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Characterization of a NO_3^--Sensitive H^+-ATPase from Corn Roots[1]

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ABSTRACT

When assayed in the presence of azide, NO_3^- was shown to be a specific inhibitor of a proton-translocating ATPase present in corn (*Zea mays* L. cv WF9 × M017) root microsomal membranes. The distribution of the NO_3^--sensitive ATPase on sucrose gradients and its general characteristics are similar to those previously reported for the anion-stimulated H^+-ATPase of corn roots believed to be of tonoplast origin. An ATPase inhibited by 20 μM vanadate and insensitive to molybdate was also identified in corn root microsomal membranes which could be largely separated from the NO_3^--sensitive ATPase on sucrose gradients and is believed to be of plasma membrane origin. Inasmuch as both ATPases most likely catalyze the efflux of H^+ from the cytoplasm, our objective was to characterize and compare the properties of both ATPases under identical experimental conditions. The vanadate-sensitive ATPase was stimulated by cations (K^+ > NH_4^+ > Rb^+ > Cs^+ > Li^+ > Na^+ > choline$^+$) whereas the NO_3^--sensitive ATPase was stimulated by anions (Cl^- > Br^- > $C_2H_3O_2^-$ > SO_4^{2-} > I^- > HCO_3^- > SCN^-). Both ATPases required divalent cations. However, the order of preference for the NO_3^--sensitive ATPase (Mn^{2+} > Mg^{2+} > Co^{2+} > Ca^{2+} > Zn^{2+}) differed from that of the vanadate-sensitive ATPase (Co^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+} > Ca^{2+}). The vanadate-sensitive ATPase required higher concentrations of Mg:ATP for full activity than did the NO_3^--sensitive ATPase. The kinetics for Mg:ATP were complex for the vanadate-sensitive ATPase, indicating positive cooperativity, but were simple for the NO_3^--sensitive ATPase. Both ATPases exhibited similar temperature and pH optima (pH 6.5). The NO_3^--sensitive ATPase was stimulated by gramicidin and was associated with NO_3^--inhibitable H^+ transport measured as quenching of quinacrine fluorescence. It was insensitive to molybdate, azide, and vanadate, but exhibited slight sensitivity to ethyl-3-(3-dimethylaminopropyl carbodiimide) and mersalyl. Overall, these results indicate several properties which distinguish these two ATPases and suggest that under defined conditions NO_3^--sensitive ATPase activity may be used as a quantitative marker for those membranes identified tentatively as tonoplast in mixed or nonpurified membrane fractions. We feel that NO_3^- sensitivity is a better criterion by which to identify this ATPase than either Cl^- stimulation or H^+ transport because it is less ambiguous. It is also useful in identifying the enzyme following solubilization.

The existence of H^+-translocating pumps in both the plasma membrane and tonoplast has been proposed based on electrophysiological evidence (29, 30). The identification of ATPase activities associated with both the plasma membrane (24) and tonoplast (33) together with electrophysiological studies have suggested that these H^+ pumps are driven by the hydrolysis of ATP. Inasmuch as both catalyze efflux of H^+ from the cytoplasm, they may be involved in cytoplasmic pH regulation in addition to their role in providing a gradient of $\Delta\bar{\mu}_{H^+}$ that is subsequently used to drive secondary transport processes (25).

The plasma membrane ATPase has been well characterized (13, 19, 24), but the tonoplast ATPase, having only recently been identified (1, 33), has not been studied so thoroughly. Inasmuch as the active sites for ATP hydrolysis of both the plasma membrane and tonoplast ATPase are exposed to the same cytoplasmic environment, we felt it was of interest to compare the activities of both ATPases under identical assay conditions in order to determine whether one might expect differential responses of the ATPases to changes in the cytoplasmic environment. In order to do this, criteria were required to assay specifically each ATPase in membrane fractions enriched in the appropriate ATPase, but not completely pure.

Because vanadate (5, 9, 13) and NO_3^- (1, 26, 33) have been reported to inhibit the plasma membrane and tonoplast ATPase, respectively, we investigated the use of these inhibitors to inhibit each ATPase selectively. Conditions were defined to inhibit specifically the tonoplast ATPase with NO_3^-. Nitrate-sensitive ATPase activity was characterized and compared with vanadate-sensitive ATPase activity in corn root membrane fractions enriched in either tonoplast or plasma membrane. Furthermore, we describe optimal conditions to measure NO_3^--sensitive ATPase activity which should be useful as a quantitative marker for those membranes believed to be of tonoplast origin in mixed membrane fractions. The low density corn root membranes (1.10–1.12 g/cc) enriched in anion-sensitive ATPase activity, and characterized in this paper, have been identified tentatively as being of tonoplast origin by several groups (12, 21). We refer to this membrane fraction as tonoplast throughout this report; however, it should be recognized that this identification is tentative and requires further confirmation.

MATERIALS AND METHODS

Membrane Isolation. *Zea mays* L. cv WF9 × M017 (Crow Hybrid Seed Co.) was used for all membrane isolations. Corn seedlings were grown for 3 d at 28°C on moistened germination paper (Anchor Paper Co., St. Paul, MN) prior to harvesting of root tips (1.5-cm apical segments). Approximately 50 g fresh weight of root tips were harvested into aerated 0.1 mM $CaCl_2$ at room temperature. Excised root tips were homogenized with a chilled mortar and pestle in 0.25 M sucrose, 25 mM Tris/Mes (pH 7.2), 2.0 mM EGTA,[2] 2 mM DTT, and 0.1% BSA. The total homogenate was subjected to one or two 10-min 10,000*g* centrifugations for continuous or discontinuous sucrose gradients, re-

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[2] Abbreviations: EGTA, ethylene glycol bis-*N,N'*-tetraacetic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; EDAC, ethyl-3-(3-dimethylaminopropyl carbodiimide); FC, fusicoccin.

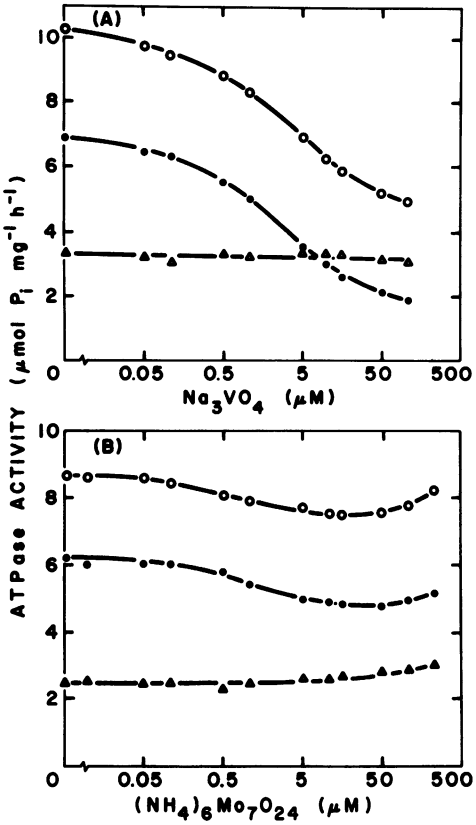

FIG. 1. The effect of NO_3^- on inhibition of ATPase activity by vanadate (A) or ammonium molybdate (B). Activity was assayed as described in "Materials and Methods" in the absence (○) or presence (●) of 50 mM NO_3^- with NO_3^--sensitive ATPase activity (△) as the difference between the two.

