

# Mechanism of Stimulation and Inhibition of Tonoplast H<sup>+</sup>-ATPase of *Beta vulgaris* by Chloride and Nitrate<sup>1</sup>

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## ABSTRACT

The H<sup>+</sup>-ATPase of tonoplast vesicles isolated from red beet (*Beta vulgaris* L.) storage tissue was studied with respect to the kinetic effects of Cl<sup>-</sup> and NO<sub>3</sub><sup>-</sup>. *N*-Ethylmaleimide (NEM) was employed as a probe to investigate substrate binding and gross conformational changes of the enzyme. Chloride decreased the *K<sub>m</sub>* of the enzyme for ATP but caused relatively little alteration of the *V<sub>max</sub>*. Nitrate increased *K<sub>m</sub>* only. Michaelis-Menten kinetics applied throughout with respect to ATP concentration. Nitrate yielded similar kinetics of inhibition in both the presence and absence of Cl<sup>-</sup>. Other monovalent anions that specifically increased the *K<sub>m</sub>* of the ATPase for ATP were, in order of increasing *K<sub>i</sub>*, SCN<sup>-</sup>, ClO<sub>4</sub><sup>-</sup>, and ClO<sub>3</sub><sup>-</sup>. Sulfate, although inhibitory, manifested noncompetitive kinetics with respect to ATP concentration. ADP, like NO<sub>3</sub><sup>-</sup>, was a competitive inhibitor of the ATPase but ADP and NO<sub>3</sub><sup>-</sup> did not interact cooperatively nor did either interfere with the inhibitory action of the other. It is concluded that NO<sub>3</sub><sup>-</sup> does not show competitive kinetics because of its stereochemical similarity to the terminal phosphoryl group of ATP. NEM was an irreversible inhibitor of the tonoplast ATPase. Both Mg·ADP and Mg·ATP protected the enzyme from inactivation by NEM but Mg·ADP was the more potent of the two. Chloride and NO<sub>3</sub><sup>-</sup> exerted little or no effect on the protective actions of Mg·ADP and Mg·ATP suggesting that neither Cl<sup>-</sup> nor NO<sub>3</sub><sup>-</sup> are involved in substrate binding.

Electrogenic H<sup>+</sup> translocation is thought to be the primary transport event across the plasma and vacuolar membranes of plant cells (18). A pH gradient (inside-acid *versus* cytoplasm) and sometimes an electrical potential difference (inside-positive *versus* cytoplasm) can be measured across the vacuolar membrane (tonoplast) of intact cells (10). Together with the demonstration of ATPase activity and ATP-dependent electrogenic H<sup>+</sup>-translocation into isolated vacuoles, these findings have established the existence of a H<sup>+</sup>-translocating ATPase in tonoplast (19).

With the development of techniques for the preparation of sealed membrane vesicles from various plant tissues, several laboratories have demonstrated an electrogenic H<sup>+</sup>-ATPase associated with low density microsomes (15). The low density microsomes are identified as tonoplast according to three primary criteria: (a) vesicles prepared from intact isolated vacuoles equilibrate at the same density (about 1.10 g/ml) upon density centrifugation and show similar enzyme (20) and H<sup>+</sup>-transloca-

tion activities (1); (b) only low activities of marker enzymes for other identifiable membranes also equilibrate at the same density (14); and (c) the ATPase and PPase activities of the low density fraction are quantitatively and qualitatively similar to those of intact vacuoles (16, 20).

The H<sup>+</sup>-ATPase of tonoplast is characterized as such on the basis of its stimulation by Cl<sup>-</sup> and its inhibition by NO<sub>3</sub><sup>-</sup> (19). However, despite the value of its anion-sensitivity as a criterion for distinguishing it from other cellular H<sup>+</sup>-ATPases, very little is known of the mechanisms of action of Cl<sup>-</sup> and NO<sub>3</sub><sup>-</sup> on this important transport enzyme. In this communication we describe experiments directed at gaining a better understanding of the mechanisms of stimulation and inhibition of the tonoplast H<sup>+</sup>-ATPase of red beet (*Beta vulgaris* L.) storage tissue by Cl<sup>-</sup> and NO<sub>3</sub><sup>-</sup>, respectively.

## MATERIALS AND METHODS

**Plant Material.** Fresh red beet (*Beta vulgaris* L.) with leaves attached were purchased commercially, stored at 4°C and used within 1 week of purchase.

**Isolation of Tonoplast Vesicles.** Vesicles identified as tonoplast in origin were isolated as described previously (16).

**ATPase Assays.** ATPase activity was measured colorimetrically as the rate of liberation of Pi from ATP by the method of Ames (16), radiometrically as the rate of liberation of <sup>32</sup>Pi from [γ-<sup>32</sup>P]ATP or spectrophotometrically by coupling ATP hydrolysis to NADH oxidation (16). The Ames method was employed for the routine determination of ATPase activity at a fixed concentration of Tris-ATP (3 mM). Radiometric determinations were performed when ADP was to be included in the assay media. And, the coupled method was used for the routine investigations of the ATP concentration-dependence of the ATPase in the absence of ADP.

