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Purification of an Ion-Stimulated Adenosine Triphosphatase from Plant Roots: Association with Plasma Membranes

(transport/phospholipid/sterols/plasma membrane stain/glucan synthetase)

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ABSTRACT **A membrane-bound adenosine triphosphatase (EC 3.6.1.3) that requires Mg^{++} and that is stimulated by monovalent ions has been purified 7- to 8-fold from homogenates of oat (*Avena sativa* L. Cult. Goodfield) roots by discontinuous sucrose-gradient centrifugation. The enzyme was substrate specific; adenosine triphosphate was hydrolyzed 25 times more rapidly than other nucleoside triphosphates. The membrane fraction containing adenosine triphosphatase was enriched in plasma membranes, which were identified by the presence of a glucan synthetase (EC 2.4.1.12), a high sterol to phospholipid ratio, and by a stain consisting of periodic acid, chromic acid, and phosphotungstic acid that is specific for plant plasma membranes. Oat-root plasma membranes and the associated adenosine triphosphatase were purified on either a 6-layer discontinuous sucrose gradient or on a simplified gradient consisting of only two sucrose layers.**

These results represent the first demonstration that plant plasma membranes contain an adenosine triphosphatase that is activated by monovalent ions, and this finding further implicates the enzyme in the absorption of inorganic ions by plant roots.

Absorption of inorganic ions by plant-root cells is an energy-requiring process dependent on aerobic respiration (1, 2). Furthermore, adenosine triphosphate (ATP) appears to be the energy source, since ion absorption by plant roots is inhibited by dinitrophenol (3, 4), arsenate (4), and oligomycin (5–7). The mechanism of energy transfer from ATP to the ion-transport system is unknown, however, and this phenomenon represents one of the major unresolved aspects of the ion-absorption process in plants.

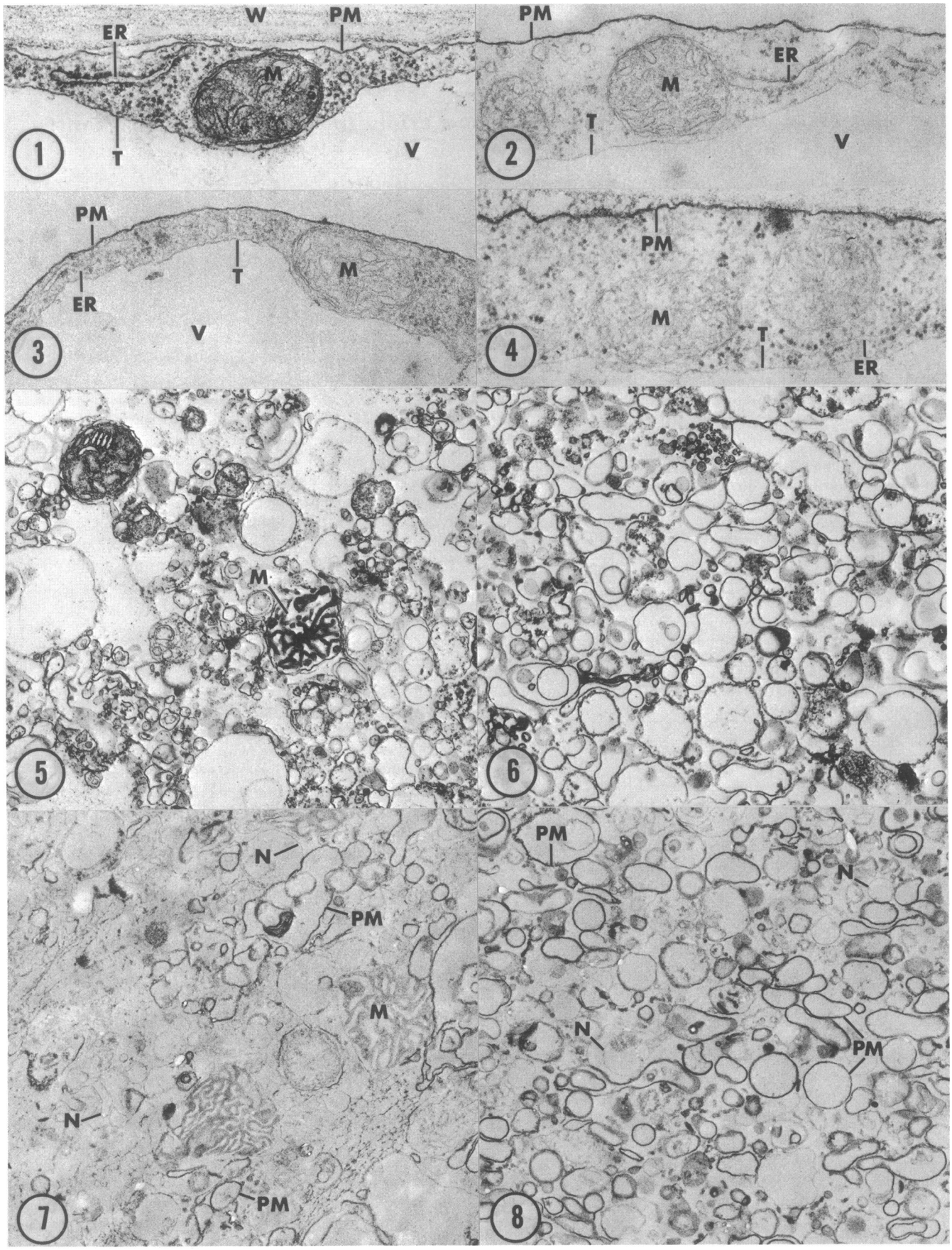
We have suggested (8, 9) that the energy transduction process involved in ion transport of plant cells involves an adenosine triphosphatase (ATPase; EC 3.6.1.3) similar to the "transport" ATPase of animal cells (10). Plant ATPase is associated with membranes, requires Mg^{++}, and is further activated by monovalent ions (8, 9, 11). A high correlation exists between the KCl- or RbCl-activated component of the ATPase and K^+ or Rb^+ absorption by root tissue (9). Also, the kinetics of monovalent-ion transport into roots and the kinetics of monovalent ion-stimulated ATPase are similar (8, 9). However, in order for this ATPase to be involved in energy transduction for ion transport, it should be associated with one or both of the membranes involved in active ion transport (i.e., either the plasma membrane or tonoplast), and this has not been demonstrated.