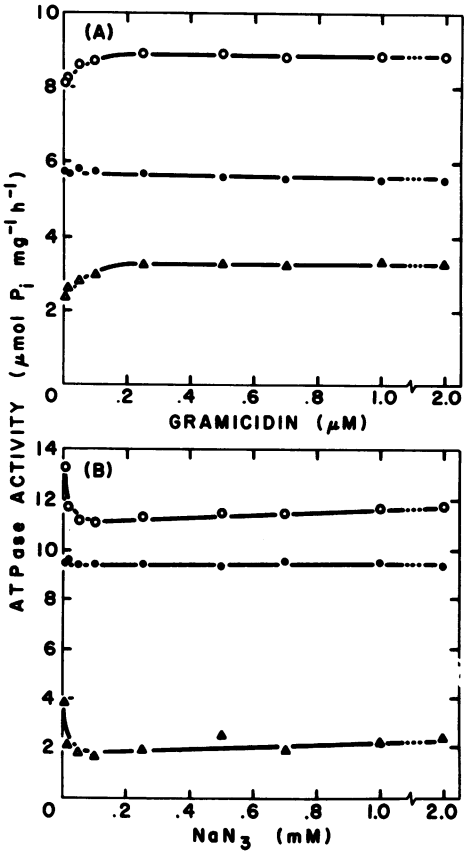

FIG. 2. The effect of NO_3^- on stimulation of ATPase activity by gramicidin (A) and on inhibition of ATPase activity by sodium azide (B). Activity was assayed as described in "Materials and Methods" in the absence (○) or presence (●) of 50 mM NO_3^- with NO_3^--sensitive activity (△) as the difference between the two.

spectively, and the pellets were discarded. The resulting supernatant was collected and centrifuged at 80,000*g* for 30 min to pellet the microsomes.

Microsomal membranes were resuspended in 0.25 M sucrose, 5 mM Tris/Mes (pH 6.8), and 1 mM DTT. For continuous gradients, the resuspended microsomes were layered on a 15 to 45% (w/w) continuous sucrose gradient and centrifuged at 80,000*g* for 3 h. Continuous gradient fractions were collected in 2-ml aliquots and assayed directly. For routine membrane preparations, microsomes were layered on a 20%/30%/38% (w/w) discontinuous sucrose step gradient and centrifuged at 80,000*g* for 2 h. All sucrose solutions contained 5 mM Tris/Mes (pH 6.75), 1 mM EDTA, and 1 mM DTT. Membrane fractions were collected from the 20%/30% (w/w) and 30%/38% (w/w) interfaces, diluted in sucrose-free resuspension buffer, and repelleted at 80,000*g* for 30 min. Pellets were resuspended in 0.25 M sucrose, 5 mM Tris/Mes (pH 6.8), and 1 mM DTT.

Transport Assays. The formation of acid interior pH gradients across vesicle membranes was monitored as quenching of fluorescence of the permeant amine dye, quinacrine, as previously described (3). Membrane vesicles were added to a reaction mix containing 0.25 M sucrose, 25 mM Tris/Mes (pH 6.75), 50 mM KCl, and 10 μM quinacrine. Following temperature equilibration of the reaction mix in the cuvette to 25°C, ATP (Tris salt) was added to a final concentration of 5 mM and equilibration again was established. Fluorescence quenching was initiated upon the subsequent addition of $MgSO_4$ to a final concentration of 5 mM. Fluorescence was measured at 25°C with a Perkin-Elmer 650-10S fluorescence spectrophotometer at excitation/emission wavelengths of 425/500 nm. Transport assays were not carried out at 38°C, the optimal ATPase assay temperature, because passive permeability of the membrane vesicles increased above 30°C (S. D. O'Neill and R. M. Spanswick, unpublished results). Approximately 200 μg of membrane protein were added per transport assay.

Biochemical Assays. ATPase activity was determined by measuring the release of Pi from ATP (Tris salt) according to the method of Ames (2). ATPase activity was assayed for 30 min at 25°C for sucrose gradient fractions and resuspended microsomes, or at 38°C for step gradient interfaces. The basic reaction mix contained 5 mM Tris-ATP (pH 6.5), 5 mM $MgSO_4$, 30 mM Tris/Mes (pH 6.5), and the appropriate salts in a final volume of 0.5 ml. For assaying ATPase activity in the absence or presence of NO_3^-, either 25 mM K_2SO_4 or 50 mM KNO_3 was added, respectively. For assaying ATPase activity in the absence or presence of 20 μM vanadate, 50 mM KCl was added to all treatments. In general, 25 mM KCl was included throughout except in the monovalent ion assay and KCl titration.

Cyt *c* oxidase activity was assayed as previously described (17) by measuring the oxidation of reduced Cyt *c* at 550 nm with a Varian 634 spectrophotometer.

Protein Determination. Proteins were determined by the method of Markwell *et al.* (22), and/or Schaffner and Weissmann (27). Fairly good agreement was obtained between these two methods at the concentrations of proteins used in our experiments.

Source of Chemicals. ATP was obtained from Boehringer Mannheim as the disodium salt and converted to the Tris salt by passage through Dowex 50W. Sodium orthovanadate was obtained from Fisher Scientific Co. and the concentration of the stock solution was verified spectrophotometrically using the ex-

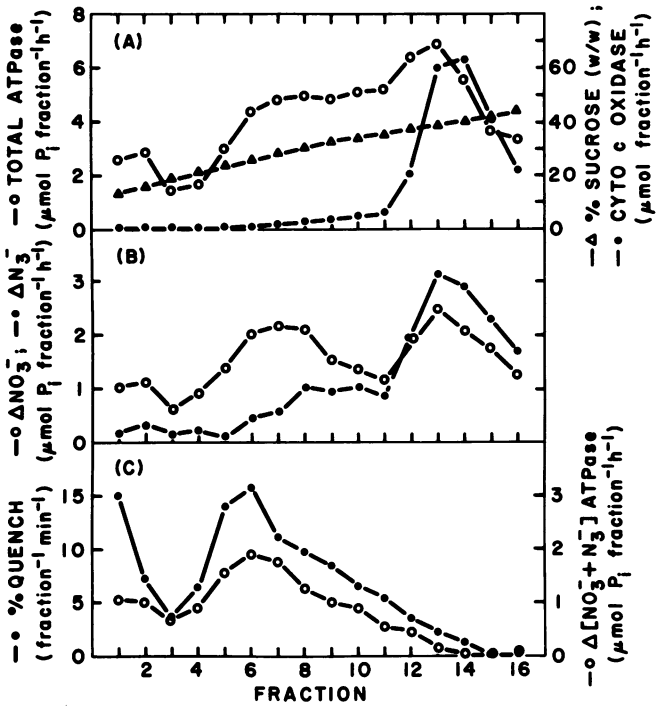

FIG. 3. Distribution of ATPase activity and subcellular markers on continuous sucrose gradients. Total ATPase (A) was assayed with 5 mM ATP, 5 mM $MgSO_4$, and 50 mM KCl. Total nitrate-sensitive (NO_3^-) and azide-sensitive (N_3^-) ATPase (B) were calculated as the difference in ATPase activity assayed with 5 mM ATP, 5 mM $MgSO_4$, 25 mM KCl (also see "Materials and Methods") and in the absence or presence of 50 mM NO_3^- or 1 mM NaN_3. Nitrate-sensitive ATPase assayed in the presence of 1 mM NaN_3 (C) was calculated as the difference in ATPase activity assayed as above and in the absence or presence of 50 mM NO_3^- and 1 mM NaN_3. Cyt *c* oxidase activity (●) and per cent sucrose (Δ) are also presented (A). Proton transport (●) shown in the lower panel was measured as initial rate of fluorescence quenching as described in "Materials and Methods."

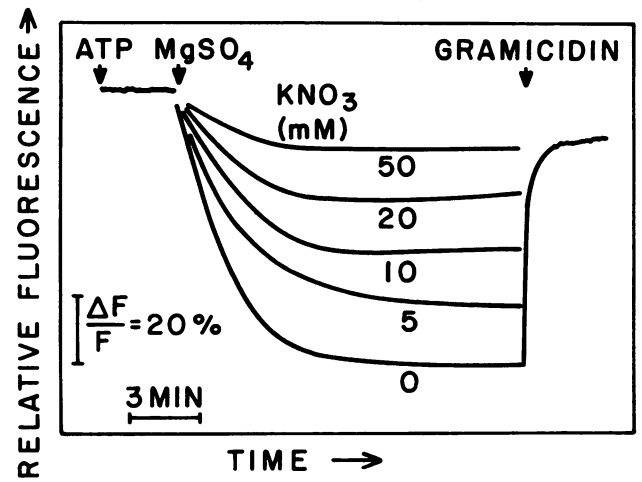

FIG. 4. Effect of increasing KNO_3 on H^+ transport measured as quenching of quinacrine fluorescence as described in "Materials and Methods." ATP, $MgSO_4$, and gramicidin were added where indicated.

tinction coefficient determined by Cantley *et al.* (8). DCCD, gramicidin, and oligomycin were obtained from Calbiochem-Behring. All other chemicals were obtained from Sigma.

