Plant Physiol. (1987) 83, 490–496
0032-0889/87/83/0490/07/$01.00/0

Decrease of pH Gradients in Tonoplast Vesicles by NO_3^- and Cl^-: Evidence for H^+-Coupled Anion Transport[1]

Received for publication March 13, 1986 and in revised form October 15, 1986

KAREN S. SCHUMAKER AND HEVEN SZE*
Botany Department, University of Maryland, College Park, Maryland 20742

ABSTRACT

Chloride or nitrate decreased a pH gradient (measured as [^{14}C]methylamine accumulation) in tonoplast-enriched vesicles. The ΔpH decrease was dependent on the anion concentration. These effects are independent of the anion-sensitive H^+-ATPase of the tonoplast, since the pH gradient (acid inside) was imposed artificially using a pH jump or a K^+ gradient and nigericin. 4,4′-Diisothiocyano-2,2′-stilbene disulfonic acid partially prevented the decrease in pH gradient induced by Cl^-. Two possible models to account for this anion-dependent decrease of ΔpH are: (a) H^+ loss is accompanied by Cl^- or NO_3^- efflux from the vesicles via H^+/anion symport systems on the tonoplast and (b) H^+ loss is accompanied by Cl^- or NO_3^- uptake into the vesicles via H^+/anion antiport systems. Depending on the requirements and conditions of the cell, these two systems would serve to either mobilize Cl^- and NO_3^- stored in the vacuole for use in the cytoplasm or to drive anions into the vacuole. Chloride or nitrate also decreased a pH gradient in fractions containing plasma membrane and Golgi, implying that these membranes may have similar H^+-coupled anion transport systems.

Inorganic anions, such as nitrate, phosphate, sulfate, and chloride, are essential for plant growth, yet we know very little about how these ions are transported into cells and compartmentalized. The membrane potential of the cytoplasm relative to the external medium is about −110 mV for most cells, thus anions are transported into cells against a steep electrochemical gradient (15, 19). Anions are thought to be accumulated in cells via H^+/anion symport systems by utilizing the proton motive force generated by H^+ extrusion pumps. Evidence supporting this model has been obtained in studies using intact cells or tissues. For example, phosphate uptake into duckweed, *Lemna gibba*, was accompanied by a transient membrane depolarization (27, 29). The depolarization was optimal at pH 5.7 to 6.0. The results suggested phosphate uptake is energized by the H^+ electrochemical gradient via a $2H^+/H_2PO_4^-$ cotransport mechanism. Similar conclusions have been reached for nitrate (28) and sulfate (12) transport in *L. gibba* and chloride transport in Chara (21). Less is known about anion transport across the tonoplast. For example, Cl^- influx into the vacuole may move down an electrochemical gradient, but in some cases, Cl^- uptake is presumed to have an active component (15, 19).

Using isolated vesicles, our laboratory and others have shown that electrogenic H^+-pumping ATPases exist on the plasma membrane as well as on the tonoplast (Sze [25] and references therein). Tonoplast vesicles (apparently right-side-out) hydrolyze ATP with formation of a proton electrochemical gradient (positive and acidic inside). Since vacuoles serve as storage sites for ions and metabolites (16), the tonoplast must regulate the transport of various nutrients across its membrane similar to transport at the plasma membrane. The H^+-pumping ATPase of the tonoplast, like that on the plasma membrane, may provide the driving force for transport of many solutes. We have used tonoplast vesicles as a model system to study H^+-coupled solute transport (23, 24).

Here we present evidence for a H^+-coupled Cl^- (H^+/Cl^-) and a H^+-coupled NO_3^- (H^+/NO_3^-) transport system. In this paper, *coupled* transport refers to an apparent interdependence of H^+ and anion movement which occur at the same time (within our detection limits). A preliminary report of this work has been presented (22).

MATERIALS AND METHODS

Plant Material. Oat seeds (*Avena sativa* L. var Lang) were germinated in the dark over an aerated solution of 0.5 mM $CaSO_4$. Roots were harvested after 4 d.

Preparation of Tonoplast-Enriched Vesicles. Tonoplast vesicles were prepared as previously reported (23) with the following modifications. All procedures were conducted at 4°C. Oat roots (20–60 g) were homogenized with a mortar and pestle in a medium containing 250 mM mannitol, 3 mM EGTA, 25 mM Hepes-BTP[2] (pH 7.4), 1 mM DTT, and 0.2% BSA at a medium-to-tissue ratio of 1.5 ml/g fresh weight. After filtration through cheesecloth, the debris was rehomogenized in 1.5 ml/g of the original tissue weight and filtered. The homogenate was centrifuged for 15 min at 13,000 *g* and the resulting supernatant was centrifuged for 30 min at 60,000 *g* (Beckman SW 28 rotor, r_{max}). The 60,000 *g* pellet (crude microsomal fraction) was resuspended in 250 mM mannitol, 2.5 mM Hepes-BTP (pH 7.2), and 1 mM DTT (resuspension buffer). Usually, 6 ml of the microsomal suspension was layered over 10 ml of a 6% (w/w) dextran cushion (made in resuspension buffer) and centrifuged for 2 h at 70,000 *g* (SW 28.1, r_{max}). A visible band at the mannitol-dextran interface was collected and is referred to as the tonoplast-enriched vesicles.

Separation of Membranes with a Three-Step Dextran Gradient. To fractionate microsomal membranes, the following modifications to the above procedure were used. The crude microsomal pellet was resuspended and layered over a three-step (4, 7, and 12%) dextran gradient. Usually 15 ml of the microsomal suspension was layered over 7 ml of each of the dextran steps and centrifuged for 2 h at 70,000 *g* (SW 28, r_{max}). Visible bands at the 0/4%, 4/7%, and 7/12% interfaces were collected

[1] Supported in part by Department of Energy grant DE-AS05-82ER13015 and by National Science Foundation grant PCM 83-04130 to H. S.

