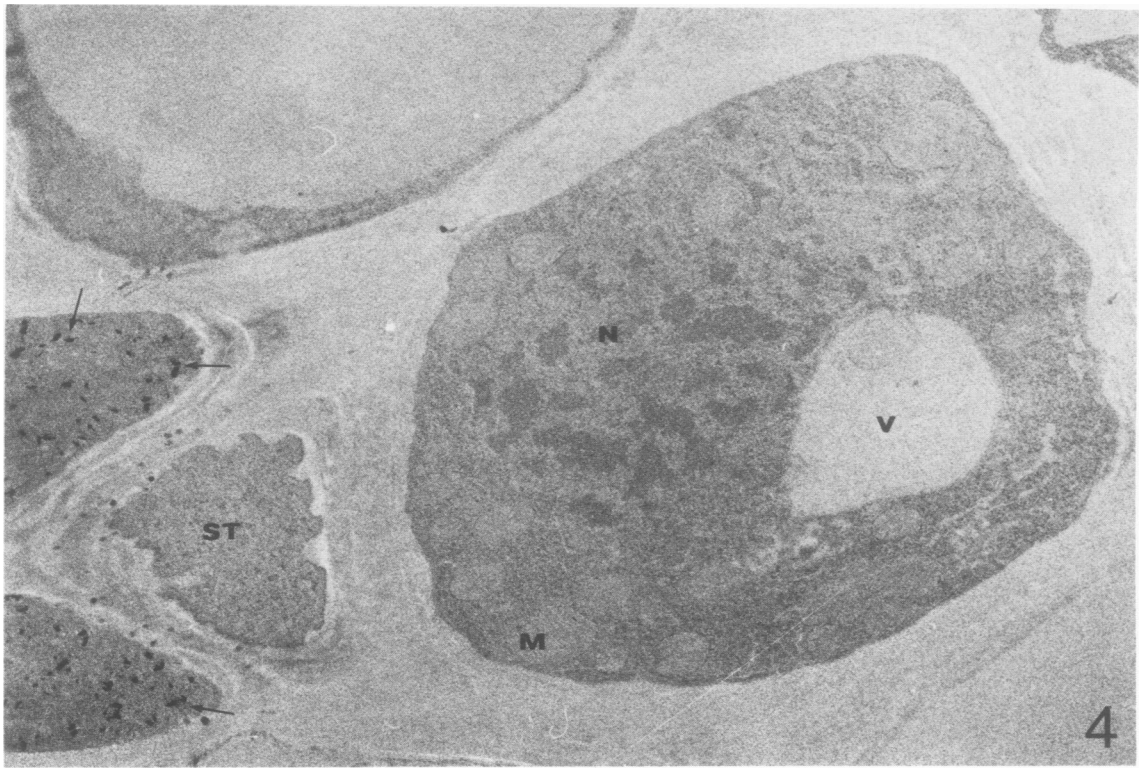


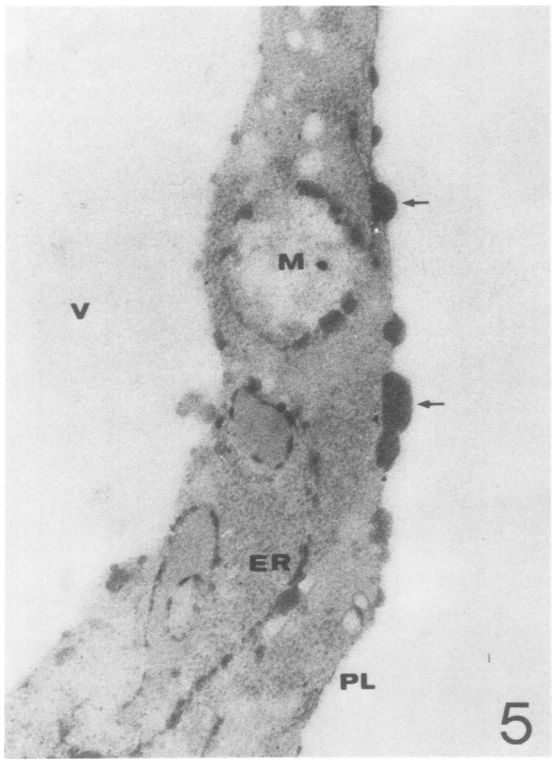
FIGS. 1-3. CW: cell wall; ER: endoplasmic reticulum, M: mitochondrion; N: nucleus; PL: plasmalemma; V: vacuole; XPC: xylem parenchyma cell.