RESULTS

Microsomal Membranes. Previous work in our laboratory showed that an ATPase activity associated with ATP-dependent

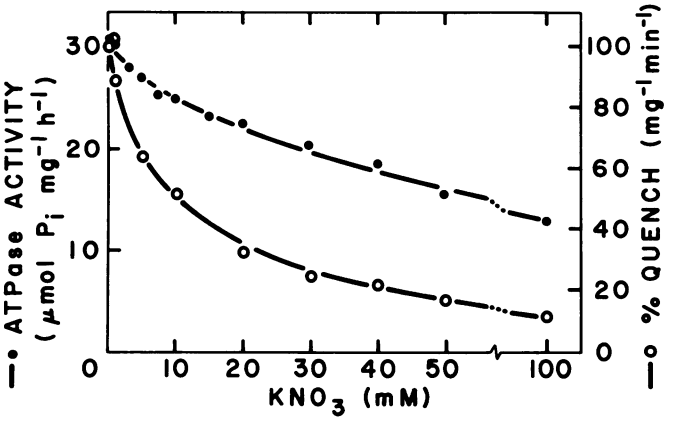

FIG. 5. Inhibition by NO_3^- of ATPase activity (●) assayed in the presence of 5 mM ATP, 5 mM $MgSO_4$, 25 mM Cl^-, 75 mM K^+, 2 μM gramicidin, and 1 mM NaN_3, and of H^+ transport (○) measured and plotted as initial rate of fluorescence quenching.

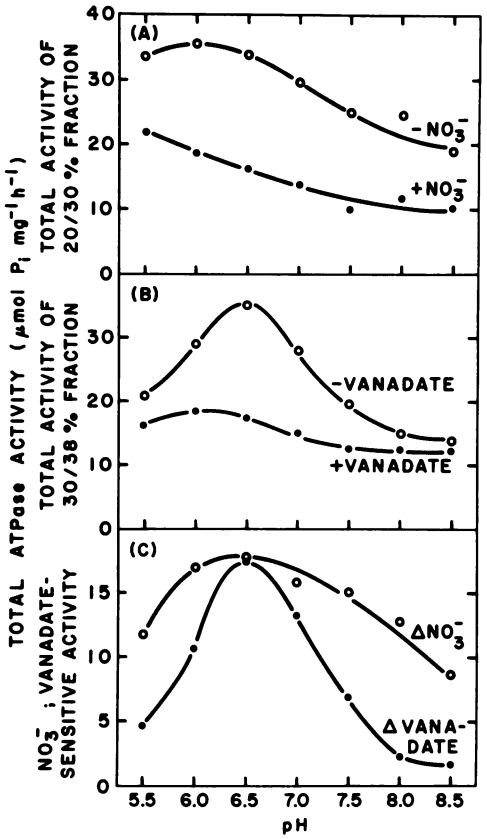

FIG. 6. Effect of pH on ATPase activity in the 20%/30% membrane fraction (A) in the absence (○) and presence (●) of 50 mM NO_3^- and on ATPase activity in the 30%/38% membrane fraction (B) in the absence (○) and presence (●) of 20 μM vanadate. The nitrate-sensitive (○) and vanadate-sensitive (●) ATPase activities (C) were calculated as the difference in activity measured in the presence and absence of 50 mM KNO_3 or 20 μM vanadate, respectively.

proton transport and gramicidin stimulation of ATPase activity was inhibited by NO_3^- (4, 14). This same activity in both native membrane vesicles and reconstituted proteoliposomes was insensitive to vanadate (4), a potent inhibitor of plasma membrane ATPases (6, 23, 24), indicating that this anion-sensitive proton-translocating ATPase is not of plasma membrane origin. In an initial attempt to characterize the NO_3^- sensitivity of this enzyme and its potential as a membrane marker, we decided to investigate

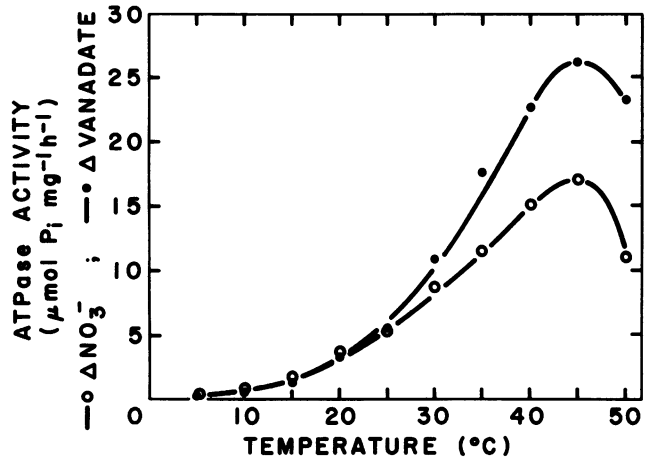

FIG. 7. Effect of temperature on nitrate-sensitive (○) and vanadate-sensitive (●) ATPase activity assayed as described in "Materials and Methods." The ΔNO_3^- activity was calculated as the difference in activity obtained in the absence and presence of 50 mM NO_3^-. The Δvanadate activity was calculated as the difference in activity obtained in the presence or absence of 20 μM vanadate. ATPase activity was assayed for 30 min.

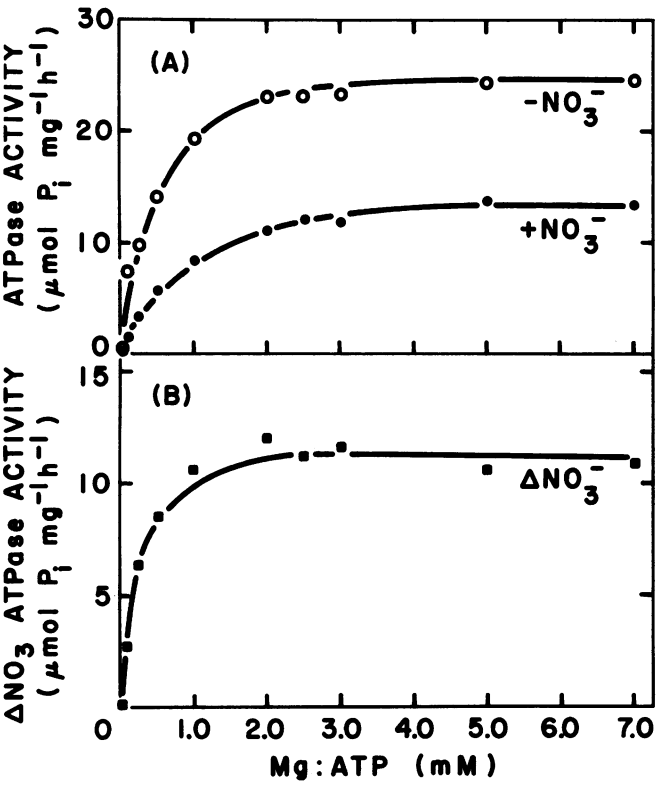

FIG. 8. Effect of Mg:ATP concentration on ATPase activity of the 20%/30% fraction (A) in the absence (○) and presence (●) of 50 mM NO_3^- and on NO_3^--sensitive activity (B) considered alone as ΔNO_3^- (■). Assays were performed as described in "Materials and Methods."

the effect of NO_3^- on ATPase activity in corn root microsomes in conjunction with various known selective inhibitors and the ionophore gramicidin.