[2] Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid BTP, bis-tris propane or 1,3-bis (tris[hydroxymethyl]methylamine) propane; IDA, iminodiacetate; MeA, methylamine; ΔpH, pH gradient; Δψ, membrane potential.

and used for transport and enzyme assays.

Protein Determination. Protein concentration was estimated after precipitation with 10% TCA by the method of Lowry *et al.* (14) with BSA as the standard.

Artificial pH Gradient Formation. In most experiments, ΔpH was imposed using a K^+ gradient and nigericin. Vesicles were loaded with KCl by incubation in 36 ml dilution buffer (50 mM mannitol, 2.5 mM Hepes-BTP at pH 7.2, and 150 mM KCl) for 1 h at 10°C (23). The vesicles were then pelleted for 30 min at 90,000 *g* (SW 28, r_{max}) and the pellet was resuspended in a small volume (usually 100–200 μl) of dilution buffer. An aliquot was diluted 50- to 200-fold into a K^+-free reaction mixture (1 ml) containing 250 mM mannitol, 25 mM Hepes-BTP (pH 7.0) with or without 5 μM nigericin. It was preferable to create pH gradients with KCl because salts such as KIDA were less effective. Apparently K^+ does not diffuse into the vesicles as well when the accompanying anion is relatively impermeant.

pH gradients could also be imposed using 'pH jumps.' Vesicles (1–2 ml) were incubated with 50 mM Hepes-BTP at pH 6.0 and 200 mM mannitol (36 ml) for 1 h at 10°C. The vesicles were pelleted at 90,000 *g* (SW 28, r_{max}) for 30 min and resuspended in a small volume of the same buffer (50–150 μl). A pH gradient was formed by diluting an aliquot 200-fold into a reaction mixture containing 200 mM mannitol and 50 mM BTP-Hepes at pH 8.0 (24).

[^{14}C]Methylamine Accumulation. Artificially formed pH gradients were measured as [^{14}C]methylamine accumulation using a filtration method (23). All assays were performed at 4°C, unless otherwise indicated. To initiate the reaction, K^+-loaded vesicles were diluted 50- to 200-fold to give a final reaction mixture (0.5–1.0 ml) of 40–100 μg/ml membrane protein, 20 μM [^{14}C]methylamine (approximately 1 μCi/ml), 250 mM mannitol, 25 mM Hepes-BTP at pH 7.0, with or without 5 μM nigericin. In pH jump experiments, vesicles preloaded with pH 6.0 buffer were diluted similarly into a mixture with 200 mM mannitol, 50 mM BTP-Hepes at pH 8.0, and 20 μM methylamine.

ATP-dependent methylamine accumulation was measured at 22°C with the following modifications. Interface vesicles were diluted in resuspension buffer and pelleted at 90,000 *g* for 30 min (SW 28, r_{max}). The pellet was resuspended in resuspension buffer and an aliquot (40–100 μg membrane protein/ml) was added to a reaction mixture containing (final concentrations) 250 mM mannitol, 25 mM Hepes-BTP (pH 7.0), 20 mM KCl, 0.2 mM NaN_3, 3 mM $MgSO_4$, 20 μM [^{14}C]methylamine (approximately 1 μCi/ml), 100 μM orthovanadate with or without 3 mM ATP.

In all assay mixtures, ionophores or inhibitors dissolved in ethanol were added to a final ethanol concentration of 0.5 to 1.0% (v/v). At specified times, aliquots of 100 μl were filtered through Millipore filters (0.45 μm pore size). The filtration procedure involved wetting a Millipore filter with 1 ml of cold (4°C) resuspension buffer, filtering an aliquot of the reaction mixture and quickly rinsing with 1 ml of cold resuspension buffer. The filters were dried and the radioactivity determined by liquid scintillation counting. Results are presented as nmol methylamine taken up per mg protein. Nigericin-dependent methylamine uptake is the difference in uptake in the presence and absence of the ionophore.

ΔpH-Dependent Ca^{2+} Transport. Ca^{2+} uptake dependent on a pH gradient was measured as $^{45}Ca^{2+}$ uptake using a filtration method. To initiate the reaction, K^+-loaded vesicles were diluted 50- to 200-fold into a reaction mixture (0.5–1.0 ml) containing (final concentrations) 250 mM mannitol, 25 mM Hepes-BTP (pH 7.0), 40 to 100 μg membrane protein/ml, 10 μM $^{45}Ca^{2+}$ (approximately 0.25 μCi/ml) with or without 5 μM nigericin. Unless otherwise indicated, filtration assays were performed at 4°C as described above for methylamine uptake. Nonradiolabeled Ca^{2+} (0.1 mM) was added to the rinse solution to exchange away any $^{45}Ca^{2+}$ bound to the filter or vesicle surface. Results are presented as nmol Ca^{2+} taken up per mg protein. Nigericin-dependent Ca^{2+} uptake refers to Ca^{2+} accumulation dependent on the ΔpH.

Enzyme Activities. ATPase activity was determined as previously described (30). The reaction mixture (0.5 ml) consisted of 30 mM Hepes-BTP (pH 7.0), 3 mM $MgSO_4$, 3 mM ATP-BTP at pH 7.0, 50 mM KCl, 10 to 30 μg protein, and 0.2 mM NaN_3. Reactions were initiated by addition of ATP and incubated at 35 C for 30 min. Pi was determined by a modified Fiske and Subbarow method (9, 10).

UDPase activity was determined in the presence of Triton (17). Vesicles were pretreated with 0.1% Triton for 5 min. The final concentrations of reaction components were 30 mM Hepes-BTP (pH 7.0), 3 mM $MgSO_4$, 3 mM UDP-Na_2, and 0.02% Triton in a volume of 0.5 ml. Reactions were initiated by addition of UDP and incubated at 35°C for 30 min. Reactions were terminated with an acid-molybdate solution containing 1% SDS, and Pi release was measured (9, 10).

Antimycin A-insensitive NADH-Cyt *c* reductase activity was assayed by monitoring the reduction of Cyt *c* spectrophotometrically at 550 nm (10, 23).