It is difficult to isolate and identify the membrane system containing the ion-stimulated ATPase because of the ubiquity of membrane-associated ATPases in plants (12–14) and the paucity of known membrane "markers" for plant cells (14). We have recently found, however, that the membrane system containing the monovalent ion-stimulated ATPase can be separated from nearly all the other membranes on either continuous or discontinuous sucrose gradients (14). In this paper, we show that this membrane system has a high sterol:phospholipid ratio, which is characteristic of animal plasma membranes, (15, 16) and that it is enriched in a glucan synthetase (glycosyl transferase, EC 2.4.1.12), which is associated with plant plasma membranes (17). In addition, the isolated membranes were stained with PACP (a special stain consisting of periodic acid, chromic acid, and phosphotungstic acid; see refs. 18, 27–29), which specifically stained oat-root plasma membranes *in situ*. These results provide the first evidence that the plant-root ATPase, which is stimulated by monovalent ions, is associated with the plasma membrane, and this finding serves to further implicate the enzyme in the absorption of inorganic ions by plant roots.

METHODS

Oat (*Avena sativa* L. Cult. Goodfield) roots were grown as described in ref. 14. Root tissue was homogenized by grinding in a mortar and pestle for 60–90 sec in an ice-cold medium of 0.25 M sucrose, 3 mM EDTA, and 25 mM Tris[tris(hydroxymethyl)aminomethane]–MES [2-(*N*-morpholino)ethane sulfonic acid] (pH 7.2). A solution:tissue ratio of 4:1 (v/w) was used. The brei was strained through four layers of cheesecloth and centrifuged at 13,000 × *g* for 15 min (13K pellet). The supernatant was then centrifuged at 80,000 × *g* for 30 min (13–80K pellet). The 13K and 13–80K pellets were suspended separately in homogenizing medium and pelleted again by the centrifugation forces used for obtaining the original pellets. 2 ml (containing 10–20 mg of protein) of the 13–80K pellet [suspended in 18%, w/w, sucrose–1 mM $MgSO_4$–1 mM Tris–MES (pH 7.2)] were layered onto a discontinuous sucrose gradient consisting of the following sucrose solutions: 4 ml of 45% (w/w), and 6.4 ml each of 38, 34, 30, 25, and 20%. Sucrose solutions contained 1 mM $MgSO_4$ and 1 mM Tris-MES (pH 7.2). Membrane-loaded gradients were centrifuged for 2 hr at 27,000 rpm (95,000 × *g*) in a Spinco SW27 rotor. Visible protein bands occurred at all sucrose

Abbreviations: PACP, special strain consisting of periodic acid, chromic acid, and phosphotungstic acid; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethane sulfonic acid.



