

Anion-Sensitive, H⁺-Pumping ATPase in Membrane Vesicles from Oat Roots¹

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ABSTRACT

H⁺-pumping ATPases were detected in microsomal vesicles of oat (*Avena sativa* L. var Lang) roots using [¹⁴C]methylamine distribution or quinacrine fluorescent quenching. Methylamine (MeA) accumulation into vesicles and quinacrine quench were specifically dependent on Mg, ATP. Both activities reflected formation of a proton gradient (Δ pH) (acid inside) as carbonyl cyanide *m*-chlorophenylhydrazone, nigericin (in the presence of K⁺), or gramicidin decreased MeA uptake or increased quinacrine fluorescence. The properties of H⁺ pumping as measured by MeA uptake were characterized. The $K_{m,app}$ for ATP was about 0.1 millimolar. Mg, GTP and Mg, pyrophosphate were 19% and 30% as effective as Mg, ATP. MeA uptake was inhibited by *N,N'*-dicyclohexylcarbodiimide and was mostly insensitive to oligomycin, vanadate, or copper. ATP-dependent MeA was stimulated by anions with decreasing order of potency of Cl⁻ > Br⁻ > NO₃⁻ > SO₄²⁻, iminodiacetate, benzene sulfonate. Anion stimulation of H⁺ pumping was caused in part by the ability of permeant anions to dissipate the electrical potential and in part by a specific requirement of Cl⁻ by a H⁺-pumping ATPase. A pH gradient, probably caused by a Donnan potential, could be dissipated by K⁺ in the presence or absence of ATP. MeA uptake was enriched in vesicles of relatively low density and showed a parallel distribution with vanadate-insensitive ATPase activity on a continuous dextran gradient. Δ pH as measured by quinacrine quench was partially vanadate-sensitive. These results show that plant membranes have at least two types of H⁺-pumping ATPases. One is vanadate-sensitive and probably enriched in the plasma membrane. One is vanadate-resistant, anion-sensitive and has many properties characteristic of a vacuolar ATPase. These results are consistent with the presence of electrogenic H⁺ pumps at the plasma membrane and tonoplast of higher plant cells.

Electrogenic H⁺ pumps are thought to be localized in plant membranes, such as the plasma membrane (H⁺ extrusion) and the tonoplast (H⁺ influx) (2, 22). *In vivo* dissipation of the electrochemical gradient of H⁺ has been correlated with the energy-dependent transport of anions, sugars, and amino acids across the plasmalemma (22) and with sucrose (7) and Cl⁻ accumulation into vacuoles (18). For several years, membrane-bound ATPases have been postulated to generate pH gradients (22) though no direct evidence demonstrated this until recently. Sze (30) found that the ATPase activity of sealed microsomal vesicles is stimulated by ionophores that increase conductance to H⁺ and K⁺. These results suggested that ATPases generate electrical and chemical (H⁺, K⁺, or both) gradients.

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Subsequently, Sze and Churchill (32) reported that ATP generated a transmembrane potential (positive inside) and a pH gradient (acid inside) in sealed microsomal vesicles of tobacco callus and oat roots, respectively. Because the membrane potential was partially vanadate-sensitive, we concluded that a KCl-stimulated, Mg²⁺-requiring ATPase of the plasma membrane (33) is an electrogenic pump, but did not eliminate the possibility of electrogenic ATPases on other subcellular membranes. Recently, several laboratories have reported *in vitro* evidence of a H⁺-pumping ATPase from microsomal vesicles with properties different from a plasma membrane ATPase (5, 8, 10, 21).

In this paper, we show that there are at least two types of H⁺-pumping ATPases in nonmitochondrial membranes of plants: one is vanadate-sensitive and one is vanadate-insensitive. Proton pumping as measured by methylamine distribution is stimulated by Cl⁻ and is insensitive to vanadate.

MATERIALS AND METHODS

Plant Material. Oats (*Avena sativa* L. var Lang) were germinated in the dark over an aerated solution of 0.5 mM CaSO₄. After 5 to 6 d of growth, the apical tips (3-4 cm) of the roots were harvested. Lang oats were generously provided by the Agronomy Department, Kansas State University.

Isolation of Sealed Microsomal Vesicles. The procedure of Sze (30) was used to isolate sealed membrane vesicles with some modifications. All procedures were conducted at 4°C. Oat roots, usually 30 g, were homogenized twice with a mortar and pestle in a medium containing 250 mM mannitol, 3 mM EGTA², 25 mM Hepes-BTP (pH 7.4), 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 0.5% BSA using a medium to tissue ratio of 3 ml/g. The brei was strained through cheesecloth and the homogenate was centrifuged for 15 min at 13,000g. The 13,000g supernatant was centrifuged for 30 min at 60,000g. The 60,000g pellet (crude microsomal fraction) was gently resuspended in a resuspension medium containing 250 mM mannitol, 2.5 mM Hepes-BTP (pH 7.3), and 1 mM DTT, layered onto a 6% (w/w) dextran cushion made in the resuspension medium (dextran average mol wt was 79,000), and centrifuged for 2 h at 70,000g. The white interface on top of the dextran cushion was collected and is referred to as the sealed microsomal vesicles.

