

Cytochemical Demonstration of a Sodium-Activated and a Potassium-Activated Adenosine Triphosphatase in Loblolly Pine Seedling Root Tips

Iola T. McClurkin and D. C. McClurkin

Department of Biology, University of Mississippi, University, Mississippi 38677

and

Southern Forest Experiment Station, Forest Service,
United States Department of Agriculture,
Oxford, Mississippi 38655

Received January 30, 1967.

Summary. Sodium stimulated ATPase activity in the nuclei of the meristematic cells, while potassium stimulated it in the mycorrhizae between root cap cells. The detection of these 2 mutually exclusive cation-stimulated ATPases, which both require magnesium-ATP in equivalents, which have a similar optimum pH of about 5.5, and which are located in entirely different parts of the root tip, suggests that particular enzyme systems can only be activated by a specific cation and that one cation cannot substitute for the other. Such a feature may explain the capacity of plants to differentiate between ions as closely similar as sodium and potassium. The similarities between the enzyme system described for salt transport in animal tissues and that depicted here cytochemically in pine roots at the most active site of salt uptake in roots indicate this may be a carrier mechanism for salt entry into plant roots. The presence of the potassium-activated enzyme only in the mycorrhizae may relate to the dependence of pine trees on mycorrhizae for growth.

Few plant processes have provoked more discussion than salt absorption by the root system. Hendricks (5) cited 3 striking characteristics of the process: A salt may be more concentrated in root exudates than in ambient solutions; plants differentiate between ions as closely similar as K^+ and Na^+ ; and, after salts enter plants, distinctions are still evident between actions of Na^+ and K^+ , or Ca^{2+} and Mg^{2+} , as examples. Since salt movement occurs against concentration gradients, there must be use of energy for osmotic work—a process known as active transport.

Skou (13, 14) demonstrated, in animal tissue, an active transport mechanism that is dependent upon sodium ions. The mechanism requires an enzyme system that can catalyze the hydrolysis of the high-energy compound, adenosine triphosphate (ATP), and then convert the energy from ATP into a movement of cations. Magnesium must be present and in equivalent concentration with ATP in the substrate before sodium can stimulate an active transport enzyme, adenosine triphosphatase (ATPase).

Hendricks (4, 5) described similarities in all salt transport, whether in animal or plant cells, and pointed out that, although a sodium-sensitive active transport ATPase was found in animal cell membranes, none could be found in the broken membranes, none could be found in the broken membranes, he reasoned that the degree of distinction that roots make between potassium and sodium must involve some carrier mechanism.

This paper presents cytochemical evidence that both a sodium-activated and a potassium-activated

ATPase are present in roots of loblolly pine seedlings (*Pinus taeda* L.), discusses a possible active transport mechanism for movement of salts into pine roots, and suggests a cause for pine dependence on mycorrhizal infection.

Materials and Methods

Thrifty loblolly pine seedlings were selected from a bale fresh from the nursery. Roots on half the seedlings were pruned to a length of 10 cm. Those on the other half were left intact. A third of the pruned and unpruned seedlings were potted in No. 10 cans containing sandy loam from a forest site, and were grown in an environmental shelter for 4 to 5 months; a third were placed in cold storage for 10 weeks; and a third were prepared for immediate sectioning. To determine whether they were dead or alive at the time of sectioning, the seedlings used at once and those stored for 10 weeks were potted and placed in the environmental shelter as soon as the root sections were removed.

On unpruned fresh, stored, or potted seedlings, root tips (approximately 1.25 cm lengths) were snipped from ends of vertical and lateral roots. Half-inch segments of woody root tissue also were snipped about 5 cm back from the tips.

On pruned seedlings, the root stubs instead of tips were taken.

Seedlings that were grown in pots prior to sampling were treated in the same fashion as those unpruned. Root-pruned seedlings had developed new root tips and were sampled identically with the unpruned plantings.

All snipped sections were promptly placed in a fixing solution of cold 4% formalin. Although formaldehyde may destroy some enzyme activity, appreciable activity survives the dilute formaldehyde treatment and there is excellent preservation of cytological detail (10). Overnight fixation (approximately 24 hr) gave the best results. The tissue must be fixed to demonstrate maximum enzyme activity. Both prolonged fixation and too-brief fixation produced artifacts.

Tissue Sectioning. All woody root segments and root tips of seedlings fresh from the planting bale or from storage were easy to section on a freeze microtome and required no special handling. Frozen serial sections as thin as 20 μ were readily obtained. Root cells typically are on the order of 40 μ in thickness.