RESULTS

Cl^- and NO_3^- Decrease an Imposed ΔpH. An artificial pH gradient can be generated by diluting KCl-loaded vesicles into a KCl-free medium in the presence of nigericin (23, 24). Nigericin exchanges K^+ inside for H^+ outside generating a pH gradient of about 2 (inside acidic) which can be measured as [^{14}C]methylamine uptake (Fig. 1A) (24) or fluorescence quenching of acridine orange (24). Triton at 0.03% collapsed the pH gradient and decreased methylamine levels to those seen in the absence of nigericin. At 4°C, the pH gradient can be maintained for at least 20 min (Figs. 1A and 2). At 10°C, the artificially induced pH gradient built up in 1 to 2 min but decreased quickly. At 20°C, the vesicles were relatively leaky to H^+ and could not hold a pH gradient (Fig. 2). Therefore most experiments were conducted at 4°C.

When K^+-loaded vesicles were diluted into a medium containing either 10 mM Cl^- or 10 mM NO_3^- with an impermeant cation, BTP, [^{14}C]methylamine accumulation was decreased. Addition of BTP-Cl or BTP-NO_3 to vesicles holding a pH gradient also induced a rapid loss of methylamine (Fig. 1, C and D). The decrease in pH gradient was dependent on the anion concentration. It was not caused by osmotic changes or the cation, BTP, as addition of 10 mM BTP-iminodiacetate, had little effect (Fig. 1B). NO_3^- dissipation of the pH gradient could not be caused by its inhibition of the tonoplast H^+-pumping ATPase, as the pH gradient was generated artificially. Since IDA^-, an impermeant anion, had little effect, the rapid loss of methylamine induced by NO_3^- or Cl^- (Fig. 1, C and D), suggests that the pH gradient was diminished by these anions via H^+-coupled anion transport.

Both Cl^- and NO_3^- decreased an imposed ΔpH under conditions that would eliminate any diffusion potential. In these experiments, pH gradients were imposed using the pH jump method in the presence of K^+ and valinomycin. Vesicles incubated in buffer at pH 6 were diluted into a buffer at pH 8. Methylamine accumulated under these conditions indicates a pH gradient (acid inside) has been formed (Table I). This ΔpH is maintained in the presence of 20 mM K^+ and 0.2 μM valinomycin. These conditions would eliminate any H^+ diffusion potential caused by the ΔpH due to valinomycin-mediated K^+ transport. Table I shows that in the absence of a diffusion potential, Cl^- and NO_3^- were still effective in decreasing the imposed ΔpH. Therefore, anion-induced ΔpH decrease is not merely due to electrical coupling of H^+ loss to anion loss. Rather, we interpret the results as chemical coupling of H^+ out of the

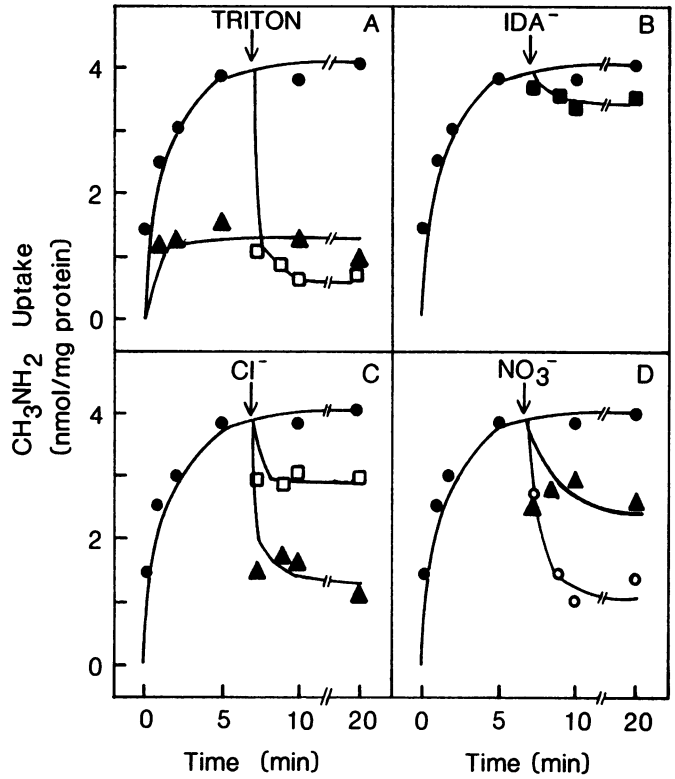

FIG. 1. Decrease of an artificially generated pH gradient by Cl^- and NO_3^-. K^+-loaded vesicles were diluted into a K^+-free medium (250 mM mannitol, 25 mM Hepes-BTP at pH 7.0, 20 μM [^{14}C]methylamine) with or without 5 μM nigericin. After 7 min at 4°C, (A) Triton X-100 (final concentration 0.03%), (B) 10 mM BTP-IDA, (C) 2.5 (□) or 5 (▲) mM BTP-Cl, or (D) 2.5 (▲) or 5 (○) mM BTP-NO_3, was added. One representative experiment of two.