Protein concentration was estimated after precipitation with 10% TCA by the Lowry method (17), with BSA as the standard.

² Abbreviations: EGTA, ethylene glycol bis(β -aminoethyl ether)-*N,N'*-tetraacetic acid; BTP, bis-tris propane (1,3-bis[tris(hydroxymethyl)-methylamino]propane); MeA, methylamine; Δ Ψ , membrane potential; EtA, ethylamine; TPMP-Br, triphenylmethylphosphonium bromide; Δ pH, proton gradient; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; IDA, iminodiacetate; DIDS, 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid.

Separation of Microsomal Vesicles by Dextran Gradient. For some experiments, the resuspended microsomal pellet was fractionated in a linear dextran gradient of 0.5 to 10% (32 ml total). The dextran solutions were made in 250 mM mannitol, 2.5 mM Hepes-BTP at 7.3. Two ml of the resuspended microsomal pellet were layered on top of the gradient and centrifuged for 2 h at 70,000g using a Beckman SW 27 rotor. Nineteen aliquots of 1.7 ml each were collected and tested for ATPase activity and MeA uptake. Sucrose density equivalence was determined using a refractometer.

Determination of ATP-Generated Proton Gradient.

[¹⁴C]Amine Distribution. ATP-dependent amine uptake was used as a relative measure of H⁺ pumping (25, 26). To initiate the reaction, sealed microsomal vesicles were added to a reaction mixture containing 175 mM mannitol, 25 mM Hepes-BTP (pH 7.5), 20 μM [¹⁴C]MeA (1–1.5 μCi/ml), 10 mM KCl (or BTP-Cl), 5 μg/ml oligomycin, 0.2 to 0.5 mg/ml membrane protein, MgSO₄ with or without ATP. The concentrations of MgSO₄ and ATP-Tris were as indicated in the legends. Ionophores and many inhibitors, dissolved in ethanol, were added to reaction mixtures to a final concentration of 0.5 to 1% ethanol which had no effect on MeA uptake. After 10 min incubation (unless otherwise indicated) at 21°C, triplicate aliquots of 50 to 100 μl were filtered through a Millipore filter (0.45 μm pore size) using either a direct or dilution filtration procedure. In the dilution method, the aliquot was diluted into 3 ml of a cold (4°C) wash medium (250 mM sucrose, 2.5 mM Hepes-BTP, pH 7.5), stirred briefly on a vortex mixer, filtered, and rinsed with 0.5 ml cold wash medium. The direct filtration procedure involved wetting a Millipore filter with 1 ml of the cold wash medium, directly filtering an aliquot of the reaction medium, and quickly rinsing with 1 ml of cold wash medium. Using the direct procedure, four to five samples could be filtered within 2 min. Inasmuch as similar results were obtained by either method (Table I), the direct filtration procedure was used for most of the experiments. The filters were dried and the associated radioactivity determined by liquid scintillation counting. Results are presented as pmol amine uptake/mg protein. Amine binding to the filters was determined by filtering an aliquot of reaction mixture without vesicles.

Quinacrine Fluorescence Quench. Quinacrine fluorescence was

measured with a Perkin-Elmer spectrofluorometer MPF-44B at 500 nm after excitation at 420 nm by a procedure slightly modified from one described before (8). The fluorescence of quinacrine is quenched when the amine moves into an acid compartment (13, 25). Either MgSO₄ or BTP-Cl was added to a reaction mixture at 25°C to initiate H⁺ pumping. The complete reaction mixture contained 10 mM Hepes-BTP (pH 7.5), 0.33 mM EGTA, 2.5 μM quinacrine, 190 mM mannitol, 1.5 mM MgSO₄, 1.5 mM ATP, 50 mM BTP-Cl, and about 150 μg membrane protein in a final volume of 1.5 ml. In the absence of MgSO₄ and Cl⁻ salt, quinacrine fluorescence was set to 90%. The initial rate of fluorescent quench is a relative measure of the rate of H⁺ pumping.

Determination of Membrane Potential and ATPase Activity. Relative membrane potential (ΔΨ) was determined from the distribution of [¹⁴C]thiocyanate into sealed microsomal vesicles using the direct Millipore filtration procedure. Protocol was basically as described by Sze and Churchill (32) except the reaction mixture contained 16 μM [¹⁴C]SCN⁻ (1 μCi/ml).

ATPase activity was determined as described (30). Reaction mixtures at pH 6.75 were incubated for 30 min at 35°C.

Chemicals. Nigericin was a generous gift from J. Berger and J. W. Westley of Hoffman-LaRoche. K-benzene sulfonate was a gift from D. L. Hendrix. Sodium ATP was purchased from Boehringer-Mannheim and converted by Dowex ion exchange to ATP-Tris. [¹⁴C]MeA, EtA, or aminopyrine was purchased from New England Nuclear; [¹⁴C]imidazole was from California Bionuclear, and [¹⁴C]SCN was obtained from Amersham. Most of the fine chemicals were purchased from Sigma. TPMP-Br was obtained from ICN, K & K Labs.