An initial screening with microsomes showed that NO_3^- inhibition could be clearly distinguished from inhibition by vanadate. Figure 1A shows the concentration dependence of vanadate inhibition of ATPase activity with and without 50 mM NO_3^-. In the absence or presence of NO_3^-, there is a progressive inhibition of ATPase activity up to 50 μM vanadate, with little additional

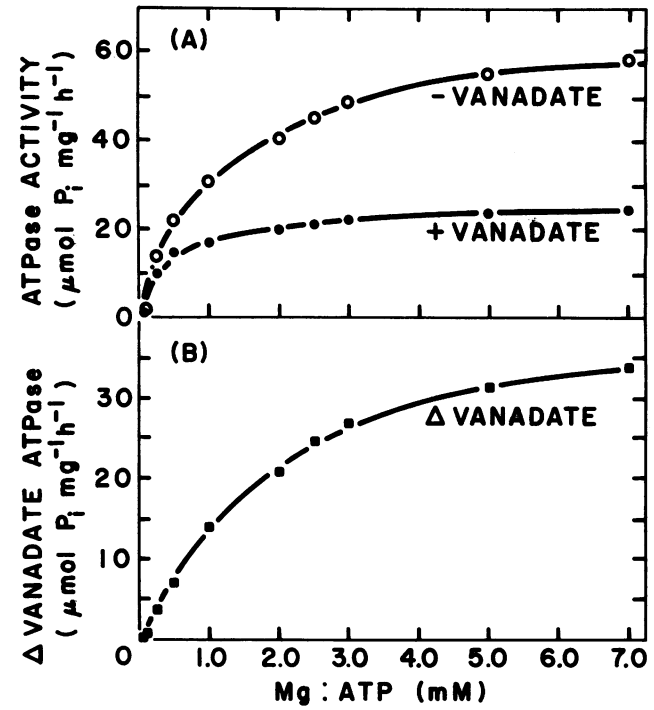

FIG. 9. Effect of Mg:ATP concentration on ATPase activity of the 30%/38% fraction (A) in the absence (○) and presence (●) of 20 μM vanadate and on vanadate-sensitive activity (B) considered alone as Δvanadate (■). Assays were performed as described in "Materials and Methods."

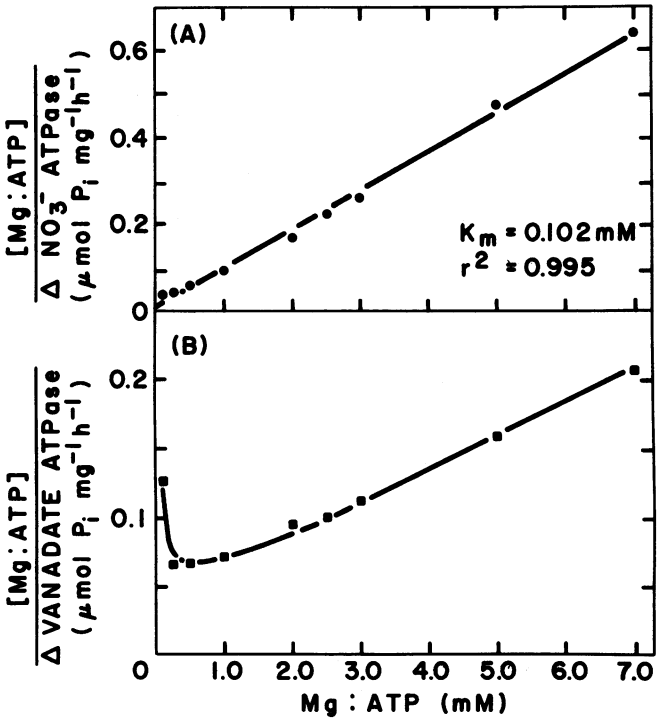

FIG. 10. Hanes-Woolf plot of (A) ΔNO_3^- ATPase activity data from Figure 8B and of (B) Δvanadate ATPase activity data from Figure 9B. Line in upper panel for ΔNO_3^- activity was fit by regression analysis.

inhibition at 100 μM vanadate. The extent of NO_3^- inhibition (ΔNO_3^-) is unaffected by vanadate, with vanadate-sensitive activity (Δvanadate) remaining constant, indicating that NO_3^- and vanadate inhibit separate enzymes. Since it is believed that vana-

Table I. *Effect of Divalent Cations on ATPase Activity of Corn Root Membranes*

ATPase activity of step gradient interfaces.

Divalent Cation (20%/30% Fraction)	Total[a]	$+NO_3^-$ [b]	ΔNO_3^- [c]	Salt Stimulation of ΔNO_3^- ATPase
	μmol Pi mg^{-1} h^{-1}			*% $MgSO_4$ stimulation*
None added	1.8	0.8	1.0	
$MgSO_4$	30.3	15.9	14.4	100
$MnSO_4$	26.8	9.7	17.1	119.5
$CoSO_4$	23.0	14.4	8.6	60.0
$ZnSO_4$	9.1	7.5	1.6	11.5
$CaCl_2$	8.8	5.0	3.8	26.0
30%/38% Fraction	Total[a]	+Vanadate[b]	ΔVanadate[c]	Salt Stimulation of ΔVanadate ATPase
None added	0.2	0	0.2	
$MgSO_4$	22.9	12.2	10.7	100
$MnSO_4$	19.7	9.8	9.9	92.7
$CoSO_4$	22.4	7.6	14.8	138.6
$ZnSO_4$	7.1	5.6	1.5	14.0
$CaCl_2$	5.2	4.3	0.9	8.1

[a] Total ATPase activity was determined in the presence of 5 mM ATP, 30 mM Tris/Mes, and either 25 mM KCl plus 25 mM K_2SO_4 (20%/30%) or 75 mM KCl (30%/38%), 1 mM NaN_3, 2 μM gramicidin at pH 6.5 and 38°C. The divalent cation salt was present at a concentration of 5 mM.

[b] NO_3^- was added to a final concentration of 50 mM as KNO_3 replacing 25 mM K_2SO_4; vanadate was added to a final concentration of 20 μM.

[c] The difference in total ATPase activity in the presence and absence of 50 mM NO_3^- or 20 μM vanadate.

Table II. *Effect of Monovalent Cations on ATPase Activity in Corn Root Membranes*

ATPase activity of step gradient interfaces.

Cation Added (20%/30% Fraction)	Total[a]	$+NO_3^-$ [b]	ΔNO_3^- [c]	Salt Stimulation of ΔNO_3^- ATPase
	μmol Pi mg^{-1} h^{-1}			*% KCl stimulation*
None	14.1	11.8	2.3	
KCl	22.6	12.5	10.1	100
RbCl	21.6	12.3	9.4	92.7
NaCl	21.6	12.6	9.0	89.7
CsCl	21.7	12.2	9.5	94.8
LiCl	21.9	12.3	9.6	95.4
Choline Cl	19.3	11.4	7.9	78.4
NH_4Cl	23.4	11.9	11.5	114.1
30%/38% Fraction	Total[a]	+Vanadate[b]	ΔVanadate[c]	Salt Stimulation of ΔVanadate ATPase
None	17.6	11.9	5.7	
KCl	26.2	12.0	14.2	100
RbCl	25.0	12.9	12.1	85.1
NaCl	24.4	18.1	6.3	44.3
CsCl	25.0	15.5	9.5	67.1
LiCl	23.9	14.9	9.0	62.9
Choline Cl	20.8	15.7	5.1	35.6
NH_4Cl	25.1	11.6	13.5	94.4

[a] Total ATPase activity was determined in the presence of 5 mM ATP, 5 mM $MgSO_4$, 30 mM Tris/Mes, 1 mM NaN_3, 2 μM gramicidin at pH 6.5 and 38°C and the added monovalent salt at a concentration of 50 mM.

[b] 50 mM NO_3^- or 20 μM vanadate were included in the reaction mix.

[c] The difference in total ATPase activity in the presence and absence of 50 mM NO_3^- or 20 μM vanadate.

date inhibits phosphohydrolases that form a phosphorylated intermediate during their reaction cycle (20), it is also suggested then that the non-vanadate-inhibited, NO_3^--sensitive ATPase is not of the 100,000-D class of enzymes that form covalent phosphoenzyme intermediates, but may be more similar in structure to the mitochondrial F_1/F_0 ATPase group.