Root tips of potted seedlings could not be sectioned unsupported on the freeze microtome. The sectioned tissue crumbled as soon as it thawed. Satisfactory sections were made by using O.C.T.¹ (optimal cutting temperature) to support the tissue.

Incubating Media. ATPases were demonstrated cytochemically by a chelate removal method (1) in which frozen tissue sections are incubated in a substrate medium containing ATP, activating ions, a buffer, and lead ions, the lead forming a chelate with ATP. When the activated enzyme breaks down ATP, the phosphate groups are liberated from the chelate as lead phosphate. As lead phosphate is not visible, the tissue is next washed in a sulfide solution. The sulfide replaces the phosphate and lead sulfide becomes visible microscopically as a black precipitate; presence of the precipitate indicates the presence of the ATPase under test.

Chelation of lead by ATP protects the ATPase from deactivation by free lead ions (1).

The chelate-removal procedure used here in preparing and testing root sections is the same as that used for demonstrating a sodium-activated ATPase in animal tissue (7). The incubating medium is patterned after that Skou used for *in vitro* studies on sodium-activated ATPase, where magnesium and ATP are in optimum, equivalent concentrations; where sodium ions are in optimal concentration for enzyme stimulation; and where the pH is 7.2 (13). This magnesium-dependent, sodium-activated ATPase is inhibited by calcium (13) and by ouabain (16). A suggested stepwise procedure, with certain pH modifications to be discussed shortly, is outlined in the next section.

Initially, root sections were tested for the effects of sodium and potassium on the magnesium-ATP equivalent medium at pH 7.2 and when calcium replaced magnesium ions in the substrate (medium I). Later the pH was lowered to 5.5 (medium II) and additional sections were tested for effects of sodium and potassium. The effect of ouabain also was tested on sodium and potassium activation.

Since most physiological studies indicate that salt transport into roots is somewhat enhanced by calcium (4), possible locations of calcium-activated ATPases in root cells were examined by incubating sections in an ATP substrate described by Wachstein and Meisel (medium III) (15). In their medium, the pH is 7.2, calcium ions can replace the usual magnesium ions, and potassium ions can replace sodium ions in the tris buffer (9).

Rains and Epstein (11) have shown that in barley roots sodium will be transported from dilute solutions containing calcium chloride, potassium chloride, and sodium chloride in specific concentrations at pH 5.7. Their work suggested the possibility that the relative concentrations of these salts might activate an ATPase in pine roots. Hence, medium IV, patterned after that of Rains and Epstein, was prepared and the effects of potassium and sodium were tested upon it. Sodium and potassium ions also were tested with medium IV with magnesium ions used in place of the calcium ions. Magnesium and ATP were not in equivalent concentrations in this medium.

Table I enumerates the 4 media and the cation combinations tested.

Fixation and Staining Procedures. 1) Fix root tips overnight in cold (2-4°) 4% formalin. 2) Wash fixed roots in cold distilled water; freeze on microtome head using O.C.T. as an embedding medium; cut sections 20 μ thick. 3) Float root sections on cold distilled water, keeping serial sections in sequence. (This can be done with plastic tissue culture assay trays.) 4) Refrigerate sections until ready to use. Enzyme action is greatly reduced and sometimes lost after 24 to 48 hours. 5) Prepare 0.01 M solution of ATP by dissolving barium salt of ATP in distilled water acidified to pH 3.1 with 0.1 N HCl.

6) Preparation of medium

	final concentration
1.5 ml ATP (barium salt),	
0.01 M (step 5 above)	
pH 3.1	3 mM
1.4 ml distilled water	
0.6 ml tris buffer pH 6.0,	
0.2 M	24 mM
0.5 ml 1% lead nitrate (0.03 M)	3 mM
1.0 ml magnesium sulfate (0.015 M)	3 mM
Final pH 5.5	
Centrifuge to remove precipitate	
(barium sulfate).	

Place half the medium in each of 2 containers. 7) Add either 15 mg of sodium chloride (final concentration 100 mM) or 5 mg KCl (final concentration 30 mM) to 1 of the containers; the other serves as a control. Then incubate serial sections for 20 to 25 minutes at 37° in each container. An additional control may be had by adding ouabain (final concentration 3×10^{-2} M) to the medium containing sodium or potassium before incubating serial sections. 8) Following incubation, rinse sections in distilled water, float in 1% ammonium

¹ Available from Scientific Products Company.