RESULTS

Amine Distribution as a ΔpH Probe. To determine if a proton gradient was generated by ATP in sealed membrane vesicles of oat roots, we measured the accumulation of ¹⁴C-amines. The distribution of weak bases, such as MeA, can be used as a pH gradient (ΔpH) probe because the unprotonated neutral form readily diffuses across biological membranes, but most membranes are relatively impermeable to the protonated form (12, 25). In the absence of a pH gradient, the concentration of the protonated and unprotonated amine within a compartment is equal to that outside

Table I. Comparison of Various Amines as Probes to Measure ATP-Driven Proton Gradient in Sealed Microsomal Vesicles of Oat Roots

Amine	pK _a	Concn.	Amine Uptake			ΔpH ^a	Assay Conditions ^b
			-ATP	+ATP	ΔATP		
		μM	nmol/mg at 10 min				
[¹⁴ C]Imidazole	7.0	190	0.41	1.28	0.87	-0.72	a
		960	2.40	5.67	3.27	-0.57	a
[¹⁴ C]Aminopyrine	5.0	8	0.035	0.050	0.015	-0.27	b
		8	0.120	0.133	0.013	-0.09	c
[¹⁴ C]Methylamine	10.6	20	0.045	0.249	0.204	-1.00	a
		20	0.038	0.647	0.609	-1.52	c
		20	0.055	0.785	0.730	-1.44	d
[¹⁴ C]Ethylamine	10.8	40	0.090	2.279	2.189	-1.70	c

^a ΔpH was estimated as described in text.
^b Assay conditions:

	Filtration Procedure	KCl	MgSO ₄	ATP-Tris	pH
			mM		
a	Dilution	50	10	3	6.75
b	Dilution	10	5	3	7.50
c	Direct	10	5	3	7.50
d	Direct	10	1.0	1.0	7.50

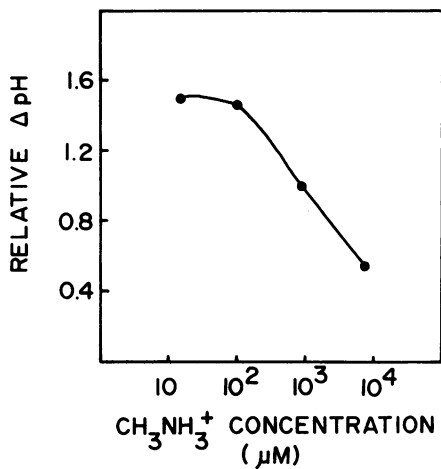
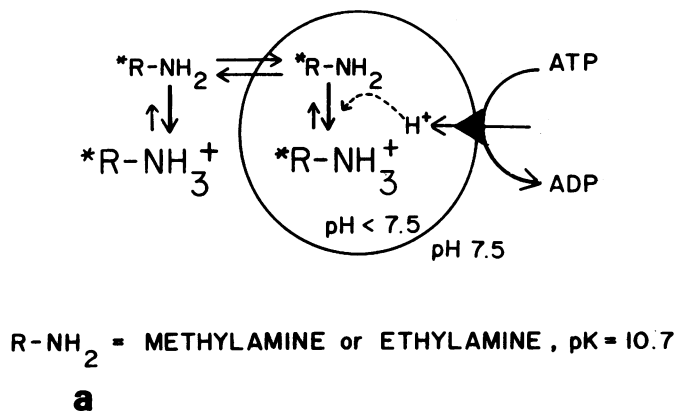


FIG. 1. Measurement of ΔpH by MeA distribution. a, Principle of MeA distribution as a ΔpH probe. Upon acidification of the interior of the vesicle, the protonated (charged) form of the amine is trapped and accumulated within the vesicle. See text for details. b, Effect of MeA concentration on ATP-generated proton gradient in sealed microsomal vesicles from oat roots. ΔpH was calculated from the concentration of MeA accumulated in the presence of ATP ($= MeA_{in}$) versus that in the absence of ATP ($= MeA_{out}$) according to the formula:

$$\Delta pH = -\log \frac{MeA_{in}}{MeA_{out}}$$

Reaction media included 50 mM KCl, 10 mM $MgSO_4$, 3 mM ADP or ATP, 25 mM Hepes-BTP (pH 7.5), 20 μM [^{14}C]MeA, and vesicles from a 10% dextran interface. Nonradioactive MeA was used to obtain the desired MeA concentration. The dilution filtration procedure was used in this study.