FIGS. 1–8. Electron micrographs of sections of intact oat-root tissues and pellets of isolated membrane fractions. (*1*) Oat-root cortical cell stained with lead citrate (×38,000). (*2*) Oat-root cortical cell stained with PACP (×38,000). (*3*) Oat-root epidermal cell

TABLE 1. *Specific activity of KCl-ATPase in various membrane fractions of oat roots*

Membrane fraction		ATPase activity (μmol/mg of protein per hr) −KCl	+KCl	Δ KCl	Enrichment
Homogenate		35.65	40.24	4.59	—
13K pellet		5.29	14.62	9.34	2.0
13–80K pellet		11.19	26.45	15.26	3.3
(13–80K overlay) Gradient fractions					
% Sucrose (w/w)	Zone*				
20	A	4.68	7.38	2.70	0.6
25	B	7.96	14.88	6.92	1.5
30	C	8.96	18.15	9.19	2.0
34	D	10.32	30.25	19.93	4.3
38	E	14.19	46.30	32.11	7.0
45	F	13.98	49.10	35.12	7.7

* The material remaining in the membrane overlay after centrifugation was removed and designated as zone A. Zones B through F refer to protein bands that occurred over the sucrose layers given in the first column.

TABLE 2. *Nucleoside triphosphate specificity (KCl-stimulated component) of an oat-root crude homogenate and membranes of zone F of discontinuous gradient*

Substrate	Nucleoside triphosphatase activity (μmol/mg of protein per hr) Homogenate	Zone F*
ATP	4.59 (100)	36.91 (100)
ITP	6.13 (134)	1.43 (4)
UTP	9.19 (200)	1.42 (4)
GTP	2.84 (62)	1.29 (3)
CTP	0.66 (14)	0.43 (1)

All nucleoside triphosphates were added as the Tris salt at a final concentration of 3 mM. Numbers in parenthesis are the percentage of activity as compared to ATP.

* See footnote of Table 1.

interfaces. They were removed with a Pasteur pipette, either assayed directly or diluted with 1 mM $MgSO_4$–1 mM Tris–MES (pH 7.2), and then pelleted and resuspended in 1 mM $MgSO_4$–1 mM Tris–MES before assay.

ATPase Assay. Adenosine triphosphatase activity was measured as described in ref. 14. The final concentration of reaction components was 3.0 mM ATP-Tris, 1.5 mM $MgSO_4$, 33 mM Tris–MES (pH 6) (or pH 9, Table 4), and when added, 50 mM KCl in a final volume of 1 ml. Reactions were initiated by addition of 20–40 μg of membrane protein. P_i release was estimated according to Fiske and SubbaRow (19). Protein was determined by the method of Lowry *et al.* (20) with care being taken to eliminate sucrose as an interfering substance in the assay (21).

Lipid Analysis. Lipids were extracted from membrane fractions and washed according to Folch *et al.* (22). Lipid-P was determined by the procedure of Rouser *et al.* (23), and total sterols were determined as described by Stadtman (24).

Electron Microscopy. Segments of oat roots and isolated membrane fractions were fixed with 3% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 6.8) and postfixed in 1% OsO_4 in buffer. Fixed specimens were soaked in 0.5% uranyl acetate, dehydrated in acetone, and embedded in Epon before sectioning. Thin sections were stained either with lead citrate or with PACP, which preferentially stains plasma membranes of plants (18, 27–29).

RESULTS

The KCl-stimulated ATPase is enriched 7- to 8-fold in zones E and F of the discontinuous gradient (Table 1). Other zones of the gradient were also enriched in the enzyme, but the highest specific activities were in zones E and F. Emphasis was placed on the KCl-stimulated activity since this was the enzyme activity previously shown to be correlated with ion transport (9), and, in addition, it is necessary in order to distinguish this enzyme from a soluble acid phosphatase (8) present in the homogenate (note homogenate ATPase activity in absence of KCl, Table 1).

ATPase of membranes collected at zone F of the discontinuous gradient was highly substrate specific (Table 2); ATP was hydrolyzed at least 25 times more rapidly than the other nucleoside triphosphates examined. By contrast, the crude homogenate exhibited little specificity for the various nucleoside triphosphates.