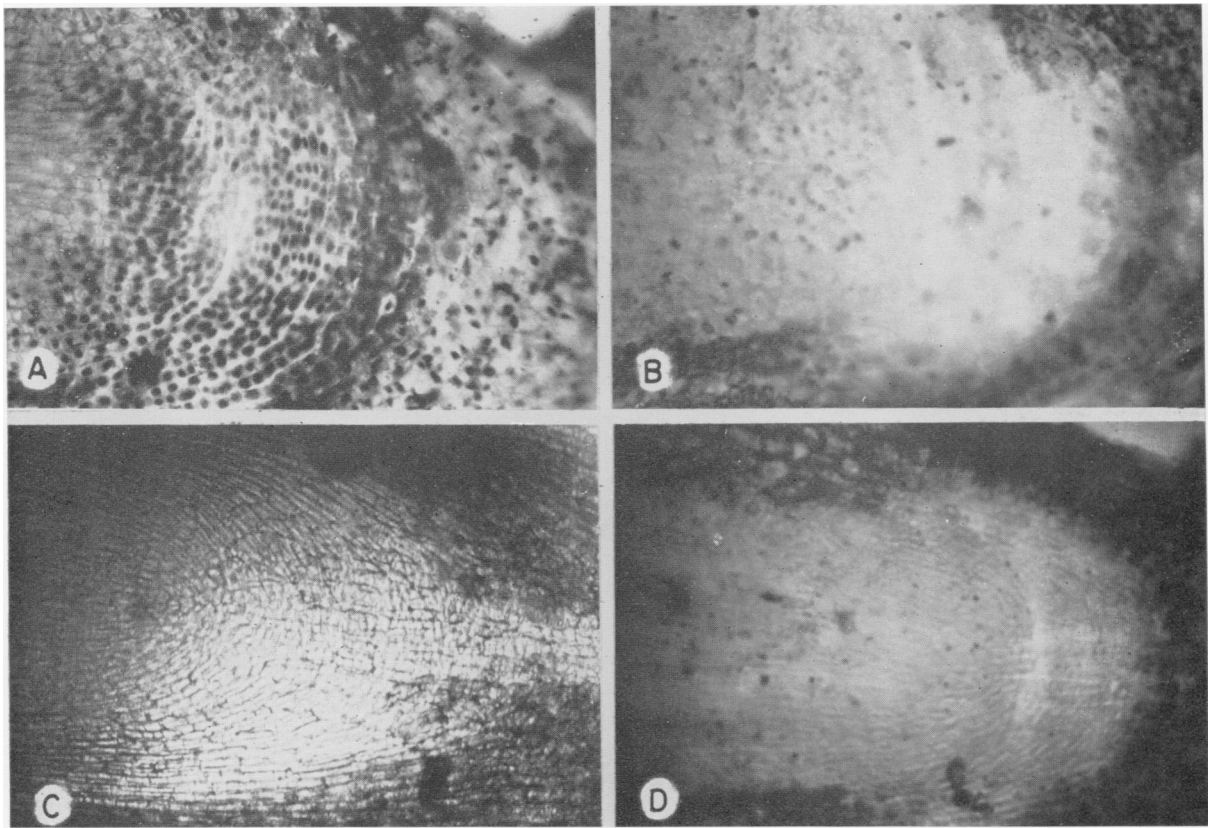


FIG. 1. Meristematic regions of root tips. All views at 85 \times . A) Unpruned, medium IIA (Na-Mg, pH 5.5). The dark spots are sites of sodium-activated ATPase. The stain is visible only in the cell nuclei and not in the membranes. B) Pruned, medium IA (Na-Mg, pH 7.2). The effect of pH on activation of the enzyme is apparent. Only a little stain is visible in the cell nuclei. C) Unpruned, serial section to A, medium IIA with omission of sodium (Mg, pH 5.5). This section served as the control for the sodium- and potassium-activated stains for medium IIA. D) Unpruned, serial section to A and C, medium IIB (Na-Mg-ouabain, pH 5.5). In the presence of ouabain, no sodium-activated stain is seen in the nuclei of the meristematic region.