The liberation of <sup>32</sup>Pi was measured in a 200 μl incubation volume containing 50 mM KCl, 2.4 mM MgSO<sub>4</sub>, 30 mM Tris-Mes buffer (pH 8.0) and the indicated concentrations of Tris-[γ-<sup>32</sup>P]ATP (4 mCi/mmol). The reaction was initiated by the addition of tonoplast (5–10 μg membrane protein) and allowed to proceed for 5 min at 37°C, after which time 775 μl of ice-cold 5% (w/v) TCA containing 15% (w/v) Norit A activated charcoal (250–350 mesh) were added. The samples were mixed thoroughly, left on ice for 1 h, and centrifuged in an Eppendorf microfuge for 3 min. Five hundred μl aliquots of the supernatants were added to 5 ml Aquasol liquid scintillation cocktail (New England Nuclear, Lachine, Quebec) and <sup>32</sup>Pi was determined by liquid scintillation counting.

The amount of <sup>32</sup>Pi liberated from [γ-<sup>32</sup>P]ATP increased linearly with time and with increase in membrane protein concentration.

The coupled assays were performed in a reaction volume of 1.0 ml containing 50 mM KCl or K-Mes, as indicated, 30 mM

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Tris-Mes (pH 8.0), 3 mM MgSO<sub>4</sub>, 3 mM PEP,<sup>2</sup> 0.184 mM NADH, 2 units pyruvate kinase, and 1 unit lactate dehydrogenase (16). The reaction was initiated by the addition of the desired concentration of Tris-ATP. Whenever KCl was omitted from the incubation medium, it was replaced by an equivalent concentration of K-Mes to ensure that the overall reaction was not rate-limited by pyruvate kinase which requires K<sup>+</sup> as cofactor.

Five μM gramicidin D was included in all of the assay media to ensure that the H<sup>+</sup>-ATPase was not rate-limited by ΔpH and/or Δψ.

**NEM Protection Experiments.** The mechanisms of action of Cl<sup>-</sup>, NO<sub>3</sub><sup>-</sup> and ADP on the tonoplast ATPase were examined by determining their capacity to alter the susceptibility of the enzyme to irreversible inhibition by the sulfhydryl reagent NEM. Since Mg·ATP and Mg·ADP protect the enzyme from inactivation by NEM (see "Results"), it was assumed that if Cl<sup>-</sup> and NO<sub>3</sub><sup>-</sup> effect substrate binding they should have an effect on the protective actions of Mg·ATP and Mg·ADP.

The DTT in the tonoplast suspension was removed by dilution of 250 μg membrane protein in 25 ml of 30 mM Tris-Mes (pH 8.0) containing 10% (w/w) glycerol ('glycerol medium'). The membranes were pelleted at 100,000g, resuspended in another 25 ml glycerol medium, pelleted again and resuspended in glycerol medium. Twenty-five μg membrane protein were added to 100 μl volumes of the NEM inhibition solutions (50 mM KCl, 1.5 mM MgSO<sub>4</sub>, 30 mM Tris-Mes, pH 8.0, containing 1–25 μM NEM, unless otherwise indicated). After incubation for 5 min on ice, the reaction was terminated by the addition of 75 μl of 750 μM DTT. The samples were then centrifuged through 1 ml Sephadex columns equilibrated with 50 mM KCl, 1.5 mM MgSO<sub>4</sub>, and 30 mM Tris-Mes buffer (pH 8.0) at 112g for 2 min. Two hundred μl volumes of the Sephadex-treated tonoplast suspensions were assayed for ATPase activity by the Ames method.

**Protein.** Protein was estimated as described previously (4).

**Chemicals.** General laboratory reagents were obtained from BDH Chemicals, Canada, Ltd., Boehringer-Mannheim, Canada, and Sigma Chemical Co. [ $\gamma$ -<sup>32</sup>P]ATP (3–4 Ci/mmol) was a kind gift from Dr. Rhoda Blostein, Montreal General Hospital.

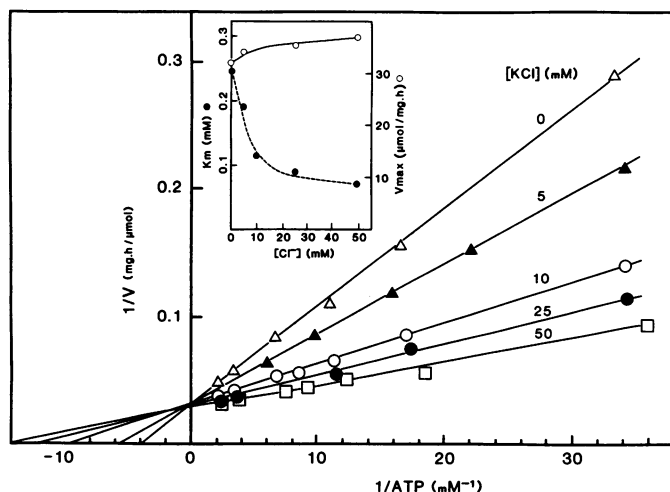


FIG. 1. Influence of Cl<sup>-</sup> on ATP concentration-dependence of tonoplast ATPase. Main figure: plot of 1/v against 1/ATP at Cl<sup>-</sup> concentrations of 0, 5, 10, 25, and 50 mM, respectively. Inset: effect of Cl<sup>-</sup> on apparent K<sub>m</sub> and V<sub>max</sub>. ATPase activity was measured by coupling ATP hydrolysis to NADH oxidation as described in "Materials and Methods."