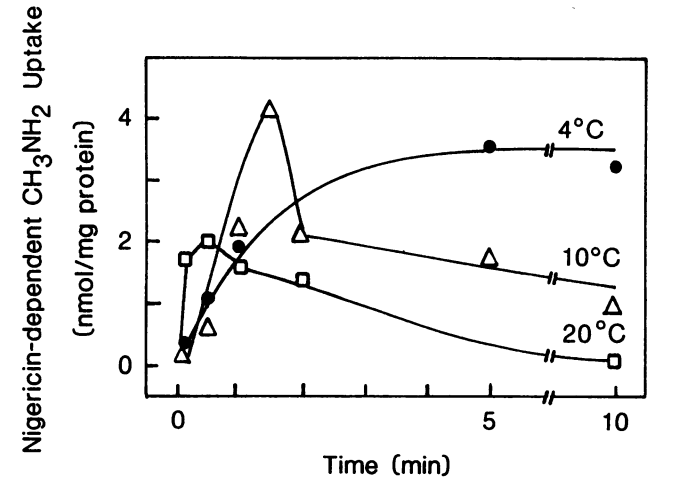

FIG. 2. Effect of temperature on artificially generated pH gradients. K^+-loaded vesicles were diluted into K^+-free media in the absence or presence of 5 μM nigericin. The reaction mixtures were incubated at 4 (●) 10 (△) or 20°C (□). Aliquots were removed and filtered at various times. Artificially-generated pH gradient is expressed as nigericin-dependent [^{14}C]methylamine uptake.

vesicles either in parallel with the anion (symport) or perhaps in exchange for anion uptake (antiport). For simplicity, we shall refer to the activity as 'H^+-coupled anion transport.'

Since the results of the pH jump experiments (in the absence or presence of K^+ and valinomycin, Table I) were similar to those conducted with a K^+ gradient and nigericin (Fig. 1), all subsequent experiments were performed with the latter method which produced a more stable ΔpH.

Table I. *Anion Decrease of ΔpH in the Presence (No K^+/Valinomycin) or Absence (+ K^+/Valinomycin) of a H^+ Diffusion Potential*

Vesicles were loaded with buffer at pH 6.0 and diluted into reaction mixtures at pH 8.0 (pH jumps) with or without anions. Diffusion potentials were eliminated by including 0.2 μM valinomycin and 20 mM KIDA. Valinomycin or KIDA alone had no effect on the amount of methylamine taken up. ΔpH ([^{14}C]MeA uptake) was measured at 4°C after 7 min. Values reported are ΔpH dependent activities which were determined by subtracting [^{14}C]MeA taken up in the absence of a pH gradient, (when $pH_i = pH_o = 6$) from the total MeA taken up in the presence of a ΔpH. ND = not determined.

Additions	[^{14}C]MeA Uptake	
	−(K^+/val)	+(K^+/val)
	nmol/mg protein (%)	
Control	3.56 (100)	2.94 (100)
Cl^- (5 mM)	2.14 (60)	1.33 (45)
(20 mM)	1.21 (34)	0.47 (16)
NO_3^- (5 mM)	1.57 (44)	1.15 (39)
(20 mM)	0.86 (24)	0.70 (24)
Triton (0.03%)	0.38 (11)	ND

H^+-Coupled Anion Transport Is Associated with Various Membranes. We fractionated the microsomal membranes using a three-step dextran gradient to determine the membrane identity of the H^+-coupled anion transport systems. ATP-dependent [^{14}C] methylamine uptake that was vanadate- and azide-insensitive was used as a marker of tonoplast vesicles (5, 23, 25). This activity was found mainly in the 0/4% dextran interface (Table II). ATP-dependent Ca^{2+} uptake (vanadate- and azide-insensitive) was similarly distributed. This Ca^{2+} uptake is mediated by a H^+/Ca^{2+} antiport system dependent on the proton motive force of the tonoplast H^+-ATPase (23). Antimycin A-insensitive NADH-Cyt *c* reductase activity, a marker of the ER, was enriched at the 4/7% dextran interface. Both the Golgi (UDPase) and the plasma membrane (vanadate-sensitive ATPase activities) markers were recovered at the 4/7% and 7/12% dextran interfaces with an enrichment of the plasma membrane at the heavier density.

All the vesicle fractions showed an ability to hold an artificial pH gradient (Table II, 'nigericin-dependent MeA uptake') though the highest specific activity was found in the vesicles at the 0/4% dextran interface. Furthermore, the pH gradients of all the fractions were sensitive to Cl^- and NO_3^- (Table III). The results suggest that the H^+-coupled anion transport systems may exist on a number of membranes including the tonoplast, ER, Golgi and plasma membranes. It would not be surprising to find many membranes with H^+-dependent anion transport systems. H^+ pumps exist on the plasma membrane and the tonoplast (25), and possibly on other endomembranes such as the Golgi (1, 2). We have chosen to study the H^+-coupled anion transport systems in the low density vesicles which are enriched in tonoplast membranes.

Cl^--Induced ΔpH Decrease Is Inhibited by DIDS. If Cl^- or NO_3^- movement is coupled to H^+ efflux, then increasing concentrations of these anions should progressively decrease the pH gradient formed. Figures 3A and 4A show that the decrease in methylamine accumulation was dependent on the Cl^- or NO_3^- concentration. The decrease was saturated by about 5 to 10 mM Cl^- or NO_3^-, suggesting the anions moved via proteinaceous porters. We have shown before that the tonoplast vesicles from oat roots possess a H^+/Ca^{2+} antiport system (23). The H^+/Ca^{2+} exchange system can be driven by the H^+ electrochemical gra-

Table II. *Relative Distribution of (A) Various Transport Systems and (B) Enzyme Activities in Membrane Fractions Obtained from a Step Dextran Gradient*

Microsomal vesicles were separated with a three-step dextran gradient. Interfaces from 0/4%, 4/7% and 7/12% were collected and half the vesicles were loaded with K^+ as described in the "Materials and Methods." Nigericin-dependent methylamine (MeA) uptake was measured at 4°C after 7 min. Vanadate- and azide-insensitive ATP-dependent transport (MeA or Ca^{2+}) was measured at 22°C after 10 min incubation. Enzyme activities were measured as described in the "Materials and Methods." Results (based on 30 g fresh weight of oat roots) are the average of two experiments.