FIG. 2. A) Root cap, pruned root tip. Medium IIA (K-Mg, pH 5.5). The dark stained areas are sites of potassium-activated ATPase. The tissue which stained is ectotrophic mycorrhizae (My) and the configuration of the stain is known as Hartig's net. No potassium ATPase is evident in the pine root tissue. 85 \times . B) Meristematic region of root tip, pruned. Medium IA (K-Mg, pH 7.2). No potassium-activated stain is evident in either the root tissue or the mycorrhizae. The black flecks are artifacts. Note the mitochondrial stain in meristematic cells (M). 85 \times . C) Root cap, unpruned root tip. Medium IIB (K-Mg ouabain, pH 5.5). In the presence of ouabain, potassium-activated ATPase appears agglutinated and dispersed. Compare with figure 2A. 85 \times .

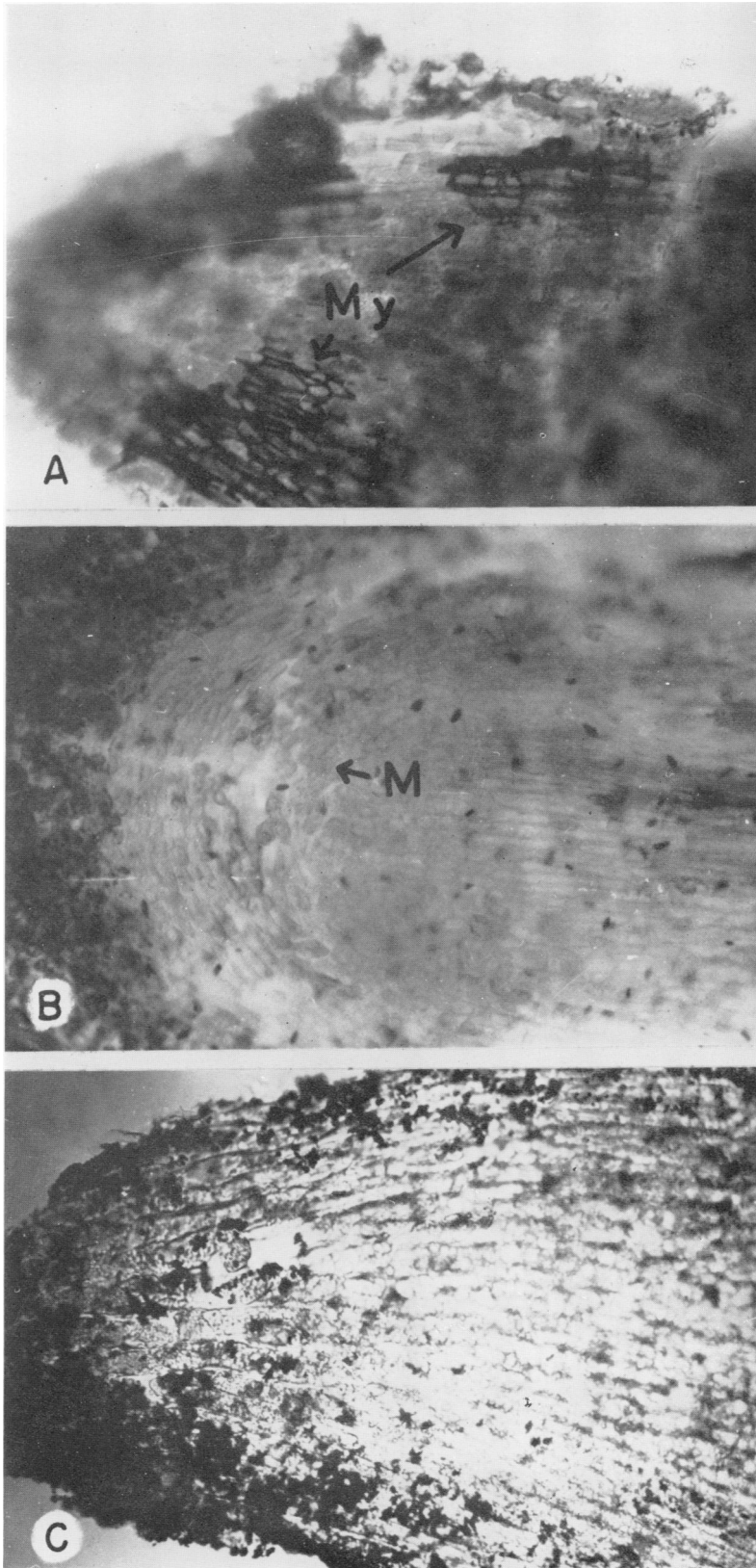


Table I. *Media, Final Concentration of Their Constituents, and Sites of ATPase Activity in Root Tip Sections*