<sup>2</sup> Abbreviations: PEP, phosphoenolpyruvate; NEM, *N*-ethylmaleimide; ΔpH, pH difference (outside-inside, where pH = -log [H<sup>+</sup>]); pmf, proton motive force; Δψ, electrical potential difference (inside-outside).

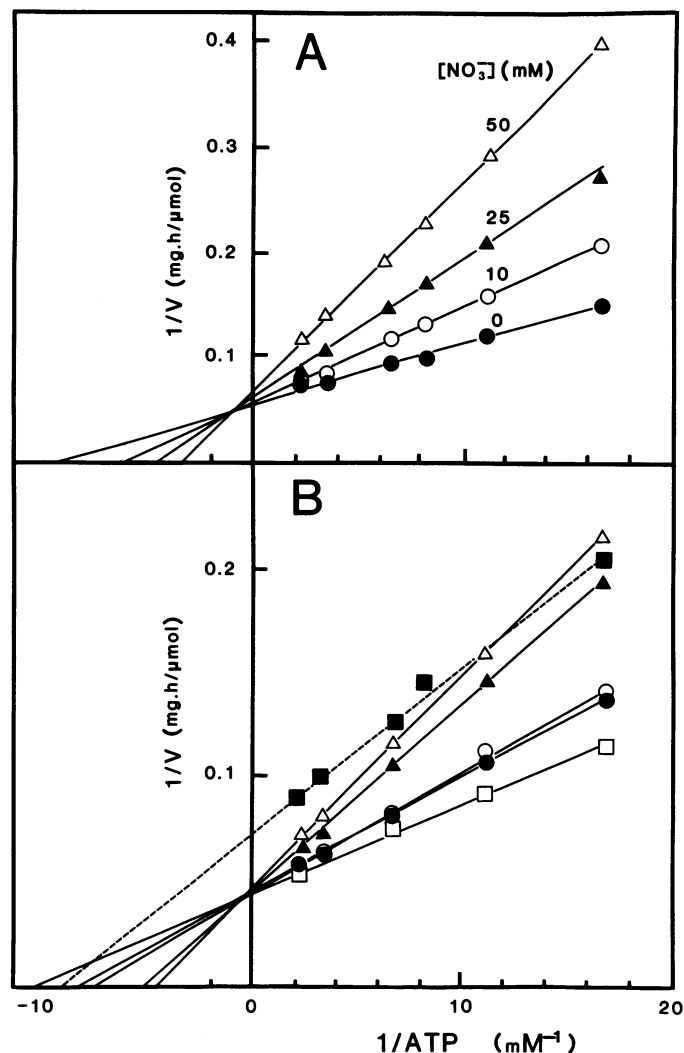


FIG. 2. Influence of NO<sub>3</sub><sup>-</sup> (A) and other anions (B) on ATP concentration-dependence of tonoplast ATPase in presence of 50 mM KCl. Nitrate was present at the concentrations indicated (A). Thiocyanate (Δ), ClO<sub>4</sub><sup>-</sup> (▲), ClO<sub>3</sub><sup>-</sup> (○) and CH<sub>3</sub>COO<sup>-</sup> (●) were present at 50 mM, while SO<sub>4</sub><sup>2-</sup> (■) was present at 25 mM; control activity (□) (B). All of the anions were added as their K<sup>+</sup>-salts. Conditions otherwise identical to Figure 1.

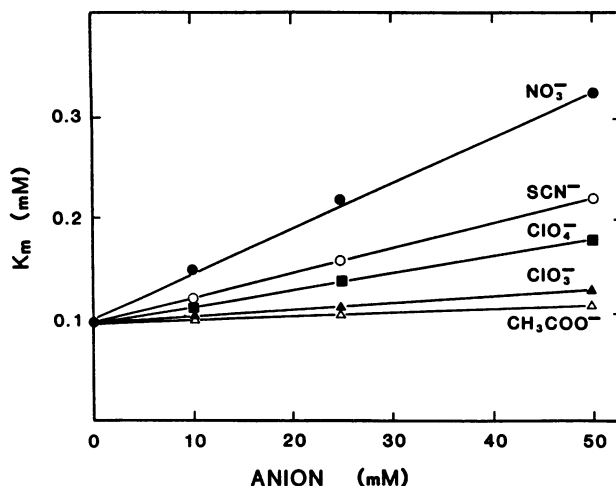


FIG. 3. Secondary plot of K<sub>m</sub>(ATP) against anion concentration for anions examined in Figure 2. Nitrate, SCN<sup>-</sup>, ClO<sub>4</sub><sup>-</sup> and ClO<sub>3</sub><sup>-</sup> yield K<sub>i</sub> values of 21.9, 38.3, 54.7, and 120 mM, respectively.