the compartment. If a transmembrane pH gradient, acidic inside, is generated across membrane vesicles, the equilibrium is shifted so that more of the protonated species is formed and thus trapped within the vesicles (Fig. 1a). Because the concentration of the neutral form remains relatively constant and is negligible if $pH_{out} < pK_a - 1$, the concentration of the protonated amine (AH^+) is approximately equal to the total amine concentration and related to the H^+ concentration within the vesicles so that:

$$\frac{[H^+]_{in}}{[H^+]_{out}} = \frac{[AH^+]_{in}}{[AH^+]_{out}}$$

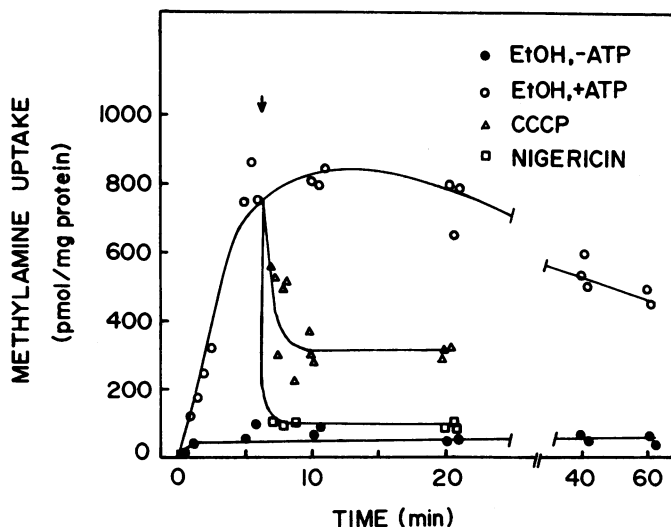


FIG. 2. Decrease of ATP-dependent MeA uptake by ionophores in sealed microsomal vesicles from oat roots. Assay conditions were: 1.0 mM $MgSO_4$, 1.0 mM ADP (●), or ATP-Tris (○), with 25 mM Hepes-BTP (pH 7.5), 175 mM mannitol, 10 mM KCl, 5 $\mu g/ml$ oligomycin, and 20 μM [^{14}C] MeA. At the time indicated by the arrow, ethanol (○, ●), CCCP (△), or nigericin (□) were added to give a final ethanol concentration of 0.5% with or without 5 μM ionophore.

and

$$pH_{in} - pH_{out} = \Delta pH = -\log \frac{[AH^+]_{in}}{[AH^+]_{out}}$$

We tested the distribution of [^{14}C]imidazole, aminopyrine, MeA, and EtA to determine which amine was the most sensitive ΔpH probe (Table I). Although ATP caused all the amines to accumulate in sealed microsomal vesicles, ATP-dependent MeA and EtA uptake gave the highest values and the most consistent results.

Amine distribution is a sensitive ΔpH probe only when the concentration of the unprotonated species of an amine is low. MeA and EtA satisfied this condition because MeA (or EtA) has a high pK_a (10.6) and was available with high specific radioactivity (50 mCi/mmol), so the final amine concentration in the reaction mixture was about 20 μM . MeA at high concentrations was unsatisfactory as a ΔpH probe because high amine levels dissipated the ΔpH (Fig. 1b). Imidazole and aminopyrine were less sensitive ΔpH probes for this reason (Table I). [^{14}C]Imidazole had a low specific radioactivity (1.3 mCi/mmol), thus it was used at a relatively high concentration (0.2–0.9 mM). Though available with high specific activity, aminopyrine had a low pK_a of 5.0, and therefore the neutral species was present at a relatively high concentration when pH_{out} was 7.5.

ATP-dependent MeA uptake into microsomal vesicles was immediately released upon addition of nigericin or CCCP plus valinomycin (Fig. 2). These ionophores, in the presence of K^+ , would dissipate proton gradients. CCCP alone was only effective in reducing about 60% of the MeA uptake, possibly either because there was no compensating charge movement or because of some residual Donnan potential. These ionophores prevented MeA accumulation when they were present during the entire course of the incubation (not shown).

Most of our data is expressed as ATP-dependent amine uptake (pmol/mg membrane protein) rather than ΔpH , because of the heterogeneous nature of the vesicles. Some vesicles might lack a proton-pumping ATPase and some could be oriented so that ATP was inaccessible to the ATPase. Assuming that half of the sealed vesicles pumped H^+ in, a relative ΔpH could be calculated without

Table II. Substrate Dependence of Methylamine Uptake into Microsomal Vesicles of Oat Roots

Substrates were present at 1.0 mM; values represent the mean of two to four experiments.

Substrate	Substrate-Dependent Methylamine Uptake	
	Net Methylamine Uptake	%
	<i>pmol/mg protein</i>	
None	30	
MgSO ₄	53	
ATP	50	
Mg, ATP	788	100
Mn, ATP	406	48
Ca, ATP	119	9
Mg, GTP	193	19
Mg, ADP	90	5
Mg, AMP	159	14
Mg, NPPi	97	6
Mg, PPi	303	34

an exact value of the internal vesicle volume. In the absence of ATP we have assumed $[AH^+]_{in} = [AH^+]_{out}$. Since the ratio of vesicle to medium volume is negligible (less than 1%), $[AH^+]_{out}$ remains constant. In the presence of ATP, the ratio of $[AH^+]_{in}/[AH^+]_{out}$ was often as much as 26, yielding an estimated ΔpH of -1.4 (Fig. 1b; Table I). This value is probably a conservative estimate.

Mg,ATP-Dependence. The proton gradient was specifically generated by Mg,ATP (Table II). GTP sustained only a small proton gradient and other nucleotides were ineffective. Interestingly, PPi generated a significant proton gradient.