Similar results were seen with ammonium molybdate (Fig. 1B), an inhibitor of nonspecific phosphatase activity (18). Below 20 μM final concentration, ammonium molybdate progressively inhibited total microsomal ATPase activity without altering the extent of NO_3^- inhibition, suggesting that ammonium molybdate and NO_3^- inhibit different ATP-hydrolyzing enzymes. A slight increase in NO_3^--sensitive ATPase is seen above 20 μM, perhaps due to an uncoupling and stimulation of the anion-sensitive ATPase by ammonium acting as a permeant base and relieving ΔpH. These two inhibitor titrations of microsomal ATPase activity permitted the separation of NO_3^--inhibitable activity from plasma membrane and nonspecific phosphatase activities.

We next examined the effect of gramicidin on NO_3^--sensitive ATPase (Fig. 2A). Gramicidin is a channel-forming ionophore that relieves both gradients of pH (ΔpH) and membrane potential ($\Delta\psi$). At concentrations up to 0.2 μM and in the absence of NO_3^-, gramicidin stimulated total ATPase activity. The addition of 50 mM NO_3^- abolished gramicidin stimulation in addition to depressing total ATPase activity over the same concentration range. The overall effect of gramicidin was to increase NO_3^--sensitive ATPase activity by >40%. Nitrate inhibition, then, is apparently associated with gramicidin-stimulated ATPase activity. It was previously shown that NO_3^- inhibited ATP-dependent proton transport associated with corn root microsomal vesicles and reconstituted vesicles (4).

These results suggested that the NO_3^--sensitive ATPase was that previously identified as an anion-sensitive H^+-ATPase (3) and agrees with the finding by Walker and Leigh (33) that NO_3^- inhibits ATPase activity associated with red beet vacuoles. The results shown here further indicate that NO_3^- does not disrupt membrane-associated ATP-hydrolyzing activity in a nonspecific manner but can be identified as a potent inhibitor of the tonoplast, but not the plasma membrane, H^+-ATPase.

In addition to tonoplast, plasma membrane ATPase and nonspecific phosphatase activities, a major ATPase activity present in corn root membranes is the mitochondrial F_1-ATPase. This ATPase is sensitive, like the tonoplast ATPase, to anions, but differs from the tonoplast ATPase in its sensitivity to azide (N_3^-) (6, 18). Titration of microsomal membranes with NaN_3 (Fig. 2B) in the absence and presence of 50 mM NO_3^- indicated that a NO_3^--sensitive ATPase activity was also inhibited by NaN_3. From this experiment alone, it was not possible to distinguish whether the mitochondrial ATPase or the tonoplast ATPase or both ATPases were inhibited by both NO_3^- and N_3^-. Previous work on both tonoplast (33) and mitochondrial ATPases (6, 16) has indicated that the tonoplast ATPase is sensitive only to NO_3^- and that the mitochondrial ATPase is sensitive to both NO_3^- and N_3^-. In order to confirm this result in corn root microsomal membranes, the distribution of ATPase activities on sucrose gradients was examined.

Sucrose Density Gradients. Total ATPase distribution on a linear sucrose gradient is shown in Figure 3A with major peaks occurring at 25 and 38% (w/w) sucrose. A peak of Cyt *c* oxidase activity (Fig. 3A), a mitochondrial marker, peaks at about 40% (w/w) sucrose and is associated with a peak of N_3^--sensitive ATPase activity (Fig. 3B). NO_3^--sensitive ATPase activity showed

Table III. *Effect of Monovalent Anions on ATPase Activity of Corn Root Membranes*

ATPase activity of step gradient interfaces.

Anion Added (20%/30% Fraction)	Total[a]	+NO_3^- [b]	ΔNO_3^- [c]	Salt Stimulation of ΔNO_3^- ATPase
	μmol Pi mg⁻¹ h⁻¹			*% KCl stimulation*
None	14.1	11.8	2.3	
KCl	22.6	12.5	10.1	100
KBr	21.8	12.6	9.2	91.3
$KC_2H_3O_2$	20.1	12.6	7.5	74.5
K_2SO_4	15.7	10.5	5.2	51.1
KI	9.0	8.3	0.6	6.7
$KHCO_3$	17.3	11.4	5.9	58.5
KSCN	3.1	3.0	0.1	1.0
KNO_3	11.8			
30%/38% Fraction	Total[a]	+Vanadate[b]	ΔVanadate[c]	Salt Stimulation of ΔVanadate ATPase
None	14.1	11.8	2.3	
KCl	22.6	12.5	10.1	100
KBr	26.2	11.4	14.8	147.3
$KC_2H_3O_2$	23.8	9.6	14.2	141.0
K_2SO_4	21.6	9.8	11.8	117.1
KI	16.5	6.0	10.5	104.2
$KHCO_3$	22.6	8.1	14.5	143.5
KSCN	6.3	1.8	4.5	44.6
KNO_3	19.0	7.0	12.0	119.2

[a] Total ATPase activity was determined in the presence of 5 mM ATP, 5 mM $MgSO_4$, 30 mM Tris/Mes, 1 mM NaN_3, 2 μM gramicidin at pH 6.5 and 38°C and the added monovalent salt at a concentration of 50 mM.

[b] 50 mM NO_3^- or 20 μM vanadate were included in the reaction mix.

[c] The difference in total ATPase activity in the presence and absence of 50 mM NO_3^- or 20 μM vanadate.

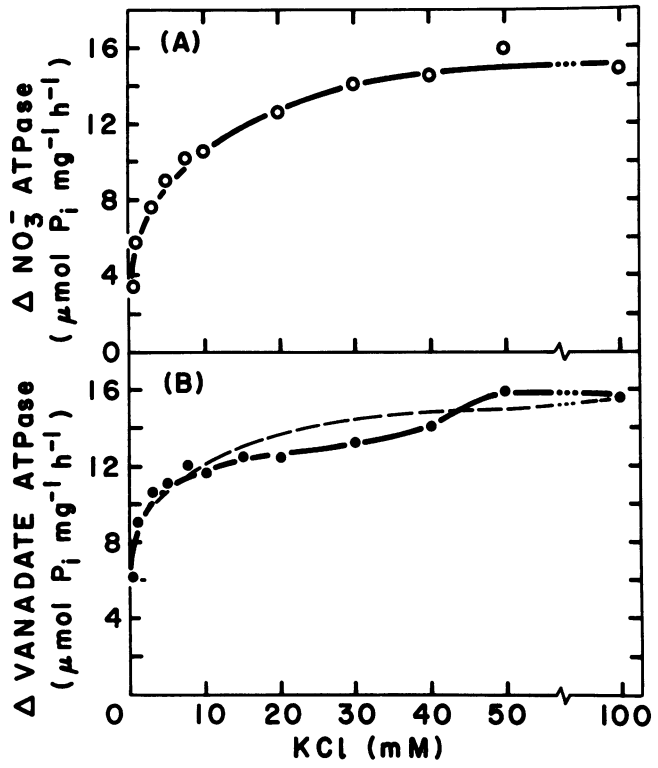

FIG. 11. Kinetics of KCl stimulation of total ΔNO_3^- ATPase activity (A) and of total Δvanadate ATPase activity (B). ATPase activity was assayed as described in "Materials and Methods." The broken line in B was calculated from the regression analysis in Figure 13B.

two distinct peaks: one at approximately 24% (w/w) sucrose and the other at 40% (w/w) sucrose, this latter peak being coincident with N_3^--sensitive ATPase. This confirmed our expectation that the mitochondrial ATPase was sensitive to both NO_3^- and N_3^-. Vanadate-sensitive ATPase had a peak density of about 35 to 37% (w/w) sucrose that fell between the two peaks of NO_3^--sensitive ATPase activity (not shown). When NO_3^--sensitive ATPase activity was assayed in the presence of N_3^-, the distribution showed one peak at 23% (w/w) sucrose and was highly correlated with the distribution of ATP-dependent H^+ transport as measured by quenching of quinacrine fluorescence (Fig. 3C). From these findings, it seemed possible to establish conditions under which inhibition by NO_3^- was specific for the anion-sensitive H^+-ATPase previously suggested to be of tonoplast origin (4, 12).