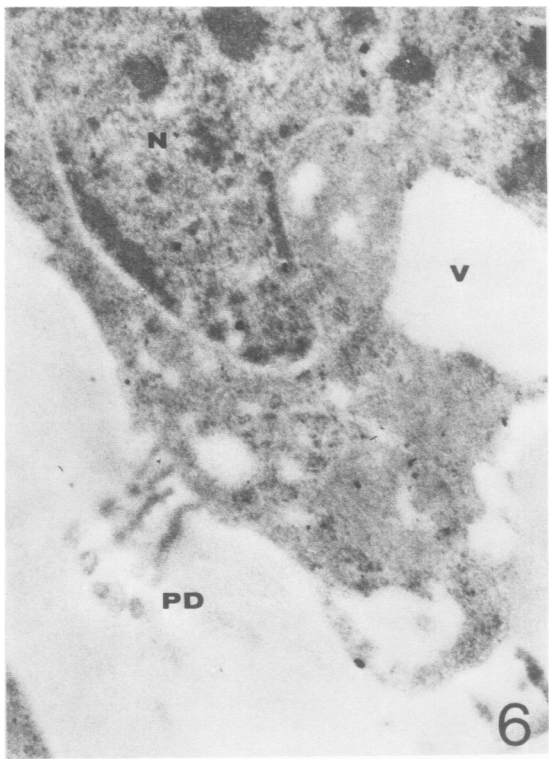
FIG. 1. XPC of barley root, about, 1.5 to 2 cm from the apex. ATPase localization with a lead precipitation technique. Arrows indicate specific precipitates. (× 23,000).

FIG. 2. XPC with specific ATPase precipitates at plasmalemma and ER. (× 45,500).

FIG. 3. XPC facing a half-bordered pit of an adjoining vessel. ATPase localization predominatly at the plasmalemma. (× 66,600).







FIGS. 4–6. PD: plasmodesmata; ST: sieve tube; other abbreviations as in Figures 1–3.

FIG. 4. XPC and part of the phloem. Incubation for ATPase activity at 0 C. Arrows indicate nonspecific precipitates. (× 22,000).

FIG. 5. XPC. Incubation for ATPase activity with additional 50 mM K^+. Arrows indicate enhanced intensity of specific precipitates at the plasmalemma. (× 39,000).

FIG. 6. XPC. Incubation for ATPase activity in the presence of 0.2 mM diethylstilbestrol. (× 37,000).

which was determined biochemically and bound to plasma membranes (11). This enzyme is thought to play an important role in cation transport across the plasma membrane. Leigh *et al.* (15) presented evidence that in addition to the plasmalemma, there may be a further K^+-stimulated ATPase associated with an undetermined membrane fraction.

Our results on the inhibition of membrane-bound ATPases by diethylstilbestrol are consistent with those described by Balke

and Hodges (2) using plasma membranes from oat roots. This effect must be treated with caution, because studies on animal mitochondria (4) and *Neurospora* (B. Bowman, personal communication) indicate that diethylstilbestrol may affect the permeability of plasma and mitochondrial membranes. The cytochemical demonstration of K^+-stimulated ATPase at the plasmalemma of xylem parenchyma cells provides additional evidence for an important role of this membrane in ion transport to the vessels. Davis and Higinbotham (6) recently presented electrochemical data on transport of K^+ and Cl^- in excised corn roots and showed that K^+ is probably transported actively into the vessels. The data presented here suggest that active K^+ transport into the vessels is located at the plasmalemma of the xylem parenchyma cells.

Acknowledgments—The authors are grateful to R. Guggenheim and L. Henning from the Department of Geology, University of Basel, for cooperation in x-ray microanalysis. They are also thankful to B. Bowman and C. Slayman, Yale University, for suggestions on the specificity of diethylstilbestrol and to H. Greenway for critical discussion of the manuscript.