To identify the membrane system containing the KCl-ATPase and to evaluate the purity of the membrane fraction, we have determined the distribution on the gradient of 25 other enzymes. All enzymes measured (including nucleoside mono-, di-, and triphosphatases, glucose 6-phosphatase, pyrophosphatase, acid- and alkaline-phosphatase, cytochrome *c* oxidase, NADH- and NADPH cytochrome *c* reductase), except for the KCl-ATPase and a glucan synthetase (14), had lower specific activities in zones E and F than in the membrane overlay. For glucan synthetase, actual values for incorporation of UDP- [U-^{14}C]glucose (1 μmol/ml) into total polysaccharide for 0-80K, 0-13K, and 13-80K fractions, and zones E and F from the gradient were 2.93, 1.60, 6.60, 14.92, and 11.60 nmol/mg of protein per min, respectively (14). Enrichment of this enzyme in zones E and F is particularly interesting because this enzyme has been reported to be as-

stained with PACP (×38,000). (*4*) Oat-root stelar parenchyma cell stained with PACP (×38,000). (*5*) Total membrane fraction (0–80K) of oat roots stained with lead citrate (×23,000). (*6*) Purified membrane fraction (zone E–F from simplified discontinuous gradient) stained with lead citrate (×23,000). (*7*) Total membrane fraction (0–80K) of oat roots stained with PACP (×23,000). (*8*) Purified membrane fraction (zone E–F from simplified discontinuous gradient) stained with PACP (×23,000).

Abbreviations: *W*, cell wall; *PM*, plasma membrane; *ER*, endoplasmic reticulum; *T*, tonoplast; *M*, mitochondria; *V*, vacuole; *N*, unidentified membranes that do not stain with PACP.

TABLE 3. *Phospholipid and sterol content in various membrane fractions of oat roots*

Membrane fraction	mg of phospholipid / mg of protein	mg of sterol / mg of protein	Sterol / phospholipid (molar basis)*
13K pellet	0.233	0.046	0.385
13–80K pellet	0.421	0.095	0.440
(13–80K overlay) Gradient fractions			
Zone†			
A	0.789	0.125	0.308
B	0.479	0.056	0.226
C	0.276	0.077	0.537
D	0.331	0.143	0.837
E	0.332	0.184	1.074
F	0.220	0.138	1.213

* Average molecular weights for phospholipids and sterols of 750 and 387, respectively, are assumed.

† See footnote of Table 1.

sociated with Golgi apparatus membranes (25) and/or plasma membranes (17) of higher plant cells. It is unlikely that zones E and F contain Golgi membranes since IDPase is a "marker" enzyme for Golgi membranes in plant cells (25, 26), and this enzyme is enriched in zone C of the gradient (14). Thus, the enrichment of both the glucan synthetase and ion-stimulated ATPase in membranes of zones E and F suggests that these fractions are rich in plasma membranes, and the low specific activities (as compared to the membrane overlay) of all other enzymes measured to date indicate that these fractions contain predominantly one type of membrane.

Another possible way of identifying the membrane system in question is by its chemical composition. In animal cells, the plasma membrane is unique (as compared to other cytoplasmic and organelle membranes) in that it has a high cholesterol:phospholipid ratio (15, 16). Since plasma membranes were the suspected site of the KCl-ATPase, we determined the total sterol and phospholipid content of various membrane fractions (Table 3). Membranes of zone E contained the most sterol per unit protein, and the membranes of both zones E and F had high sterol:phospholipid ratios. The types of sterols and phospholipids in plant membranes are unknown, but if we assume average molecular weights of 387 (cholesterol) and 750, respectively, molar ratios of sterol:phospholipid of 1.1 and 1.2 were obtained for the membranes of fractions E and F. Typical molar ratios of cholesterol:phospholipid for purified animal plasma membranes range from 0.7 to 1.2 (ref. 16 and literature cited within). These results further support the contention that the membranes collected at zones E and F are plasma membranes.

Membranes containing the KCl-ATPase can be purified by a simpler discontinuous gradient than the one previously used. The simplified gradient consists of 30 ml of 45% (w/w) sucrose, 6 ml of 34% (w/w) sucrose, and 2 ml of membrane overlay. Basically, this gradient combines zones E and F of the more complex gradient (i.e., Tables 1 and 3) into a single zone referred to as zone E–F (Table 4). Specific activities of the KCl-ATPase and other enzymes of zone E–F of the simplified gradient are similar to those found in zones E and F of the more complex gradient. Thus, the two gradients gave nearly identical purification of the KCl-ATPase. By the simplified gradient, about 20% of the total KCl-ATPase was recovered in zone E–F (Table 4). By comparison, only 4% of the cytochrome oxidase activity (a mitochondrial marker) and 3% of a pH 9, KCl-ATPase activity, which is a measure of other membrane ATPases (14), were recovered in this fraction. Thus, with this simple isolation procedure the KCl-ATPase was enriched 7- to 8-fold, and about 20% of the enzyme was recovered.