Inasmuch as tonoplast ATPase is insensitive to azide, NO_3^- inhibition measured in the presence of NaN_3 seems to represent exclusively an inhibition of this low density ATPase. Similarly, inhibition of ATPase activity by vanadate has been shown to represent an inhibition of plasma membrane ATPase (5, 6, 8) in fungi and animals. Using these two inhibitors and membrane fractions enriched in either tonoplast or plasma membrane, it was possible to characterize independently the activities of these two putative, ion-translocating ATPases from plant cells.

As indicated by Figure 3 and as previously demonstrated by Bennett and Spanswick (4) using sucrose gradients, corn root membranes collected at either the 20%/30% (w/w) or 30%/38% (w/w) interface from discontinuous sucrose gradients (see "Materials and Methods") were enriched in either anion-sensitive H^+-ATPase (tonoplast ATPase) or in cation-sensitive ATPase (plasma membrane ATPase), respectively. One of the major difficulties in working with both interfaces, though, is the problem of cross-contamination between interfaces. The criteria we used to measure selectively ATPase activity associated with either tonoplast or plasma membrane were sensitivity of the activity to NO_3^- (in the presence of N_3^- and gramicidin) or vanadate, respectively. We have used these criteria to characterize the ATPase activities associated with tonoplast and plasma membrane fractions collected from a 20%/30% (w/w) or 30%/38% (w/w) sucrose interface. This has allowed us to determine the characteristics of the respective ATPases even in the presence of contaminating activities.

Our choice of vanadate as an inhibitor of plasma membrane ATPase activity deserves some justification in light of the recent report by Gallagher and Leonard (15) that vanadate also inhibits a soluble phosphatase activity associated with corn roots. In a separate study, we have determined a K_I for vanadate inhibition of total ATPase activity in the 30%/38% membrane fraction of 15 μM vanadate (supplied as sodium orthovanadate or vanadium oxide). In this membrane preparation, 20 μM vanadate routinely results in 50% inhibition of ATPase activity whereas 1 mM sodium molybdate inhibits less than 7% of the ATPase activity. The phosphohydrolase activity associated with this membrane fraction is also highly substrate specific for ATP (S. D. O'Neill and R. M. Spanswick, unpublished results). Taken together, these results indicate that the 30%/38% membrane fraction is substantially free of nonspecific phosphatase activity and that the vanadate inhibition of ATPase activity represents the sensitivity of the plasma membrane ATPase to vanadate.

The approach we have taken in assessing plasma membrane ATPase, that is, one based on vanadate-sensitive ATPase activity, may have some limitations in that the sensitivity of the enzyme to vanadate may vary with ionic conditions as with the (Na^+/K^+)-

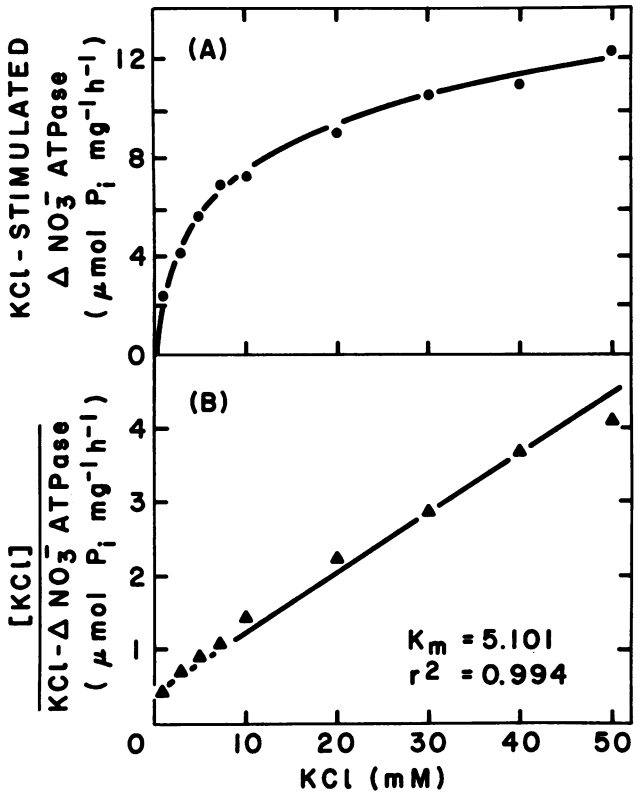

FIG. 12. A, Data from Fig. 11A of ΔNO_3^- ATPase activity replotted after subtraction of the activity measured in the absence of added KCl labeled as KCl-stimulated ΔNO_3^- ATPase activity; B, Hanes-Woolf plot of same with line fit by regression analysis.

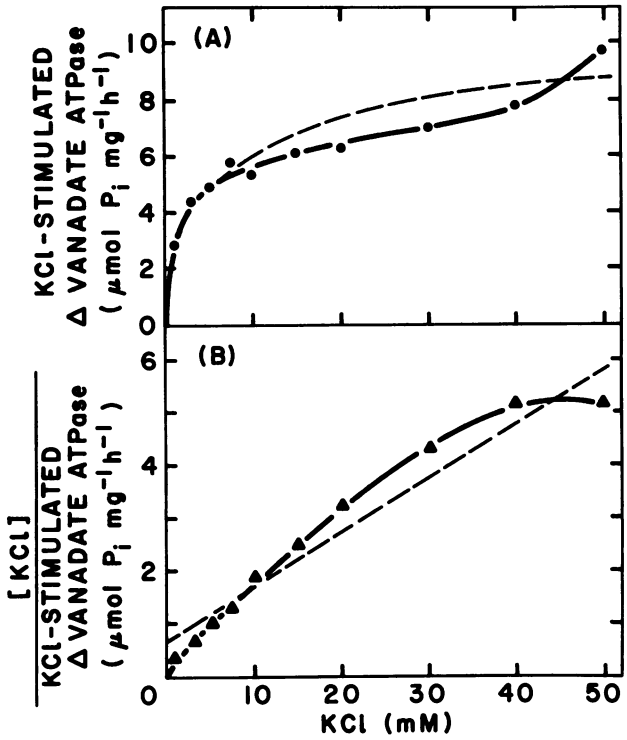

FIG. 13. A, Data from Figure 11B of Δvanadate ATPase activity replotted after subtraction of the activity measured in the absence of added KCl and labeled as KCl-stimulated Δvanadate ATPase activity; B, Hanes-Woolf plot of same. Broken line was fit by regression analysis; solid line was fit by eye.

ATPase of animal cells (8), the Ca^{2+}-ATPase of the sarcoplasmic reticulum (23), and the H^+-ATPase of *Neurospora* plasma membrane (5). Thus, in looking at the vanadate-sensitive components, it should be kept in mind that different conditions may favor vanadate inhibition without actually stimulating the ATPase. For these reasons, we have presented our data on ATPase activity in the presence and absence of vanadate whenever possible.

Kinetics of NO_3^- Inhibition of H^+ Transport and ATPase Activity. Preliminary to a detailed characterization of NO_3^--sensitive ATPase activity, the kinetics of NO_3^- inhibition of ATPase activity in the 20%/30% (w/w) membrane fraction were determined and compared with the kinetics of NO_3^- inhibition of H^+ transport. Quenching of quinacrine fluorescence was used to measure H^+ transport. Transport was dependent on Mg:ATP as substrate, stimulated by Cl^-, inhibited by NO_3^-, insensitive to 100 μM vanadate, and abolished by 2 μM gramicidin. As shown in Figure 4, Mg:ATP-dependent fluorescence quenching is inhibited progressively by increasing concentrations of NO_3^-, with half-maximal inhibition at approximately 10 mM NO_3^-. It is apparent that NO_3^- inhibits both the initial rate and the extent of fluorescence quenching. When the initial rates of fluorescence quenching are plotted as a function of NO_3^- concentration (Fig. 5), it can readily be seen that H^+ transport is nearly abolished at 50 to 100 mM NO_3^-, indicating that nearly all the ATPase involved in H^+ translocation in this 20%/30% membrane fraction is inhibitable by high concentrations of NO_3^-.