A. Transport Activities

Dextran Interface	Protein	Nigericin-dependent MeA Uptake		ATP-dependent			
				MeA uptake		Ca^{2+} uptake	
%	*mg (%)*	*Sp.*[a]	*total (%)*	*Sp.*	*total (%)*	*Sp.*	*total (%)*
0/4	0.37 (28)	3.74	1.38 (48)	0.99	0.37 (64)	4.95	1.83 (88)
4/7	0.46 (35)	1.82	0.84 (29)	0.36	0.16 (29)	0.42	0.19 (9)
7/12	0.49 (37)	1.36	0.67 (23)	0.08	0.39 (7)	0.13	0.06 (3)

B. Relative Total Enzyme Activities of Subcellular Membranes[b]

Dextran Interface	Tonoplast[c]	ER[d]	Golgi[e]	Plasma Membrane[f]
%		%		
0/4	89	23	14	7
4/7	11	63	46	43
7/12	0	14	40	50

[a] Sp. refers to specific activity in nmole per mg protein per time. Total refers to total activity in nmole per fraction. [b] The total activity of each marker enzyme in the three interfaces was set to 100%. All enzyme activities are expressed as percent of the total. [c] Nitrate-sensitive ATPase activity (100% = 4.67 μmol Pi/h). [d] Antimycin A-insensitive NADH-Cyt *c* reductase (100% = 222 nmol Cyt *c* reduced/min). [e] UDPase activity in the presence of Triton (100% = 10.34 μmol Pi/h). [f] Vanadate-sensitive ATPase activity (100% = 5.63 μmol Pi/h).

Table III. *Decrease of pH Gradients by Cl^- and NO_3^- in Membrane Fractions Obtained from a Dextran Step Gradient*

Microsomal vesicles were separated with a three-step dextran gradient. Interfaces from 0/4%, 4/7% and 7/12% were collected and loaded with K^+ as described in "Materials and Methods." The K^+-loaded vesicles were diluted 200-fold in a medium containing 5 μM nigericin with various amounts of BTP-Cl or BTP-NO_3. Duplicate aliquots were removed and filtered at 7 min, and 0.03% Triton was added at 8 min. The artificially formed pH gradient was expressed as Triton-sensitive methylamine uptake.

Anion	Concn.	Triton-Sensitive [^{14}C]Methylamine Uptake		
		0/4%	4/7%	7/12%
	mM		%	
Cl^-	0	100 (3.74)[a]	100 (1.82)	100 (1.36)
	10	50	35	82
	20	41	19	54
NO_3^-	0	100	100	100
	10	54	40	86
	20	27	20	55

[a] Relative ΔpH expressed in specific activity (nmol/mg protein · 7 min).

dient of the tonoplast H^+-pumping ATPase or by an artificially-generated pH gradient (23, 24). Since Ca^{2+} uptake via the antiport reflects the magnitude of the pH gradient, we also tested the effect of Cl^- and NO_3^- on ΔpH-dependent Ca^{2+} uptake. As with methylamine, Ca^{2+} uptake was decreased by increasing concentrations of BTP-Cl and BTP-NO_3 (Figs. 3B and 4B).

We tested whether the two anion transport systems might be inhibited by DIDS, an anion transport inhibitor in red cells (4) and corn protoplasts (13). Vesicles were preincubated with or without DIDS for 10 min at 4°C before pH gradient formation in the presence or absence of anions. DIDS partially prevented the decrease of the pH gradient by Cl^- (Fig. 5A). In the absence of added Cl^-, DIDS also showed some protective action. This can be explained by the presence of residual KCl in the reaction mixture. After dilution of the KCl-loaded vesicles by 100- or 200-fold, we estimate that 0.75 to 1.5 mM KCl remains in the final reaction mixture. This amount plus the KCl inside the vesicles were sufficient to decrease the pH gradient slightly (about 20%) in the absence of DIDS. At higher concentrations of Cl^-, the drop in pH gradient as measured by Ca^{2+} uptake was also decreased by DIDS (Fig. 5B). However, DIDS could not prevent the decrease of Ca^{2+} uptake induced by NO_3^- (Fig. 6). The results suggest the H^+-coupled Cl^-, but not the H^+-coupled NO_3^-, transport system is inhibitable by DIDS.

DIDS inhibition of the pH gradient collapse was concentration-dependent. Protection was observed at 3 and 5 μM (Table IV). Higher DIDS concentrations decreased methylamine uptake. Furthermore, a preincubation period with the vesicles for at least 10 min was preferred. Apparently, time is required for effective modification of a Cl^- transporter by DIDS. The low concentration needed suggests DIDS acts specifically on a Cl^- transporter. High DIDS levels might unspecifically modify other proteins resulting in membrane leakage.

To provide direct evidence for the coupling of the movement of H^+ and Cl^-, we have attempted to measure $^{36}Cl^-$ flux in the presence of a pH gradient. Preliminary results suggest that a ΔpH dependent $^{36}Cl^-$ loss is decreased by DIDS, however, the fluxes were rapid and therefore difficult to measure. We have also tested

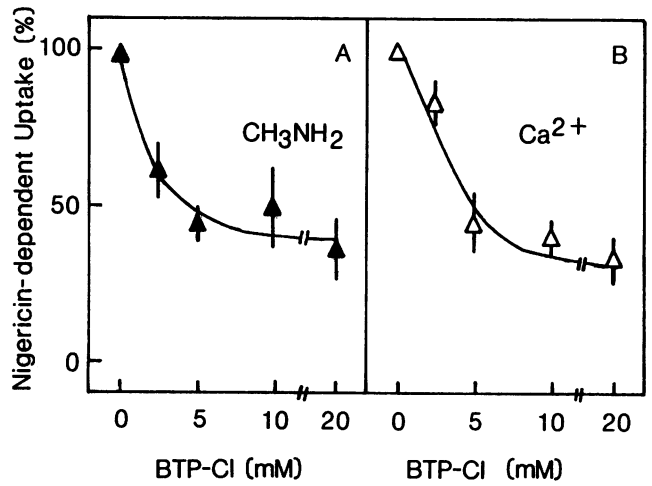

FIG. 3. Cl^--induced pH gradient decrease is dependent on Cl^- concentration. K^+-loaded vesicles were diluted into a K^+-free medium containing various concentrations of BTP-Cl at 4°C. Duplicate aliquots were removed and filtered after 7 min. A, Nigericin-dependent [^{14}C]methylamine uptake. Activity in the absence of BTP-Cl (2.14 ± 0.9 nmol/mg protein) was set to 100%. Results are the average of 2 to 7 experiments. B, Nigericin-dependent $^{45}Ca^{2+}$ uptake. Activity in the absence of BTP-Cl (17.4 ± 0.8 nmol/mg protein) was set to 100%. Data from 1 to 5 experiments.