MeA uptake was dependent on Mg,ATP concentration between 0.05 and 0.50 mM (Fig. 3). A Lineweaver-Burk analysis yielded a $K_{m,app}$ for Mg,ATP of about 0.1 mM in the presence of 10 mM KCl (Fig. 3) or BTP-Cl. In the absence of salt, the K_m was about 1.4 mM. Similar results were obtained using MeA influx (initial rate) to express relative ΔpH instead of net MeA uptake. The reason for the apparent inhibition by equimolar concentrations of Mg,ATP greater than 3 mM is not understood. At high concentrations, either Mg or ATP could bind to the enzyme, thus acting as an inhibitor for Mg,ATP, the true substrate.

pH Dependence. ATP-dependent MeA uptake exhibited a broad pH dependence, dropping off sharply above pH 8.5 (Fig. 4). Since the highest activity was detected at pH 8 to 8.5 in the presence or absence of oligomycin, mitochondrial ATPase could not be responsible for the proton gradient. No difference in MeA uptake was observed at buffer concentrations of 5, 10, or 25 mM. To minimize the concentration of the unprotonated species of MeA we routinely assayed ATP-dependent MeA uptake at pH 7.5.

Whether the broad pH optimum of MeA uptake reflected the optimum pH required for one or more H⁺-pumping ATPase activity is unclear. The internal buffering capacity of the vesicles could influence the results. As shown by Rottenberg *et al.* (26), the extent of H⁺ uptake into chloroplasts is a measure of the internal buffering capacity and only indirectly related to ΔpH .

Inhibitor Sensitivities. DCCD, an inhibitor of proton-translocating ATPases (16), almost totally inhibited ATP-dependent MeA accumulation (Table III).

The mitochondrial ATPase inhibitors oligomycin and azide generally inhibited about 20% of ATP-dependent MeA uptake (Table III). To eliminate the possibility of mitochondrial ATPase or other oligomycin-sensitive ATPase activity, oligomycin was routinely added to the reaction mixture unless otherwise indicated.

Copper has been reported as an inhibitor of a yeast vacuolar

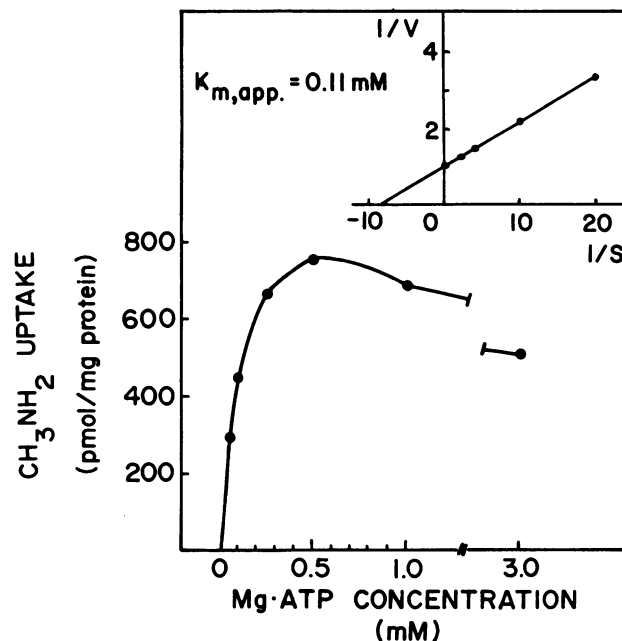


FIG. 3. Mg,ATP concentration dependence of MeA uptake into sealed microsomal vesicles from oat roots. Inset shows Lineweaver-Burk plot of the same data for Mg,ATP concentrations. Results represent the average of four experiments

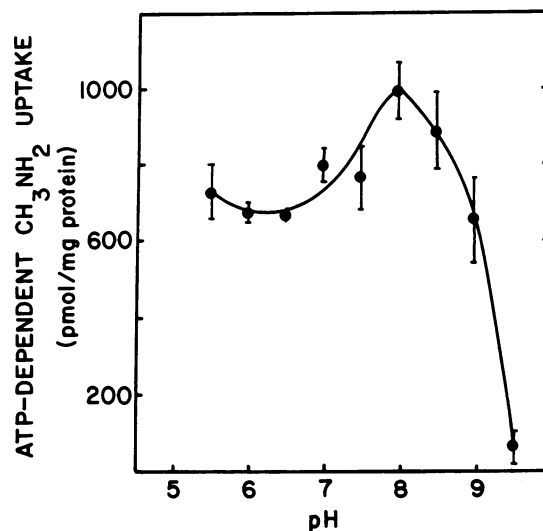


FIG. 4. Effect of pH on ATP-dependent MeA uptake into sealed microsomal vesicles from oat roots. Assay conditions were as outlined in legend to Figure 2, with 25 mM Hepes-BTP buffers at pH 5.5 to 9.5. Results are the average of five experiments.

ATPase (11) but it had no effect in this study (Table III).

Surprisingly, H⁺ pumping as determined by MeA uptake was insensitive to vanadate at concentrations that inhibited membrane potential generation in sealed membrane vesicles from tobacco callus (32) or oat roots (K. Churchill, unpublished). ATP-dependent MeA uptake was slightly (but consistently) stimulated by 200 μM vanadate; 500 to 1000 μM inhibited about 20 to 30% (Table III). Insensitivity to vanadate was not altered by the absence of Cl⁻ salts or the presence of NO₃⁻ (data not shown).