When we compared the kinetics of NO_3^- inhibition of ATPase activity in the 20%/30% (w/w) fraction with the kinetics of NO_3^- inhibition of H^+ transport (Fig. 5), it could be seen that only 60% of the ATPase activity was inhibited up to 100 mM NO_3^-. These data collectively suggest that, although nearly all the ATPase involved in H^+ transport is inhibited by NO_3^-, only about 60% of the ATPase in the 20%/30% (w/w) fraction is of tonoplast origin, the remainder being plasma membrane and/or nonspecific phosphatase contaminants. For experimental purposes of characterizing NO_3^- sensitivity, we chose a concentration of 50 mM NO_3^- for determining NO_3^- inhibition of ATPase activity (see "Materials and Methods"). This concentration should be adequate for nearly complete inhibition of the anion-sensitive ATPase of the 20%/30% (w/w) fraction.

Table IV. *Effect of Inhibitors on NO_3^-- and Vanadate-Sensitive ATPase*

Inhibitor	Assay Concn.	NO_3^--Sensitive ATPase Activity[a] of 20%/30% Interface		Vanadate-Sensitive ATPase[a] Activity of 30%/38% Interface	
		μmol Pi mg^{-1} h^{-1}	% Control	μmol Pi mh^{-1} h^{-1}	% Control
Control		9.4	100	18.8	100
DCCD	50 μM	0.4	4.0	6.6	35.1
DES	50 μM	5.5	58.3	10.6	56.1
Oligomycin	5 μg/ml	9.3	98.4	19.6	104.2
Gramicidin	2 μM	16.0	170.2	20.0	106.3
FC	5 μM	8.7	92.0	19.5	103.7
EDAC	1 mM	8.0	58.1	17.4	92.6
Mersalyl	50 μM	5.5	84.3	18.4	97.6
NaN_3	1 mM	10.2	107.6	21.8	116.0
Na_2MoO_4	0.5 mM	10.0	106.4	17.3	91.7
Vanadate	50 μM	9.7	102.4		
KNO_3	50 mM			21.6	115.0

[a] Total NO_3^-- or vanadate-sensitive ATPase activity was determined in the presence of 5 mM ATP, 5 mM $MgSO_4$, 30 mM Tris/Mes, 50 to 100 mM K^+, 50 mM Cl^- at pH 6.5 and 38°C and ±50 mM NO_3^- (20%/30%) or 20 μM vanadate (30%/38%).

General Characteristics of ΔNO_3^- and ΔVanadate ATPase. The characteristics of the NO_3^--sensitive ATPase of the 20%/30% (w/w) fraction were compared throughout with those of the vanadate-sensitive ATPase of the 30%/38% (w/w) fraction. Figure 6 shows the effect of pH on total ATPase in the absence and presence of NO_3^- or vanadate with the ΔNO_3^- and Δvanadate activities considered separately. Both enzymes exhibit a pH optimum of 6.5 at 38°C. The profile of the vanadate-sensitive enzyme is sharper than that of the NO_3^--sensitive enzyme with maximum activity occurring only within a narrow range. The profile of the NO_3^--sensitive enzyme is broad and resembles that of the gramicidin-stimulated enzyme previously reported by DuPont *et al.* (14). The same broad pH profile of ΔNO_3^- activity was seen at 25°C (not shown). Given these results, we chose to assay the ATPase activities of both enzymes at pH 6.5.

The temperature profiles of the two ATPase activities were likewise similar (Fig. 7). Both ΔNO_3^- and Δvanadate ATPase activities increased to a maximum at 45°C, abruptly declining by 50°C. The specific activity of the ΔNO_3^- ATPase was similar to that of the Δvanadate ATPase up to 25°C, above which temperature differences in specific activity became apparent with ΔNO_3^- activity much lower. An assay temperature of 38°C was chosen for both enzymes for subsequent experiments since at this temperature activities were high, and measurements at 38°C are comparable with previous characterization of the plasma membrane ATPase.

Mg:ATP Kinetics. We next examined the Mg:ATP kinetics of the 20%/30% (w/w) and 30%/38% (w/w) enzymes at pH 6.5 and 38°C. Figure 8 shows total ATPase activities in the absence and presence of 50 mM NO_3^- (Fig. 8A) and ΔNO_3^- activity alone (Fig. 8B) for the 20%/30% fraction. Total activity is depressed by NO_3^- over the entire range of Mg:ATP concentrations, with maximal inhibition above 3.0 mM Mg:ATP. When examined separately, ΔNO_3^- activity saturates at 2.5 to 3.0 mM Mg:ATP. A Hanes-Woolf plot of the NO_3^--sensitive activity (Fig. 10A) is linear and indicates a K_m of 0.10 mM Mg:ATP.

The Mg:ATP kinetics of the 30%/38% interface are presented in Figure 9. Vanadate-sensitive ATPase activity saturates at about 5 mM Mg:ATP (Fig. 9B). A Hanes-Woolf plot (Fig. 10B) of the data in Figure 9B is nonlinear in a manner indicating positive cooperativity of Mg:ATP binding. Similar Mg:ATP kinetics have been reported for the purified plasma membrane ATPase of *Neurospora* (7). However, previous studies of corn root plasma membrane ATPase have indicated simple Michaelis-Menten kinetics with respect to Mg:ATP (13, 19). More sensitive methods are needed to confirm the sigmoid dependence of the plasma membrane ATPase for Mg:ATP. The Mg:ATP concentration giving half-maximal activity is about 1 mM, indicating that the plasma membrane ATPase reaches saturation at higher concentrations of Mg:ATP than the tonoplast ATPase.

Divalent Cation Requirements. Divalent cation requirements were also examined (Table I). Total ATPase activity of the 20%/30% fraction was negligible in the absence of added divalent cations and highest in the presence of 5 mM $MgSO_4$, exhibiting a 17-fold increase in total activity. Ca^{2+} and Zn^{2+} were considerably less effective. When NO_3^--sensitive activity was considered separately, differences became apparent that had been masked by total activities. Divalent cation stimulation showed an order of preference of $Mn^{2+} > Mg^{2+} > Co^{2+} > Ca^{2+} > Zn^{2+}$.

As with the 20%/30% membrane fraction, total ATPase activity of the 30%/38% fraction in the absence of vanadate (20 μM) was greatest in the presence of $MgSO_4$. Negligible activity was seen in the absence of added divalent cations. Striking differences though in the pattern of divalent cation stimulation for the plasma membrane ATPase became apparent when the vanadate-sensitive activity was considered separately. Divalent cation stimulation showed preferential stimulation by $Co^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+} > Ca^{2+}$. This series is quite different from that of the NO_3^--sensitive enzyme, whereby Mn^{2+} is the preferred divalent cation and not Co^{2+}, as seen here for the vanadate-sensitive enzyme.

Monovalent Salt Stimulation. With respect to monovalent ion requirements, the two ATPase activities showed even greater differences with respect to salt specificity. Table II shows the effects of various monovalent cations on total and NO_3^-- or vanadate-sensitive ATPase activity. The ΔNO_3^- activity, believed to represent only anion-sensitive tonoplast ATPase activity, showed the highest stimulation by NH_4Cl (500% stimulation). The chloride salts NH_4Cl, CsCl, LiCl, NaCl, and RbCl all supported activity that was 90% or greater of the KCl-stimulated activity. Slightly lower activity (78%) was seen with choline chloride as the added salt. These data suggest that the Cl^- anion, present in each treatment, was the stimulatory species. Bennett and Spanswick (3) have reported previously that the Cl^- anion, acting as a permeant anion, reduces the membrane potential and allows development of a greater ΔpH across the membrane. Chloride is also believed to activate the ATPase directly.

Activity of the vanadate-sensitive component showed a different pattern of stimulation (Table II). Greatest Δvanadate activity was seen in the presence of KCl; however, unlike the ΔNO_3^- pattern, a specificity for cations was evident with KCl > NH_4Cl > RbCl > CsCl > LiCl > NaCl > choline chloride.