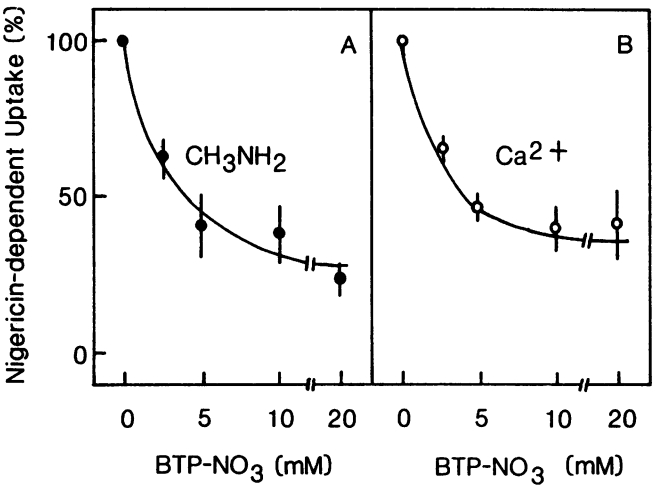

FIG. 4. Nitrate-induced pH gradient decreases is dependent on NO_3^- concentration. K^+-loaded vesicles were diluted into a reaction mixture containing various concentrations of BTP-NO_3 at 4°C. Aliquots were removed and filtered after 7 min incubation. A, Nigericin-dependent [^{14}C]methylamine uptake. Activity in the absence of BTP-NO_3 (2.51 ± 0.6 nmol/mg protein) was set to 100%. Average of one to three experiments. B, Nigericin-dependent $^{45}Ca^{2+}$ uptake. Activity in the absence of BTP-NO_3 (14.2 ± 1.3 nmol/mg protein) was set to 100%. Data from one to four experiments.

whether DIDS might block $^{36}Cl^-$ diffusion into the vesicles and found no evidence for such effects (not shown). These results are consistent with the lack of DIDS effect on Cl^- dissipation of the membrane potential (11).

DISCUSSION

We have found that chloride or nitrate decreased an artificially generated pH gradient (acid inside) in tonoplast-enriched vesicles. One or a combination of the following mechanisms could account for the anion-induced pH gradient decrease: (a) Movement of undissociated acid (HCl or HNO_3) across the lipid bilayer down a pH gradient as shown in egg phosphatidylcholine vesicles (18). (b) Electrical coupling of H^+ to anion efflux. Passive H^+ efflux becomes limited by the H^+ diffusion potential (negative inside) (8). Anions stimulate H^+ efflux by dissipating this potential as they move out of the vesicle via a lipid pathway or a separate porter. (c) H^+ and anions move out together via one

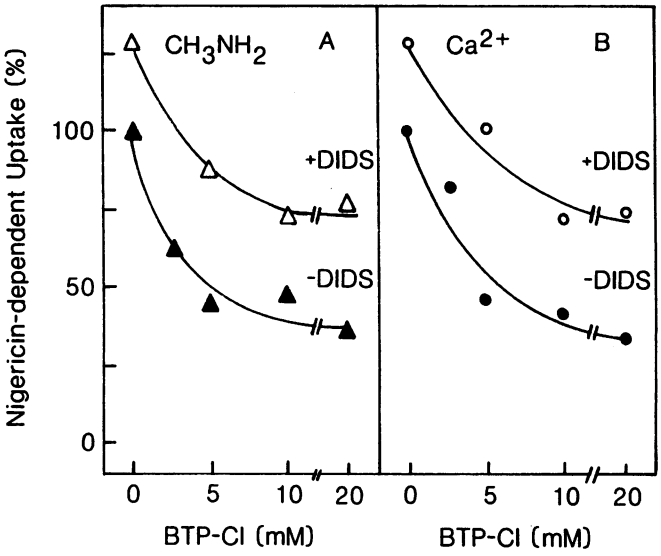

FIG. 5. Protection by DIDS of the Cl^--induced pH gradient decrease. K^+-loaded vesicles were preincubated in the absence or presence of 3 μM DIDS for at least 10 min at 4°C. The vesicles were then diluted into K^+-free medium containing various concentrations of BTP-Cl. Duplicate aliquots were filtered at 7 min. A, Nigericin-dependent [^{14}C]methylamine uptake. Activity in the absence of added Cl^- and DIDS (2.14 ± 0.9 nmol/mg protein) was set to 100%. Data from one to five experiments. B, Nigericin-dependent $^{45}Ca^{2+}$ uptake. Activity in the absence of added Cl^- and DIDS (11.2 ± 2.0 nmol/mg protein) was set to 100%. Average of two to six experiments.

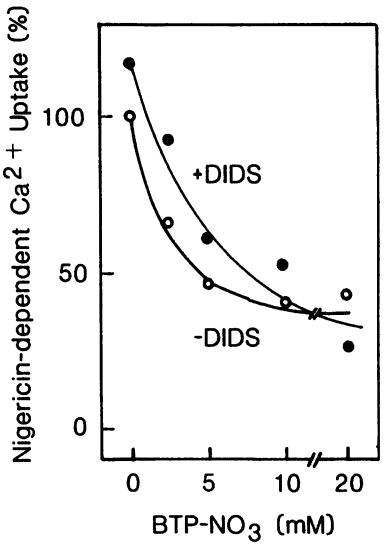

FIG. 6. Lack of DIDS effect on NO_3^--induced Ca^{2+} decrease. K^+-loaded vesicles were preincubated with or without 3 μM DIDS for 10 min at 4°C. The vesicles were then diluted into a K^+-free medium containing various concentrations of BTP-NO_3 at 4°C. Duplicate aliquots were removed and filtered at 7 min. Nigericin-dependent $^{45}Ca^{2+}$ uptake in the absence of DIDS and NO_3^- (18.55 ± 3.2 nmol/mg protein) was set to 100%. Average of two experiments.