Medium number & author	Sodium	Potassium	Onabain	Magnesium	Calcium	ATP	Final pH	Sites stained
McClurkin					mmoles/ml			
Ia	100	0		3		3	7.2	Meristematic nuclei (faint), mitochondria
	100	30		3		3	7.2	Meristematic nuclei (faint), mitochondria
	0	30		3		3	7.2	Mitochondria
Ib	100	0			3	3	7.2	None
	100	30			3	3	7.2	None
	0	30			3	3	7.2	None
McClurkin								
IIa	100	0		3		3	5.5	Meristematic nuclei, mitochondria
	0	30		3		3	5.5	Mycorrhizae in root cap, mitochondria
	0	0		3		3	5.5	Mitochondria
IIB	100	0	3×10^{-2}	3		3	5.5	Mitochondria
	0	30	3×10^{-2}	3		3	5.5	Mitochondria, dispersed stain in root cap
	0	0	3×10^{-2}	3		3	5.5	Mitochondria
IIC	100	0		3	3	3	5.5	Mitochondria
	0	30		3	3	3	5.5	Mitochondria
	0	0		3	3	3	5.5	Mitochondria
Modification of Wachstein and Meisel								
III	100	0			0.01	0.0083	7.2	None
	0	30			0.01	0.0083	7.2	None
	0	0			0.01	0.0083	7.2	None
	100	0			0.01		7.2	None
	0	30			0.01		7.2	None
Modification of Rains and Epstein								
IVa	100	1			0.5	3	5.5	None
	100	30			0.5	3	5.5	None
	0	0			0.5	3	5.5	None
IVb	100	1		0.5		3	5.5	Mitochondria
	100	30		0.5		3	5.5	Mitochondria
	0	0		0.5		3	5.5	Mitochondria

sulfide solution for 1 minute, and mount in Kaiser's glycerogel. 9) Compare sites of enzyme activity in each section. Those structures which stain only in the presence of sodium or potassium are sites of sodium- or potassium-activated ATPase.

Results and Discussion

Enzyme-activated stains were restricted to the growing root tips, and none were detected in the woody sections taken 2 inches back from the tips. No differences were noted in staining characteristics of tips from fresh, stored, or potted seedlings.

Results summarized in table I show that with medium IA, sodium, with or without potassium, produced a faint stain in the meristematic nuclei

(fig 1B). No potassium-activated ATPase was detected (fig 2B). Neither sodium nor potassium produced staining when calcium ions were substituted for magnesium ions in equivalent concentrations with ATP (medium IB).

With medium IIA, distinct enzyme activation was observed both with sodium and with potassium. Sodium-activation was restricted to the nuclei of meristematic cells (fig 1A), while potassium-activation was found in the mycorrhizae between root cap cells (fig 2A). Control for medium IIA (omission of sodium and potassium) produced stain only in the mitochondria of meristematic cells (fig 1C).

Tests at pH 5.5 in the magnesium-ATP equivalent medium IIB indicated that ouabain inhibited sodium-activated ATPase (compare figs 1A and

1D), but in the potassium-activated ATPase ouabain appeared to have fragmented and dispersed mycorrhizae throughout the root cap (compare figs 2A and 2C). Ouabain did not affect staining in the mitochondria.

Addition of calcium to the magnesium-ATP equivalent medium IIC inhibited sodium- and potassium-activated stain. Mitochondrial stain was unaffected.

When calcium and ATP were not equivalent and magnesium ions were absent (media III and IVA), no stain occurred anywhere. When magnesium and ATP were not equivalent (medium IVB), staining occurred in mitochondria but addition of sodium and potassium failed to produce stain at any other sites.

The mitochondria in the meristematic cells stained when the incubating medium contained magnesium and ATP. The stain was not dependent upon an equivalent relationship between ATP and magnesium, nor upon the presence of sodium or potassium. Mitochondria did not stain when calcium was substituted for magnesium in the medium.

These results show that a cytochemical approach may reveal the characteristics of salt carrier mechanisms in plant roots as postulated earlier by Hendricks (5). Further, the sodium-activated enzyme depicted here cytochemically in pine roots has characteristics similar to the active transport enzyme described by Skou (13) in animal tissues, i.e., the enzymes are stimulated by sodium when magnesium and ATP are in equivalent concentration; potassium cannot substitute for sodium in either enzyme; and both enzymes are inhibited by either calcium or ouabain. They differ in that the enzyme activation has an optimum pH near 5.5 in pine root tips as opposed to 7.2 in animal tissue.