Anion and Cation Effects on ΔpH (MeA Uptake), $\Delta \Psi$, and ATPase Activity. ATP-dependent proton translocation (MeA uptake) was stimulated by anions in the sequence: Cl⁻ > Br⁻ > NO₃⁻ > SO₄²⁻ = iminodiacetate (Fig. 5). Sulfate, benzene sulfonate, or iminodiacetate had little or no effect on proton pumping.

Table III. Effect of Inhibitors on ATP-Dependent Methylamine Uptake into Microsomal Vesicles of Oat Roots

Reaction mixture was as described in Figure 2.

Addition	ATP-Dependent Methylamine Uptake (%)
None	100
Vanadate	
200 μM	112
500 μM	81
1000 μM	70
DCCD, 50 μM	15
Oligomycin, 5 $\mu\text{g/ml}$	78
NaN_3 , 1 mM	79
CuSO_4 , 100 μM	105
DIDS	
5 μM	76
10 μM	55
NH_4Cl , 40 μM	98

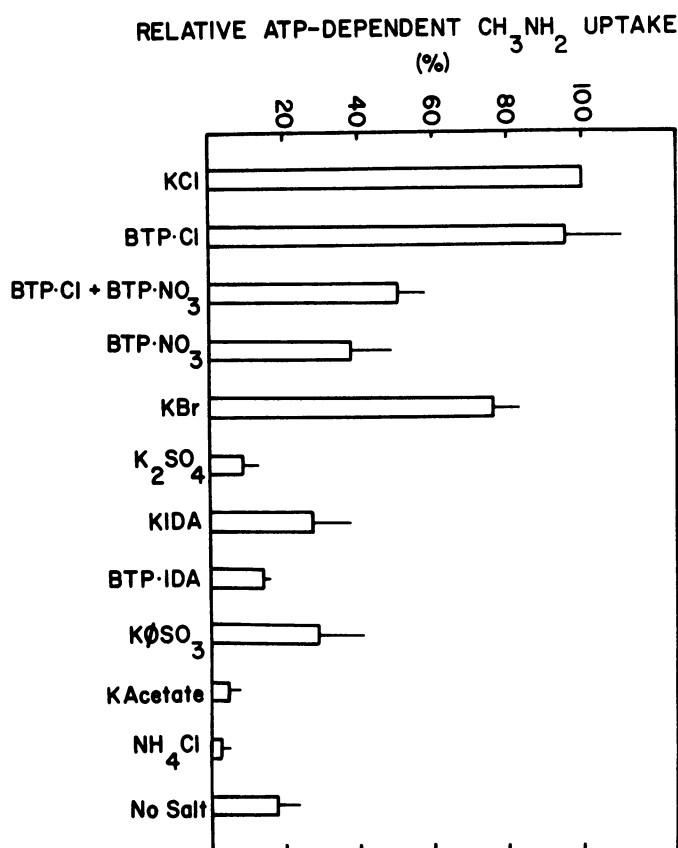


FIG. 5. Effect of salts on ATP-dependent MeA uptake into sealed microsomal vesicles prepared from oat roots. Final concentration of salts was 10 mM. MeA uptake in the presence of KCl (0.57 nmol/mg protein) was set to 100%. Assay conditions are as outlined in legend to Figure 2. Results are averages of two to ten experiments. Deviations are SE.

The pH gradient formed was dependent on Cl^- concentration (Fig. 6). Inasmuch as 10 mM Cl^- could generate the maximum pH gradient, experiments were conducted with KCl or BTP-Cl at 10 mM. Nitrate inhibited the Cl^- -stimulated pH gradient (Fig. 5).

Generation of an ATP-dependent proton gradient was also sensitive to cations (Fig. 7). At concentrations above 10 mM, BTP-Cl consistently generated a larger ΔpH than KCl (Fig. 7a).

Even in the absence of ATP, there was a sizable pH gradient of about 200 pmol MeA/mg protein. Permeable cations, such as K^+

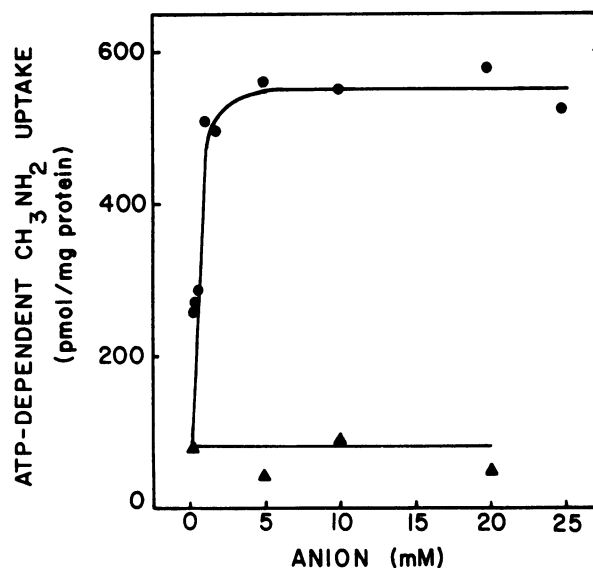


FIG. 6. Cl^- stimulation of ΔpH formation in sealed microsomal vesicles of oat roots. Assay conditions as described in legend to Figure 2, except BTP-Cl (●) and BTP-IDA (▲) concentrations ranged between 0.5 and 25 mM. Triplicate samples were taken after 3 min incubation. Mean of two to three experiments.