Table IV. *Protection of Cl^--Induced pH Gradient Decrease Is Dependent on DIDS Concentration*

K^+-loaded vesicles were preincubated with various concentrations of DIDS for at least 10 min at 4°C. They were then diluted 50-fold into K^+-free medium with or without 10 mM BTP-Cl at 10°C. Aliquots were removed and filtered at 2 min.

DIDS Concn.	Nigericin-Dependent [^{14}C]Methylamine Uptake	
	$-Cl^-$	$+Cl^-$
μM	%	
0	100 (3.81)[a]	100 (1.79)
3	128	155
5	130	102
10	99	91
20	90	80
50	44	77

[a] Relative ΔpH expressed in specific activity (nmol/mg protein · 2 min).

porter (symport), and the H^+/anion cotransport could be electroneutral or electrogenic. (d) H^+ loss from the vesicles is coupled to uptake of anions via a H^+/anion antiporter.

Two lines of evidence suggest H^+-coupled Cl^- or NO_3^- transport is via proteinaceous porters: (a) Cl^--induced ΔpH decrease was inhibited by DIDS (Fig. 5), and (b) the decrease of the steady state pH gradient was saturated by 5 to 10 mM Cl^- or NO_3^- (Figs. 3 and 4). While movement of undissociated acids, HCl or HNO_3, may be possible in pure phosphatidylcholine membranes (18), it is unlikely to be significant in biological membranes. The intravesicular pH of the vesicles is estimated to be 5 (24). At this pH, all the nitric acid or hydrochloric acid would be completely dissociated. The results in Table I would argue against the possibility of an electrical coupling between H^+ and anion efflux. Table I showed that anions dissipated a ΔpH under conditions when little or no diffusion potential existed. Taken together, the results are most consistent with a model where H^+ are chemically coupled to Cl^- and NO_3^- transport. Anion-induced ΔpH decrease might be caused by a H^+/A^- symport or a H^+/A^- antiport or both. Our present studies do not allow us to differentiate between these two models. Preliminary results indicate that there is $^{36}Cl^-$ efflux which is ΔpH dependent suggesting that there may be some activity due to a H^+/Cl^- symport. The presence of a H^+/Cl^- antiporter on the tonoplast has recently been suggested based on ATP-stimulated Cl^- influx into vacuoles of permeabilized cells in *Chara* (26).

To establish unambiguously that H^+ are chemically coupled to Cl^- or NO_3^- transport, it will be necessary to show that H^+ are translocated in a stoichiometric manner with the anions, and that the free energy available from the proton electrochemical gradient is adequate to account for the translocation of anions (32). Furthermore, it is important to show that H^+-coupled Cl^- movement is through a single porter by demonstrating transport activity in a purified and reconstituted protein (32). DIDS could be used to identify the H^+-coupled Cl^- transport system, if it covalently modified this transport protein. Although we have not reached the stage for quantitative biophysical and biochemical experimentation, the ability to detect H^+-coupled anion transport systems in tonoplast vesicles is the first step toward the characterization and identification of such transport proteins.

This is the first demonstration of a H^+-coupled Cl^- transport activity in isolated tonoplast vesicles. This was possible mainly because a pH gradient was established artificially, uncomplicated by the direct anion effects on the tonoplast H^+-pumping ATPase (*e.g.* Cl^- stimulation and NO_3^- inhibition) (6). We suggest that in the presence of ATP, Cl^- enhanced pH gradient formation because Cl^- stimulated ATPase activity and the rate of the inwardly directed H^+ pump is faster than the rate of Cl^--dependent H^+ efflux. However, with an artificially generated pH gradient, the rate of H^+ uptake at steady state is apparently much slower than the rate of the H^+-coupled Cl^- transport, therefore the pH gradient dropped.

Our results also show the presence of a H^+-coupled NO_3^- transport system. Using an artificially generated pH gradient, we clearly show that the decrease of the ΔpH by NO_3^- is not caused by the inhibition of the tonoplast H^+-ATPase. Blumwald and Poole (3) have suggested recently that a H^+/NO_3^- symport system exists in tonoplast vesicles from red beets. They were able to distinguish this system from the NO_3^- effects on the ATPase because low NO_3^- levels (<5 mM) decreased ΔpH formation but did not inhibit the ATPase activity. In oat roots, the tonoplast ATPase is sensitive to 1 to 2 mM NO_3^- (31). Thus the presence of a H^+-coupled NO_3^- system could not be detected with an ATP-generated pH gradient, but it could be seen with PPi under certain conditions (30). PPi can generate a proton electrochemical gradient in tonoplast vesicles. However, unlike the ATPase, the H^+-PPase is insensitive to NO_3^- (30). Thus, if the inward H^+ pumping by the PPase is slower than the outward movement of H^+ caused by NO_3^-, a drop in ΔpH could be observed (20, 30). With an artificially formed pH gradient, the activity of the H^+-coupled NO_3^- transport is consistently observed.

The H^+-coupled transport systems described here may be two of many anion transport systems that exist on the vacuolar membrane. Depending on the anion availability (*i.e.* concentration in the cytoplasm and vacuole) and the demands of cellular metabolism, these transport systems may serve to mobilize Cl^- and NO_3^- stored in the vacuole for use in the cytoplasm (symporters) or to drive these anions into the vacuole (antiporters). The ΔpH component of the proton motive force generated by the tonoplast H^+-ATPase or the H^+-PPase could provide the energy for the movement. However, in order to prevent an apparent cycling of anions, cells must regulate the uptake of anions into the vacuole and the subsequent transport into the cytoplasm. There is evidence that anion transport is regulated at the tonoplast (7), however the regulatory mechanisms of these transporters are not yet understood.