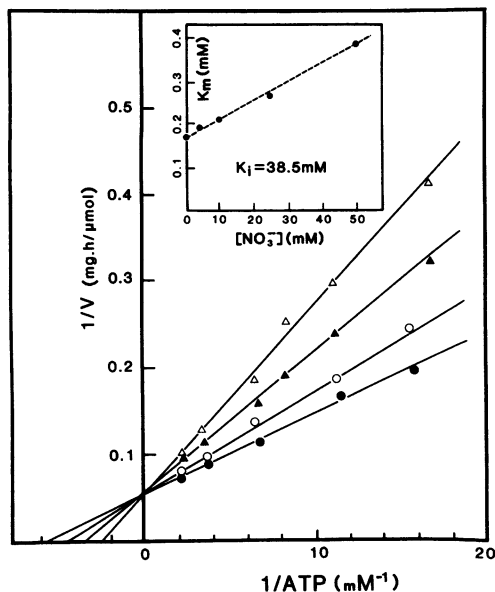


FIG. 4. Influence of  $\text{NO}_3^-$  on ATP concentration-dependence of tonoplast ATPase in the absence of  $\text{Cl}^-$ . Nitrate ( $\text{KNO}_3$ ) was employed at concentrations of 0 ( $\bullet$ ), 5 (primary data not shown), 10 ( $\circ$ ), 25 ( $\blacktriangle$ ), and 50 mM ( $\triangle$ ), respectively. Inset: secondary plot of  $K_m$  values derived from double-reciprocal plot.

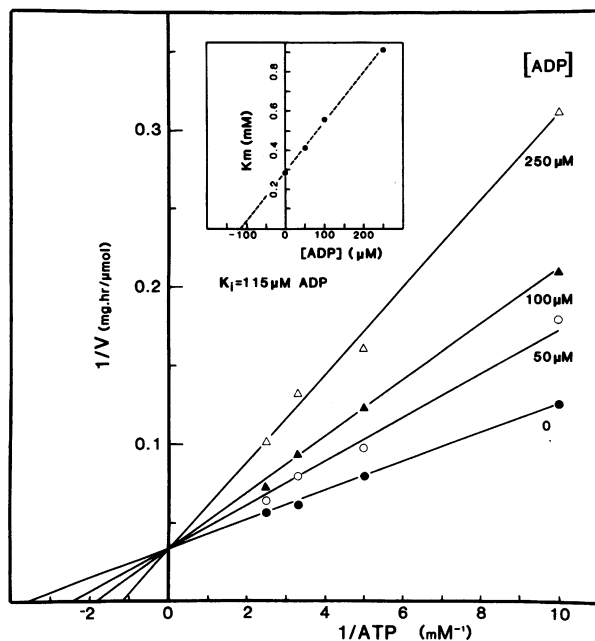


FIG. 5. ATP concentration-dependence of tonoplast ATPase in the presence of 0, 50, 100, and 250  $\mu\text{M}$  ADP. Inset: secondary plot of  $K_m$  values derived from double-reciprocal plot. ATPase activity was measured by monitoring the release of  $^{32}\text{P}$ i from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  as described in "Materials and Methods."

## RESULTS

**Kinetics of Activation by Chloride and Inhibition by  $\text{NO}_3^-$ .** The activity of the tonoplast ATPase approximated Michaelis-Menten kinetics with respect to ATP concentration in both the presence and absence of  $\text{Cl}^-$  (Fig. 1). Providing that uncoupler (5  $\mu\text{M}$  gramicidin D) was included in the assay media, the predominant effect of  $\text{Cl}^-$  on catalysis was to decrease the  $K_m$  of the enzyme for ATP (Fig. 1 and inset).

Nitrate increased the  $K_m$  of the enzyme for ATP (Fig. 2A).

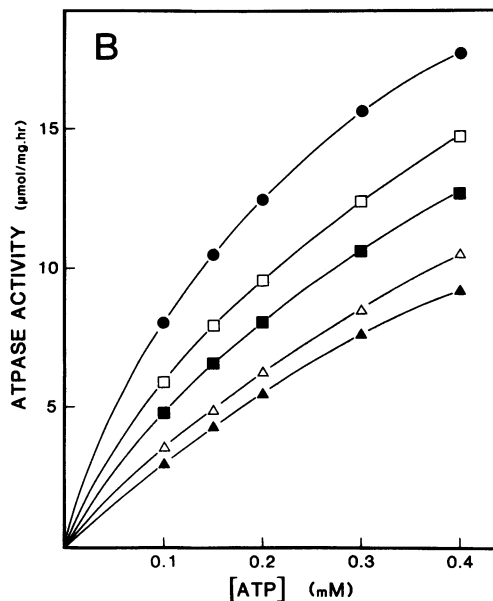
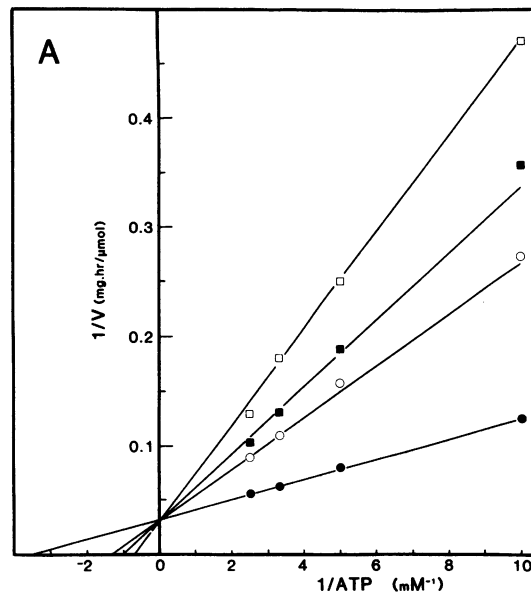