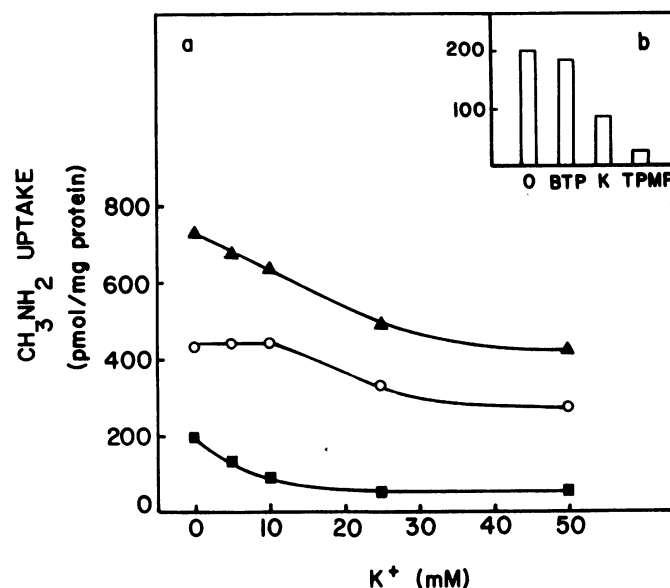


FIG. 7. Cation-induced dissipation of proton gradients in sealed microsomal vesicles of oat roots. a, Effect of K^+ concentration on CH_3NH_2 uptake. Various concentrations of KIDA were added to a reaction mixture containing 10 mM BTP-Cl as described in Figure 2. Total uptake in the presence of ATP (▲), in the absence of ATP (■), ΔATP (○). Triplicate samples were taken after 3 min incubation. b, Effect of cations on MeA uptake in the absence of ATP. Ten mM BTP-Cl, KCl, or TPMP-Br were included in the standard reaction medium.

and TPMP^+ , could dissipate this gradient (Fig. 7b) which was probably due to a Donnan potential (6, 20). Although we do not know what caused this apparent Donnan effect, proper controls must be run when the effect of salts is studied.

At high concentrations (10 mM), ammonium ion completely dissipated the proton gradient (Fig. 5) consistent with the uncoupler action of ammonium ion (Fig. 1b).

To understand the anion and cation effects on ΔpH formation, salt effects on ATPase and membrane potential activities were

Table IV. Salt Effects on ATPase Activity, Membrane Potential, and pH Gradient Generation in Sealed Microsomal Vesicles of Oat Roots

Salt	ATPase ^a	$\Delta\Psi^b$	ΔpH^c
		%	
MgSO ₄	100	100	18 ± 5
MgSO ₄ + KCl	171 ± 6	25 ± 14	100
MgSO ₄ + BTP-Cl	162 ± 7	34 ± 4	96 ± 15
MgSO ₄ + KNO ₃	78 ± 3	0	38
MgSO ₄ + BTP-NO ₃	67	0	38 ± 11
MgSO ₄ + KIDA	131 ± 6	100 ± 5	27 ± 11
MgSO ₄ + BTP-IDA	127 ± 0	90	14 ± 2
MgSO ₄ + K ₂ SO ₄	111 ± 10	74	9 ± 2
MgSO ₄ + (NH ₄) ₂ SO ₄	152	ND ^d	ND
MgSO ₄ + NH ₄ IDA	ND	85 ± 2	ND
MgSO ₄ + NH ₄ Cl	204	ND	3 ± 2

^a ATPase activity: $9.05 \pm 1.7 \mu\text{mol Pi/mg protein}\cdot\text{h}$ was set to 100%. Concentration of MgSO₄ and salt were 3 and 50 mM, respectively.

^b Relative membrane potential: ATP dependent SCN⁻ uptake of 0.30 nmol/mg protein at 10 min was set to 100%. Salt and MgSO₄ concentration were 10 and 1 mM, respectively.

^c Relative ΔpH : ATP-dependent MeA uptake of 0.57 nmol/mg protein at 10 min was set to 100%. MgSO₄ and salt concentration were 1 and 10 mM, respectively.

^d ND, not determined.

qualitatively compared to ΔpH activities (Table IV). The properties of ATPase and membrane potential activities in oat roots were similar to those found in tobacco callus (31, 32). In general, cations or anions that dissipated $\Delta\Psi$, ΔpH , or both, stimulated ATPase activity. Cl⁻ dissipated $\Delta\Psi$ and stimulated ΔpH formation. IDA or SO₄²⁻ did not dissipate $\Delta\Psi$ and could not stimulate ΔpH generation.