The plasma membranes of oat root cortical (Fig. 2), epidermal (Fig. 3), and stelar parenchyma (Fig. 4) cells were specifically stained by PACP, a stain that is known to be specific for plasma membranes in plants (18, 27–29). No other cell membranes were stained with PACP (compare Fig. 1, which is a cortical cell stained with lead citrate). Occasionally, the plasma membranes of some cortical cells showed little or no staining with PACP, but in no case was PACP staining observed in membranes other than the plasma membrane.

Isolated membranes collected in a 0–80K fraction (total membrane fraction) and in zone E–F (purified membrane fraction) of the simplified gradient were stained with lead citrate (Figs. 5 and 6) and PACP (Figs. 7 and 8). The 0–80K fraction contained various membrane structures as expected (Fig. 5) and a few membrane vesicles and pieces that stained with PACP (Fig. 7); the latter are assumed to be plasma-membrane fragments. The purified fraction (zone E–F) contained mostly vesicles of various sizes (Fig. 6), and most of these structures were stained by PACP (Fig. 8). By visual inspection of many micrographs and by actual counting of stained and nonstained membrane intersections with parallel lines drawn on a transparency and laid over electron micrographs (28, 30), we estimate that over 75% of the membranes in the purified fraction are stained by PACP. Thus, the membrane fraction enriched in KCl-ATPase is also highly enriched in plasma membranes.

DISCUSSION

A monovalent ion-stimulated ATPase of oat roots has been extensively purified, and the characterization studies re-

TABLE 4. *Amount of KCl-ATPase (pH 6 and 9) and cytochrome c oxidase of oat roots in 13K, 13–80K, and zone E–F fractions of simplified gradient*

Membrane fraction	pH 6 KCl-ATPase µmol/hr	pH 6 KCl-ATPase % of total	pH 9 KCl-ATPase µmol/hr	pH 9 KCl-ATPase % of total	Cytochrome *c* oxidase µmol/hr	Cytochrome *c* oxidase % of total
13K pellet	103.1	33.1	326.1	67.4	12.9	89.5
13–80K pellet	208.6	66.9	157.4	32.6	1.5	10.5
Total	311.7	100.0	483.5	100.0	14.4	100.0
Zone E–F (13–80K simplified gradient)	61.8	19.8	15.4	3.2	0.6	4.2

ported here indicate that this enzyme is associated with the plasma membrane.

Purification of plasma membranes from plant roots has not been reported previously. There are only two reports dealing specifically with the purification of plant plasma membranes (27, 31). Morré *et al.* (27) obtained a membrane fraction enriched by "not more than 50%" from onion-shoot meristems (more recent studies indicate that the purified fractions contain 65–75% plasma membranes; Morré, personal communication). Lai and Thompson (31) claimed to have purified plasma membranes from bean cotyledons, but they gave no estimate of the purity of the plasma membrane fraction. The procedure used in this study yields a membrane fraction enriched in plasma membranes (in excess of 75%), and it is simple and rapid. The method is also satisfactory for isolation of plasma membranes from corn and barley roots, but with these tissues, membranes frequently aggregate on the gradient. This problem can be eliminated by omission of $MgSO_4$ from gradient solutions. Thus, with only minor modifications, the procedure described here for isolation of oat-root plasma membranes should be useful for the isolation of plasma membranes from other plant tissues.

We have previously shown that a high correlation exists between the KCl- or RbCl-stimulated component of the ATPase and K^+ or Rb^+ absorption by roots of several plant species (9). The demonstration here that this ATPase is associated with plasma membranes further implicates the enzyme in inorganic ion transport. This situation is similar to that of the Na^+- + K^+-stimulated ATPase of animal plasma membranes and the participation of this enzyme in Na^+ and K^+ transport (10). However, the oat-root plasma membrane ATPase is quite different from the animal ATPase in that it is neither synergistically stimulated by Na^+ and K^+ nor inhibited by ouabain (8). The lack of ouabain inhibition of the ATPase is not surprising, however, since ouabain does not inhibit ion transport in plant roots (5). Preliminary results with the purified membrane fraction confirm our earlier findings (8) that the ATPase is stimulated to different extents by different monovalent salts and that both the cation and anion contribute to the stimulation. Thus, it appears that plasma membranes of plant roots possess a mechanism of energy transduction for active ion transport similar to that of animal cells, but the ATPases themselves have some important differences.

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