Acknowledgments—We want to thank especially E. A. C. MacRobbie, E. Blumwald, Clifford Slayman, and Mark Tester for the stimulating discussions in Sydney.

LITERATURE CITED

1. Ali MS, T Akazawa 1986 Association of H^+-translocating ATPase in the Golgi membrane system from suspension-cultured cells of sycamore (*Acer pseudoplatanus*). Plant Physiol 81: 222–227
2. Binari LLW, RH Racusen 1983 Membrane-associated ATPases in isolated secretory vesicles. Plant Physiol 71: 594–597
3. Blumwald E, RJ Poole 1985 Nitrate storage and retrieval in *Beta vulgaris*: effects of nitrate and chloride on proton gradients in tonoplast vesicles. Proc Natl Acad Sci USA 82: 3683–3687
4. Cabantchik ZI, PA Knauf, A Rothstein 1978 The anion transport system of the red blood cell. The role of membrane proteins evaluated by the use of "probes." Biochim Biophys Acta 515: 239–302
5. Churchill KA, B Holaway, H Sze 1983 Separation of two types of electrogenic H^+-ATPases from oat roots. Plant Physiol 73: 921–928
6. Churchill KA, H Sze 1984 Anion-sensitive H^+-pumping ATPase of oat roots: direct effects of Cl^-, NO_3^- and a disulfonic stilbene. Plant Physiol 76: 490–497
7. Cram WJ 1983 Sulphate accumulation is regulated at the tonoplast. Plant Sci Lett 31: 329–338
8. Deamer DW, JW Nichols 1983 Proton-hydroxide permeability of liposomes. Proc Natl Acad Sci USA 80: 165–168
9. Fiske CH, Y Subbarow 1925 The colorimetric determination of phosphorus. J Biol Chem 66: 375–400
10. Hodges TK, RT Leonard 1974 Purification of a plasma membrane-bound adenosine triphosphatase from plant roots. Methods Enzymol 32: 392–406
11. Kaestner KH, H Sze 1987 Potential-dependent anion movement into tonoplast vesicles from oat roots. Plant Physiol 83: 483–489
12. Lass B, CI Ullrich-Eberius 1984 Evidence for proton/sulfate cotransport and its kinetics in *Lemna gibba* G1. Planta 161: 53–60
13. Lin W 1981 Inhibition of anion transport in corn root protoplasts. Plant

Physiol 68: 435–438
14. LOWRY OH, NJ ROSEBROUGH, AL FARR, R. RANDALL 1951 Protein measurement with the Folin reagent. J Biol Chem 193: 265–275
15. MACROBBIE EAC 1970 The active transport of ions in plant cells. Q Rev Biophys 3: 251–294
16. MACROBBIE EAC 1979 Vacuoles: the framework. *In* E Reid, ed, Plant Organelles. Ellis Horwood, Chichester, pp 62–68
17. NAGAHASHI J, AP KANE 1982 Triton-stimulated nucleoside diphosphate activity: subcellular localization in corn root homogenates. Protoplasma 112:167–173
18. NOZAKI Y, C TANFORD 1981 Proton and hydroxide ion permeability of phospholipid vesicles. Proc Natl Acad Sci USA 78: 4324–4328
19. PIERCE WS, N HIGINBOTHAM 1970 Compartments and fluxes of K^+. Na^+, and Cl^- in *Avena* coleoptile cells. Plant Physiol 46: 666–673
20. REA PA, RJ POOLE 1985 Proton-translocating inorganic pyrophosphatase in red beet (*Beta vulgaris* L.) tonoplast vesicles. Plant Physiol 77: 46–52
21. SANDERS D 1980 The mechanism of Cl^- transport at the plasma membrane of *Chara corallina*. I. Cotransport with H^+. J Membr Biol 53: 129–141
22. SCHUMAKER KS, H SZE 1985 Anion inhibition of Ca^{2+} accumulation in tonoplast vesicles from oat roots: evidence for a H^+/anion symport system. Plant Physiol 77: s–97
23. SCHUMAKER KS, H SZE 1985 A Ca^{2+}/H^+ antiport system driven by the proton electrochemical gradient of a tonoplast H^+-ATPase from oat roots. Plant Physiol 79: 1111–1117
24. SCHUMAKER KS, H SZE 1986 Calcium transport into the vacuole of oat roots: characterization of H^+/Ca^{2+} exchange activity. J Biol Chem 261: 12172–12178
25. SZE H 1985 H^+-translocating ATPases: advances using membrane vesicles. Annu Rev Plant Physiol 36: 175–208
26. TESTER M, T SHIMMEN, MJ BEILBY, EAC MACROBBIE 1986 The transport of ions across the tonoplast of charophytes: a study using permeabilized cells. *In* NA Walker, ed, Plant Membrane Transport. Sydney. In press
27. ULLRICH-EBERIUS CI, A NOVACKY, E FISCHER, U LUTTGE 1981 Relationship between energy-dependent phosphate uptake and the electrical potential in *Lemna gibba* G1. Plant Physiol 67: 797–801
28. ULLRICH WR, A NOVACKY 1981 Nitrate-dependent membrane potential changes and their induction in *Lemna gibba* G1. Plant Sci Lett 22: 211–217
29. ULLRICH-EBERIUS CI, A NOVACKY, AJE VAN BEL 1984 Phosphate uptake in *Lemna gibba* G1: energetics and kinetics. Planta 161: 46–52
30. WANG Y, R LEIGH, KH KAESTNER, H SZE 1986 Electrogenic H^+-pumping pyrophosphatase in tonoplast vesicles from oat roots. Plant Physiol 81: 497–502
31. WANG Y, H SZE 1985 Similarities and differences between the tonoplast-type and the mitochondrial H^+-ATPases of oat roots. J Biol Chem 260: 10434–10443
32. WEST IC 1980 Energy coupling in secondary active transport. Biochim Biophys Acta 604: 91–126