The effect of monovalent anions on ATPase activity of both enzymes (Table III) further supports the monovalent cation data analysis. Nitrate-sensitive ATPase activity of the 20%/30% fraction was highest in the presence of Cl^- as added anion. Anion stimulation showed an order of preference of $Cl^- > Br^- > C_2H_3O_2^- > SO_4^{2-} > I^- > HCO_3^- > SCN^-$, with no effect of K^+ on ΔNO_3^- activity. Both HCO_3^- and $C_2H_3O_2^-$ (bicarbonate and acetate, respectively), although both stimulatory anions for total ATPase activity, were less effective in stimulating ΔNO_3^- ATPase activity relative to the KCl-stimulated level. DuPont *et al.* (14) report no effect of acetate and bicarbonate on ATPase in the absence or presence of gramicidin although NO_3^- did inhibit. The effects of acetate and bicarbonate reported here agree with those of Walker and Leigh (33) for red beet tonoplast ATPase but not with those of DuPont *et al.* (14) for corn root microsomal ATPase, probably because the latter used $MgCl_2$ in place of the $MgSO_4$ used in the current investigation. Also of interest was the effect of the SCN^- anion, which had the most inhibitory effect on total and ΔNO_3^- ATPase activities. It should be noted that the effects of SCN^- here are at a 500-fold greater concentration than that used by Sze and others (31, 32) to monitor membrane potential and therefore should not be compared directly. At very low concentrations of SCN^- (100 μM), SCN^- is probably not interfering with activity of the ATPase and so can be useful as an indicator of electrogenic activity.

Vanadate-sensitive activity was relatively unaffected by the anion present, except in the case of SCN^-, which appeared to have an inhibitory effect on the ATPase. It is apparent that the Δvanadate ATPase is not inhibited by NO_3^- and I^-, unlike the anion-sensitive ATPase of the 20%/30% fraction. In the case of KBr, $KHCO_3$, and $KC_2H_3O_2$, there appears to be an enhancement of vanadate sensitivity. This is seen to a lesser extent with K_2SO_4 and KNO_3.

The kinetics of KCl stimulation of both NO_3^-- and vanadate-sensitive ATPase activities are shown in Figure 11. The data are replotted in the upper panels of Figures 12A and 13A after subtracting the basal activity measured in the absence of KCl. Hanes-Woolf plots are shown in the lower panels (Figs. 12B and 13B). The vanadate-sensitive ATPase activity deviates from linearity in the Hanes-Woolf plot in a manner suggesting negative cooperativity. This agrees with the complex kinetics previously described for K^+ stimulation of the plasma membrane ATPase of oat roots (19). The NO_3^--sensitive ATPase fits more closely a

Michaelis-Menten relationship, although the data do deviate slightly from linearity in the Hanes-Woolf plot. The K_m for KCl stimulation of NO_3^--sensitive ATPase activity (5.1 mM KCl) is similar to that previously reported for a H^+-translocating ATPase from corn roots (3).

Inhibitors. The effect of inhibitors on NO_3^-- and vanadate-sensitive ATPase is presented in Table IV. Both enzymes were strongly inhibited by 50 μM DCCD, generally regarded as a nonspecific inhibitor of membrane-bound ATPases (6, 28), with inhibition greater for ΔNO_3^- activity. DES, another inhibitor of membrane-bound ATPases, inhibited both ATPases by about 40% when present at a final concentration of 50 μM. Oligomycin, an inhibitor of the coupled F_1/F_0-ATPase complex of mitochondria, had no effect on either enzyme at a final concentration of 5 μg/ml. Gramicidin (final concentration of 2 μM) stimulated ΔNO_3^- ATPase approximately 70% but had little or no effect on Δvanadate ATPase. FC at a final concentration of 5 μM had little effect on the ATPase activity of either enzyme although slight variations are apparent in the data. EDAC at 1 mM final concentration appeared to inhibit ΔNO_3^- activity by about 42% with only slight inhibition of Δvanadate activity, suggesting selective inhibition of the tonoplast enzyme by this inhibitor. Walker and Leigh (33) have reported the opposite result, with EDAC at 1 mM only slightly inhibiting the tonoplast ATPase of beets. Mersalyl had the same effect but to a lesser extent. NaN_3 (1 mM final), Na_2MoO_4 (0.5 mM final), and vanadate (50 μM final) had no inhibitory effect on NO_3^--sensitive ATPase, and NaN_3 (1 mM), Na_2MoO_4 (0.5 mM), and KNO_3 (50 mM) had little if any inhibitory effect on vanadate-sensitive ATPase activity. The slight stimulation of both enzyme activities in the presence of NaN_3 may be the result of an uncoupling effect of N_3^-. The slight stimulation of Δvanadate ATPase activity in the presence of KNO_3 is attributed to the increase in salt concentration from 50 to 100 mM K^+.

The only major differences in inhibitor responses presented in Table IV are the stimulatory effect of gramicidin and the inhibitory effect of EDAC on ΔNO_3^- ATPase activity. Nonetheless, no inhibitor tested in our experiments was as effective an inhibitor of the anion-stimulated ATPase of the 20%/30% fraction as NO_3^-. Likewise no inhibitor of the cation-stimulated ATPase of the 30%/38% fraction was as effective as vanadate even at the relatively low concentration of vanadate (20 μM final) used throughout this investigation.

DISCUSSION

In this paper, we have presented evidence that the mitochondrial and tonoplast, but not the plasma membrane, ATPases are inhibited by NO_3^-. We have also shown that the tonoplast and mitochondrial ATPases can be distinguished by their differential sensitivity to N_3^- so that when assayed in the presence of N_3^-, NO_3^--sensitive ATPase activity exclusively reflects the activity of the tonoplast ATPase. The lack of sensitivity of the plasma membrane ATPase to NO_3^- confirms the results of Perlin and Spanswick (24) but not of Leonard and Hodges (19) who report moderate inhibition of the plasma membrane ATPase of oat roots by NO_3^-. Given the broad distribution of the NO_3^--sensitive tonoplast ATPase on sucrose gradients, this latter finding may have resulted from tonoplast contamination of the oat root plasma membrane fraction.

In comparing the vanadate-sensitive and NO_3^--sensitive ATPase activities, many characteristics which distinguish these two ATPases are apparent. Both have a pH optimum of 6.5 but the shape of the pH profile is quite different for the two ATPases so that at pH 8.0 only the activity associated with the NO_3^--sensitive ATPase is apparent. Both enzymes are stimulated by KCl. However, on closer examination, the vanadate-sensitive ATPase is responding to K^+ while the NO_3^--sensitive enzyme is responding to Cl^-. A distinguishing feature of physiological importance is the lower requirement for Mg:ATP by the NO_3^--sensitive ATPase than by the vanadate-sensitive ATPase. This could be of physiological significance under conditions of reduced cellular ATP and would imply the continued operation of the tonoplast, but not the plasma membrane, ATPase. Furthermore, differences in the shape of the kinetic curves for Mg:ATP reflect major differences in the reaction mechanisms of these two enzymes. Other differences include the ATPases' preference for divalent cations and slight differences in the sensitivity of the ATPases to the water-soluble carbodiimide EDAC and the water-soluble mercurial mersalyl.

The apparent specificity of NO_3^- for the inhibition of the tonoplast ATPase under the conditions described here suggested that NO_3^- inhibition may be useful as a quantitative marker for these membranes in mixed membrane fractions or as a criterion for identifying the anion-sensitive ATPase during purification. We feel that NO_3^- sensitivity of the ATPase is a better criterion than either Cl^- stimulation ATPase or H^+ transport since it is less ambiguous and is also useful in identifying the enzyme following solubilization. The optimal conditions for assaying NO_3^--sensitive ATPase activity associated with tonoplast membranes from corn roots are at pH 6.5 and 38°C in the presence of 2 to 5 mM Mg:ATP, 25 mM KCl, 1 mM NaN_3, and either 25 mM K_2SO_4 or 50 mM KNO_3. Higher pH optima have been reported for other tonoplast preparations from storage tissues (26, 33) so that assays at higher pH may be appropriate for assaying tonoplast ATPase from these other tissues.

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