LITERATURE CITED

1. Anderson PJ 1967 Purification and quantitation of glutaraldehyde and its effects on several enzyme activities in skeletal muscle. J Histochem 15: 652–661
2. Balke NE, TK Hodges 1975 Inhibition of the plasma membrane ATPase by phenolics. Plant Physiol 56: S-41
3. Balke NE, TK Hodges 1975 Plasma membrane ATPase of oat roots. Activation and inhibition by Mg^{2+} and ATP. Plant Physiol 55: 83–86
4. Byington KH, JM Smoly, AV Morey, DE Green 1968 On the fragmentation of mitochondria by diethylstilbestrol. Arch Biochem Biophys 128: 762–773
5. Coulomb P, C Coulomb 1972 Localisation cytochimique ultrastructurale d'une ATPase Mg^{2+} dépendante dans des cellules de méristèmes radiculaires de la courge (*Cucurbita pepo* L.). CR Acad Sci Paris 275: 1035–1038
6. Davis RF, N Higinbotham 1976 Electrochemical gradients and K^+ and Cl^- fluxes in excised corn roots. Plant Physiol 57: 129–136
7. Epstein E 1972 Mineral Nutrition of Plants: Principles and Perspectives. John Wiley & Sons, New York
8. Hall JL 1973 Enzyme localization and ion transport. *In* WP Anderson, ed, Ion Transport in Plants. Academic Press, London, pp 11–24
9. Hall JL, TJ Flowers 1976 Properties of membranes from the halophyte *Suaeda maritima*. I. Cytochemical staining of membranes in relation to the validity of membrane markers. J Exp Bot 27: 658–671
10. Hodges TK 1973 Ion absorption by plant roots. Adv Agron 25: 163–207
11. Hodges TK 1976 ATPases associated with membranes of plant cells. *In* U Lüttge, MG Pitman, eds, Encyclopedia of Plant Physiology, New Series Vol 2 Part A. Springer, Heidelberg, pp 260–283
12. Läuchli A 1976 Symplasmic transport and ion release to the xylem. *In* IF Wardlaw, JB Passioura, eds, Transport and Transfer Processes in Plants. Academic Press, New York, pp 101–112
13. Läuchli A, D Kramer, MG Pitman, U Lüttge 1974 Ultrastructure of xylem parenchyma cells of barley roots in relation to ion transport to the xylem. Planta 119: 85–99
14. Läuchli A, D Kramer, E Sluiter, J Gullasch 1977 Function of xylem parenchyma cells in ion transport through barely roots: localization of ions and of ATPases. *In* M Thellier, J Dainty, eds, Transmembrane Ionic Exchanges in Plants. Centre National de la Recherche Scientifique, Paris, pp 469–476
15. Leigh RA, FA Williamson, RG Wyn Jones 1975 Presence of two different membrane-bound, KCl-stimulated ATPase activities in maize roots. Plant Physiol 55: 678–685
16. Leonard RT, CW Hotchkiss 1976 Cation-stimulated ATPase activity and cation transport in corn roots. Plant Physiol 58: 331–335
17. Nagahashi G, WW Thomson, RT Leonard 1975 Isolation of plasma membranes from roots of barley. Plant Physiol 56: S-41
18. Pitman MG 1977 Ion transport into the xylem. Annu Rev Plant Physol 28: 71–88
19. Robards AW, P Kidwai 1969 Cytochemical localization of phosphatase in differentiating secondary vascular cells. Planta 87: 227–238
20. Schaefer N, RA Wildes, MG Pitman 1975 Inhibition by *p*-fluorophenylalanine of protein synthesis and of ion transport across the roots in barley seedlings. Aust J Plant Physiol 2: 61–73
21. Sluiter E 1975 Cytochemische Lokalisation von ATPasen in Gerstenwurzeln und ihre mögliche Bedeutung beim Ionentransport. Diplomarbeit, Technische Hochschule Darmstadt
22. Spurr AR 1969 A low viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26: 31–43
23. Stelzer R, A Läuchli, D Kramer 1975 Pathways of intercellular chloride transport in roots of intact barley seedlings. Cytobiologie 10: 449–457
24. van Steveninck RFM 1976 Cytochemical evidence for ion transport through plasmodesmata. *In* BES Gunning, AW Robards, eds, Intercellular Communication in Plants: Studies on Plasmodesmata. Springer, Heidelberg, pp 131–147
25. Wachstein M, E Meisel 1957 Histochemistry of hepatic phosphatases at a physiologic pH. With reference to the demonstration of bile calculi. Am J Clin Pathol 27: 13–23