FIG. 6. ATP concentration-dependence of tonoplast ATPase in the presence of different combinations of ADP and  $\text{NO}_3^-$ . A, Double-reciprocal plots of the activities obtained in the absence of ADP and  $\text{NO}_3^-$  ( $\bullet$ ), in the presence of 50  $\mu\text{M}$  ADP + 25 mM  $\text{NO}_3^-$  ( $\circ$ ), 100  $\mu\text{M}$  ADP + 10 mM  $\text{NO}_3^-$  ( $\blacksquare$ ), and 250  $\mu\text{M}$  ADP + 25 mM  $\text{NO}_3^-$  ( $\square$ ). The data shown correspond to those in Table I. B, Arithmetic plot of ATPase activity against ATP concentration. Activities in the absence of ADP and  $\text{NO}_3^-$  ( $\bullet$ ); activities in the presence of 10 mM  $\text{NO}_3^-$  ( $\square$ ), 100  $\mu\text{M}$  ADP ( $\blacksquare$ ), and 100  $\mu\text{M}$  ADP + 10 mM  $\text{NO}_3^-$  ( $\blacktriangle$ ), respectively. The theoretical relationship between activity and ATP concentration ( $\triangle$ ) was calculated by assuming that the inhibitions exerted by 100  $\mu\text{M}$  ADP and 10 mM  $\text{NO}_3^-$ , individually, interact in a simple additive manner (see Table I for method of calculation). Fifty mM KCl were present throughout and ATPase activity was measured by monitoring the release of  $^{32}\text{P}$ i from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ .

Michaelis-Menten kinetics with respect to ATP concentration were approximated at all of the  $\text{NO}_3^-$  concentrations employed and a secondary plot of the results yielded a straight-line relationship with a  $K_i$  value of 21.9 mM (Fig. 3). Chlorate, a putative analog of  $\text{NO}_3^-$  (6) was markedly less effective as an inhibitor but yielded competitive kinetics ( $K_i$  about 120 mM; Figs. 2B and

Table I. Influence of Different Combinations of ADP and NO<sub>3</sub><sup>-</sup> on Activity of Tonoplast ATPase

ATP, Mg<sup>2+</sup> and KCl were present at concentrations of 0.15, 1.5, and 50 mM, respectively. The theoretical activities for different combinations of ADP and NO<sub>3</sub><sup>-</sup> were calculated by assuming that the inhibitions exerted by ADP and NO<sub>3</sub><sup>-</sup> in combination were equivalent to the sum of their inhibitions in isolation: theoretical activity,  $\mu\text{mol}/\text{mg}\cdot\text{h} = (\% \text{ control activity}_{(\text{ADP})}) \cdot (\% \text{ control activity}_{(\text{NO}_3^-)}) \times \text{control activity}, \mu\text{mol}/\text{mg}\cdot\text{h}$ .

Effector	Concentration <i>mm</i>	ATPase Activity $\mu\text{mol}/\text{mg protein}\cdot\text{h}$	Percent of Control
None (control)		10.53	100.0
Nitrate	10	7.92	75.2
	25	6.61	62.7
	50	5.56	52.8
ADP	0.05	8.10	76.9
	0.1	6.53	62.0
	0.25	4.64	44.1
ADP + nitrate (experimental)	0.05:25	5.33	50.6
	0.1:10	4.29	40.7
	0.25:25	3.08	29.2
ADP + nitrate (theoretical)	0.05:25	5.08	48.2
	0.1:10	4.91	46.6
	0.25:25	2.91	27.7