In pines, sodium-activated ATPase is located in the region of the root most effective in salt uptake (5,6), but in the nuclei rather than in the membrane fraction of the cells. Previous unsuccessful attempts to demonstrate a sodium-magnesium dependent, ouabain sensitive, ATPase in this region were restricted to examination of the membrane fraction (5) or the endoplasmic reticulum (2).

The striking parallels between the enzyme system described for sodium transport into animal cells and that described here cytochemically in pine roots, plus the presence of this sodium-activated enzyme at the site of most active salt uptake in roots, indicate this may be a carrier mechanism for salt entry into plant roots. The fact that potassium cannot substitute for sodium in activating this pine root enzyme may explain how plant roots are able to differentiate between ions as closely similar as sodium and potassium.

The potassium-activated ATPase in the mycorrhizae infecting the pine root tips, like its sodium-activated counterpart in meristematic cells, also requires magnesium and ATP in equivalent concentration before potassium can stimulate ATP

hydrolysis, has an optimum pH near 5.5, is inhibited by calcium ions, and fails if sodium ions are substituted for potassium ions. Ouabain has an effect that is not understood.

Other researchers have shown that seedlings infected with mycorrhizae appear to absorb potassium more easily than uninfected seedlings (3,8,12). That a potassium-activated ATPase with such precise enzymatic requirements is present in the mycorrhizae but not in pine root cells suggests that dependence of pines upon mycorrhizal infections for growth may be related to the uptake of potassium by these fungi.

Literature Cited

1. BERG, G. G. 1964. The staining of triphosphatases by the chelate removal method. *J. Histochem. Cytochem.* 12: 341-58.
2. CHATTOPADHYAY, S. K. AND H. D. BROWN. 1966. Ouabain-inhibited ATPase from barley root. *Ann. J. Botany* 53: 618-19.
3. HARLEY, J. L. AND J. M. WILSON. 1959. The absorption of potassium by beech mycorrhizae. *New Phytologist* 58: 281-89.
4. HENDRICKS, S. B. 1964. Salt transport across cell membranes. *Am. Scientist* 52: 306-33.
5. HENDRICKS, S. B. 1966. Salt entry into plants. *Soil Sci. Soc. Am. Proc.* 30: 1-7.
6. KRAMER, P. J. 1949. *Plant and Soil Water Relationships*. McGraw-Hill, New York, p 257-58.
7. McCLURKIN, I. T. 1964. A method for the cytochemical demonstration of sodium-activated adenosine triphosphatase. *J. Histochem. Cytochem.* 12: 654-58.
8. MACDOUGAL, D. T. AND J. DUFRENOY. 1944. Mycorrhizal symbiosis in *Aplectrum*, *Corallorhiza* and *Pinus*. *Plant Physiol.* 19: 440-65.
9. NOVIKOFF, A. B., J. DRUCKER, W. Y. SHIN, AND S. GOLDFISCHER. 1961. Further studies of the apparent adenosine triphosphate activity of cell membranes in formol-calcium-fixed tissues. *J. Histochem. Cytochem.* 9: 434-51.
10. NOVIKOFF, A. B., S. GOLDFISCHER, AND E. ESSNER. 1961. The importance of fixation in a cytochemical method for the Golgi apparatus. *J. Histochem. Cytochem.* 9: 459-60.
11. RAINS, D. W. AND E. EPSTEIN. 1965. Transport of sodium in plant tissue. *Science* 148(3677): 1611.
12. ROUTIEN, J. B. AND R. F. DAWSON. 1943. Some interrelationships of growth, salt absorption, respiration, and mycorrhizal development in *Pinus echinata* Mill. *Am. J. Botany* 30: 440-51.
13. SKOU, J. C. 1957. The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim. Biophys. Acta* 23: 394-401.
14. SKOU, J. C. 1965. Enzymatic basis for active transport of Na⁺ and K⁺ across cell membrane. *Physiol. Rev.* 45: 596-617.
15. WACHSTEIN, M. AND E. MEISEL. 1957. Histochemistry of hepatic phosphatases at a physiologic pH. *Am. J. Clin. Pathol.* 27: 13-23.
16. WHITTAM, R. AND K. P. WHEELER. 1961. The sensitivity of a kidney ATPase to ouabain and to sodium and potassium. *Biochim. Biophys. Acta* 51: 622-24.