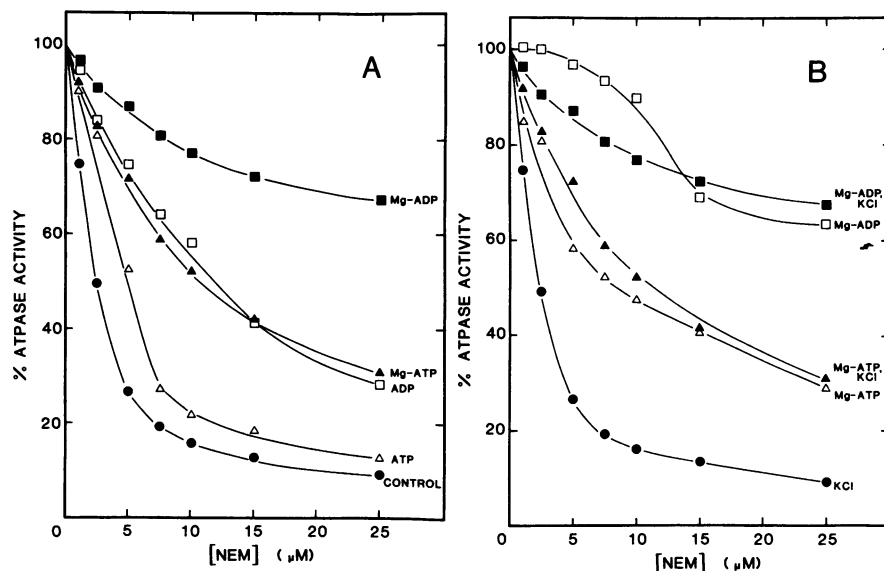


FIG. 7. Influence of Mg-ATP and Mg-ADP in the presence or absence of Cl<sup>-</sup> on inhibition of the tonoplast ATPase by NEM. A, Incubation with NEM in the presence of 50 mM KCl and 1.5 mM Mg<sup>2+</sup> (●); 50 mM KCl and 0.5 mM ATP (Δ); 50 mM KCl, 0.5 mM ATP, and 1.5 mM Mg<sup>2+</sup> (▲); and 50 mM KCl, 0.5 mM ADP, and 1.5 mM Mg<sup>2+</sup> (■), respectively. B, Incubation with NEM in the presence of 50 mM KCl (●); 0.5 mM ATP and 1.5 mM Mg<sup>2+</sup> (Δ); 50 mM KCl, 0.5 mM ATP, and 1.5 mM Mg<sup>2+</sup> (▲); 0.5 mM ADP and 1.5 mM Mg<sup>2+</sup> (□); and 50 mM KCl, 0.5 mM ADP, and 1.5 mM Mg<sup>2+</sup> (■), respectively. ATPase activity was measured by monitoring the release of Pi from ATP by the Ames method and the vesicles were treated as described in "Materials and Methods."

3). Other monovalent anions that exhibited similar kinetics of inhibition to those seen with NO<sub>3</sub><sup>-</sup> were SCN<sup>-</sup> and ClO<sub>4</sub><sup>-</sup> with *K<sub>i</sub>* values of 38.3 and 54.7 mM, respectively (Figs. 2B and 3). Acetate was without effect (Figs. 2B and 3) and SO<sub>4</sub><sup>-</sup>, although inhibitory, gave noncompetitive kinetics (Fig. 2B).

**Inhibition of Basal ATPase Activity.** The kinetics of inhibition observed above (Figs. 2 and 3) were obtained in the presence of 50 mM KCl. Similar kinetics of inhibition by NO<sub>3</sub><sup>-</sup> were also obtained in the absence of KCl (Fig. 4). The *K<sub>i</sub>* value obtained in the absence of Cl<sup>-</sup>-stimulation (38.5 mM; Fig. 4) was approximately 1.8-fold greater than that obtained in the presence of Cl<sup>-</sup> (21.9 mM above) suggesting that the Cl<sup>-</sup>-stimulated component of ATPase activity is more sensitive to inhibition by NO<sub>3</sub><sup>-</sup> than the basal component.

**Lack of Interaction between NO<sub>3</sub><sup>-</sup> and ADP.** ADP, like NO<sub>3</sub><sup>-</sup>, is a competitive inhibitor of the ATPase and yields a *K<sub>i</sub>* value of 0.12 mM (Fig. 5). However, ADP and NO<sub>3</sub><sup>-</sup> neither interact cooperatively nor negatively during inhibition. The competitive effects of ADP and NO<sub>3</sub><sup>-</sup> were additive (Fig. 6 and Table I). The inhibitions found when ADP and NO<sub>3</sub><sup>-</sup> were present together were similar to the theoretical sum of the inhibitions obtained

with each inhibitor alone, where theoretical activity,  $\mu\text{mol}/\text{mg}\cdot\text{h} = (\% \text{ control activity with ADP}) \cdot (\% \text{ control activity with NO}_3^-) \times \text{control activity}, \mu\text{mol}/\text{mg}\cdot\text{h}$ .

**Protection from Irreversible Inhibition by NEM.** NEM, a sulfhydryl reagent, inhibited the tonoplast ATPase with a *k*<sub>50</sub> of 2.5 μM but the inclusion of Mg-ADP or Mg-ATP in the NEM-inhibition solutions significantly decreased the degree of inhibition exerted (Fig. 7A). Mg-ADP was more effective than Mg-ATP as a protectant and ADP and ATP, alone, were less effective than their corresponding Mg<sup>2+</sup>-salts (Fig. 7A). The protective actions of Mg-ADP and Mg-ATP indicate the presence of essential sulfhydryl groups at the active site of the ATPase whereas the greater efficacies of Mg-ADP and Mg-ATP over ADP and ATP alone suggest that Mg<sup>2+</sup> is required for efficient nucleotide binding.

Neither Cl<sup>-</sup> nor NO<sub>3</sub><sup>-</sup> had a marked effect on the protective actions of Mg-ADP and Mg-ATP (Figs. 7B and 8A). The most marked anion effect observed was a decrease in the protective action of Mg-ADP at low NEM concentrations when Cl<sup>-</sup> was included in the incubation media (Fig. 7B). It was also found that Cl<sup>-</sup> and NO<sub>3</sub><sup>-</sup>, alone and together, enhanced the inhibitory

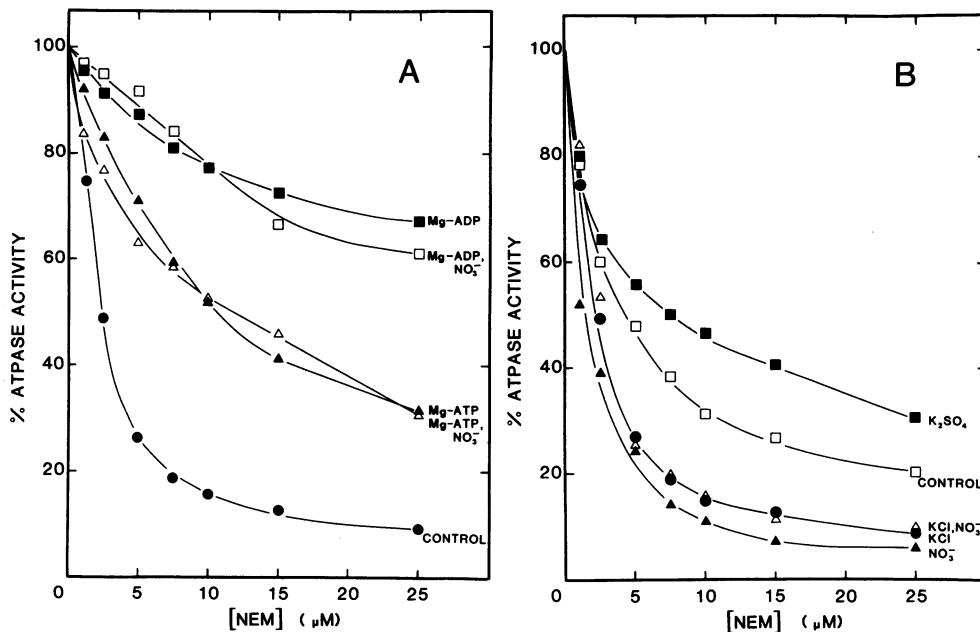


FIG. 8. A, Influence of Mg·ATP and Mg·ADP in the presence or absence of NO<sub>3</sub><sup>-</sup> on inhibition of tonoplast ATPase by NEM. Incubation with NEM in the presence of 50 mM KCl (●); 0.5 mM ATP, and 1.5 mM Mg<sup>2+</sup> (▲); 0.5 mM ATP, 1.5 mM Mg<sup>2+</sup> and 50 mM KNO<sub>3</sub> (Δ); 0.5 mM ADP and 1.5 mM Mg<sup>2+</sup> (■); and 0.5 mM ADP, 1.5 mM Mg<sup>2+</sup>, and 50 mM KNO<sub>3</sub> (□), respectively. B, Influence of anions alone on inhibition of tonoplast ATPase by NEM. Control represents incubation in the absence of anions. Where indicated, KCl and KNO<sub>3</sub> were employed at concentrations of 50 mM. Potassium sulfate was employed at a concentration of 25 mM. Conditions otherwise identical to Figure 7.

effect of NEM over that seen in their absence whereas SO<sub>4</sub><sup>2-</sup> had a protective action (Fig. 8B). Anions are apparently capable of increasing or decreasing the accessibility of essential sulfhydryl groups on the ATPase but do not appear to influence substrate binding.

## DISCUSSION

In the experiments described above the predominant effect of Cl<sup>-</sup> was to decrease the  $K_m$  of the tonoplast ATPase for ATP (Fig. 1). While several authors (3, 5, 7, 21) have found that Cl<sup>-</sup> primarily effects the  $V_{max}$  component of ATPase activity, to our knowledge, none have examined the effect of Cl<sup>-</sup> on the ATP concentration-dependence of catalysis in uncoupled tonoplast vesicles where complications due to  $\Delta pH$  and  $\Delta\psi$  formation are excluded. Permeant anions, such as Cl<sup>-</sup>, alleviate the development of an inside-positive  $\Delta\psi$  upon H<sup>+</sup> translocation and thereby increase the  $\Delta pH$  component of the pmf (3, 9). And, as shown by the stimulatory effects of ionophores, such as gramicidin D, nigericin or *m*-chlorocarbonyl cyanide phenylhydrazone in combination with K<sup>+</sup>-valinomycin, on ATPase activity (14), ATP-dependent H<sup>+</sup> translocation is subject to stalling by the pmf developed during the course of translocation. Thus, providing that the magnitude of the pmf is the determining factor (*i.e.* the vesicles are tightly coupled), the diminution of  $\Delta\psi$  by the influx of counter-anions will cause a corresponding increase in the rate of H<sup>+</sup> translocation. Consequently, it would be expected that in the coupled condition the principal effect of Cl<sup>-</sup> is to increase pump turnover, and thence  $V_{max}$ , even if Cl<sup>-</sup> decreases  $K_m$  in the uncoupled condition, as found here.

The mechanism of action of Cl<sup>-</sup> remains elusive. The NEM protection experiments indicate that Cl<sup>-</sup> is not required for Mg·ADP or Mg·ATP binding in so far as Cl<sup>-</sup> does not facilitate the protective action of either nucleotide (Fig. 7). It therefore appears that the influence of Cl<sup>-</sup> on the  $K_m$  of the ATPase for ATP is exerted at some point in the catalytic cycle after substrate binding. However, interpretation of the NEM protection experiments is complicated by the fact that Cl<sup>-</sup> or NO<sub>3</sub><sup>-</sup>, alone, increase the sensitivity of the enzyme to NEM over that seen in their absence, whereas SO<sub>4</sub><sup>2-</sup> has a protective action (Fig. 8A).

It has been known for some time that the H<sup>+</sup>-ATPase of tonoplast is inhibited by NO<sub>3</sub><sup>-</sup> (7, 20) but its mode of action is unknown. Although it has previously been shown that ADP is a competitive inhibitor of the tonoplast ATPase (Ref. 2 and Fig.

5), our demonstration of competitive kinetics for NO<sub>3</sub><sup>-</sup> has not been reported before (Figs. 2–4). However, NO<sub>3</sub><sup>-</sup> and ADP do not appear to have similar mechanisms of action. Mg·ADP protected the ATPase from inhibition by NEM but NO<sub>3</sub><sup>-</sup> exerted no protective action (Fig. 8). The protective actions of Mg·ADP and Mg·ATP are consistent with the presence of essential sulfhydryl groups at the active site of the ATPase which are rendered inaccessible to NEM by substrate or product. By the same token, the lack of protection by NO<sub>3</sub><sup>-</sup> is consistent with the interaction of this anion with a site distinct from the substrate binding site of the enzyme.

At the outset of this investigation we postulated that NO<sub>3</sub><sup>-</sup> exerts its effect in a competitive manner relative to ATP because of its trigonal planar geometry and therefore its structural similarity to the terminal phosphoryl group of ATP, by analogy with the mechanism proposed for the inhibition of creatine kinase by NO<sub>3</sub><sup>-</sup> (13). According to the scheme proposed by Milner-White and Watts (13) for creatine kinase, Mg·ADP and NO<sub>3</sub><sup>-</sup> can both occupy the active site to form a dead-end complex that stereochemically simulates the transition-state complex of Mg·ATP. In agreement with their scheme, NO<sub>3</sub><sup>-</sup> in combination with Mg·ADP potentiated the protection of creatine kinase from inhibition by the sulfhydryl reagent iodoacetamide. We, however, could not find any evidence for such a mechanism for the tonoplast ATPase. Nitrate and ADP did not cooperatively inhibit ATP hydrolysis (Table I and Fig. 6) and NO<sub>3</sub><sup>-</sup> did not potentiate the protective action of Mg·ADP against inhibition by NEM (Fig. 8).

All of the anions capable of increasing the  $K_m$  of the ATPase for ATP (SCN<sup>-</sup>, ClO<sub>4</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup>) are chaotropic whereas those which do not yield competitive kinetics (CH<sub>3</sub>COO<sup>-</sup> and SO<sub>4</sub><sup>2-</sup>) are antichaotropic (Figs. 2 and 3). Chaotropes decrease water structure and, in agreement with the concept that the apolar groups of macromolecules form hydrophobic bonds because of their thermodynamically unfavorable interaction with water, the relative water structure-breaking properties of chaotropic anions are correlated with their ability to destabilize membranes and enzyme complexes (8). Recent investigations demonstrate that the tonoplast ATPase is a multimeric complex comprising at least three different polypeptides (11, 12) and preliminary experiments show that this phosphohydrolase is markedly more sensitive to irreversible inhibition by 0.3 to 0.9 M concentrations of chaotropic anions than the tonoplast PPase (PA Rea, C. Bunyon,

CJ Griffith, RJ Poole, unpublished data). Since the tonoplast PPase is not subject to reversible inhibition by millimolar concentrations of NO<sub>3</sub><sup>-</sup> (16, 17), it is possible that reversible inhibition of the ATPase by comparatively low concentrations of chaotropic anions and irreversible inhibition by high concentrations are related.

The reversible inhibitions exerted by the anions tested fall in the sequence NO<sub>3</sub><sup>-</sup> > SCN<sup>-</sup> > ClO<sub>4</sub><sup>-</sup> ≫ CH<sub>3</sub>COO<sup>-</sup> (Figs. 2 and 3), whereas their chaotropic potencies fall in the sequence SCN<sup>-</sup> > ClO<sub>4</sub><sup>-</sup> > NO<sub>3</sub><sup>-</sup> ≫ CH<sub>3</sub>COO<sup>-</sup> (8). Nitrate is ostensibly an anomalously potent inhibitor if chaotropism is the principal cause of the inhibitions seen. There are, however, numerous examples of weak chaotropes, such as NO<sub>3</sub><sup>-</sup>, being more effective destabilizing agents than potent chaotropes (8). The chaotropic potency of anions is inversely related to their charge densities, whereas their capacity to destabilize electrostatic interactions is directly related to their charge density. Thus, if both hydrophobic and electrostatic interactions participate in the maintenance of enzyme functional integrity, the destabilizing effect of a weak chaotrope on both types of interaction might be greater, in balance, than that of a strong chaotrope. A chaotropic mechanism of this type could help explain the differential sensitivities of the ATPase and PPase to NO<sub>3</sub><sup>-</sup>; irreversible inhibition of the ATPase but not the PPase by high concentrations of chaotropic anions; the capacities of SCN<sup>-</sup> and ClO<sub>4</sub><sup>-</sup>, but not the NO<sub>3</sub><sup>-</sup>-analog ClO<sub>3</sub><sup>-</sup>, to simulate the effects of NO<sub>3</sub><sup>-</sup>; the inhibitory effects of chaotropic anions but not neutral chaotropes (*e.g.* urea; data not shown); the sensitivities of both the Cl<sup>-</sup>-stimulated and basal ATPase activities to NO<sub>3</sub><sup>-</sup>; and the two-fold greater susceptibility of the solubilized, partially purified ATPase to NO<sub>3</sub><sup>-</sup> (12). We are currently investigating this hypothesis systematically.

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