Two Types of H⁺-Pumping ATPases. An experiment was conducted to determine whether MeA accumulation was catalyzed by a vanadate-insensitive ATPase. The microsomal vesicles were separated with a dextran gradient, and ATPase activity and ATP-dependent H⁺ pumping (MeA uptake) were measured. MeA uptake was enriched in vesicles of relatively low density (Fig. 8a).

We have assumed that ATP hydrolysis in the presence of 200 μM vanadate is catalyzed by a vanadate-insensitive ATPase. This assumption was initially based on the observation that a H⁺-pumping ATPase from *Neurospora* plasma membrane was completely inhibited by less than 50 μM vanadate (28), whereas microsomal ATPase activity of higher plants was partially inhibited by 200 μM vanadate (31). Vanadate-sensitive ATPase activity can then be determined from the difference between the total and the vanadate-insensitive Mg,KCl-ATPase activities. Using this assumption, we found that total Mg,KCl-ATPase activity was broadly distributed along the dextran gradient and the vanadate-resistant and vanadate-sensitive ATPases were enriched at 2 to 3% and 4 to 8% dextran, respectively (Fig. 8b).

The distribution of vesicles (equivalent to 2 to 3% dextran) showing MeA uptake correlated with the distribution of a vanadate-insensitive ATPase activity. No H⁺ pumping activity was detected in vesicles (equivalent to 4 to 8% dextran) enriched in vanadate-sensitive ATPase activity. Thus, MeA uptake is catalyzed mainly by a vanadate-insensitive, H⁺-pumping ATPase.

Since MeA may not be a good ΔpH probe in plant cells with ammonium porters (12) we used quinacrine fluorescent quenching to determine whether a vanadate-sensitive ATPase pumped H⁺. Quinacrine fluorescence was quenched by Mg,ATP in the presence of Cl⁻ (Fig. 9). The relative rate and net amount of quinacrine quenching was dependent on the concentration of vesicles. Quinacrine fluorescence quenching reflected formation of a pH gradient as gramicidin (a H⁺ and alkali-cation conductor) reversed

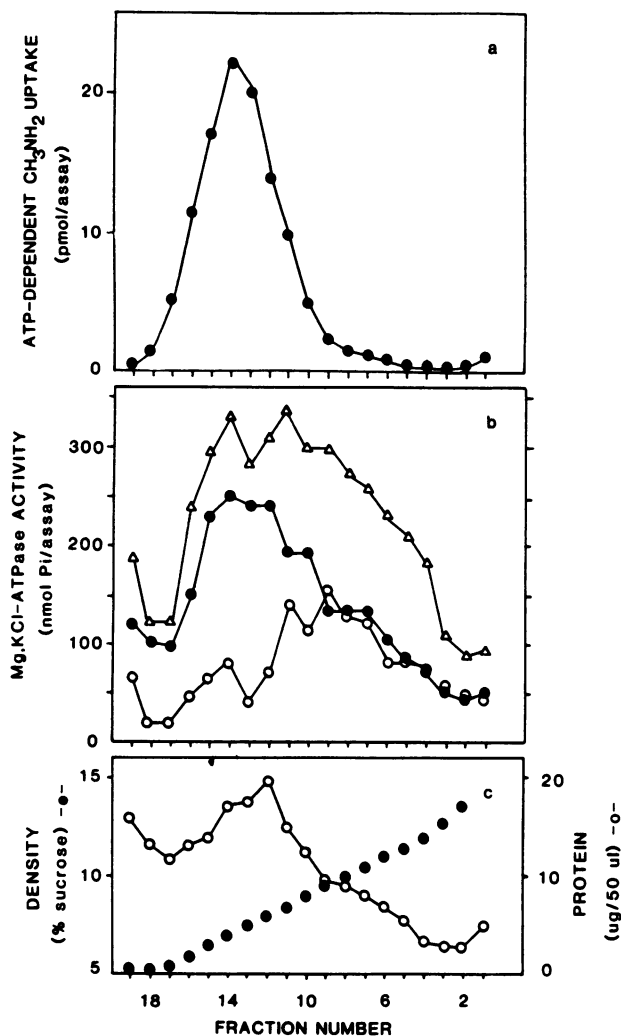


FIG. 8. Distribution of MeA uptake and ATPase activity in microsomal vesicles from oat roots separated with a dextran gradient. a, Distribution of MeA uptake in microsomal vesicles. MeA uptake was performed as described in legend to Figure 2. b, Separation of vanadate-insensitive (●) and vanadate-sensitive (○) ATPases. Vanadate-insensitive Mg,KCl-ATPase activity was obtained by measuring ATP hydrolysis in the presence of Na-vanadate. Vanadate-sensitive ATPase activity was obtained by subtracting the vanadate-insensitive activity from the total Mg,KCl-ATPase activity (Δ). Reaction mixtures contained 30 mM Hepes-BTP at pH 6.75, 3 mM MgSO₄, 50 mM KCl, 3 mM ATP-Tris, 2 μM nigericin with or without 200 μM Na-orthovanadate. c, Distribution of proteins on a linear dextran gradient (0.5–10%) made up in 250 mM mannitol and 2.5 mM Hepes-BTP at pH 7.3. The relative densities are expressed as percent sucrose.

the fluorescence quench. The ΔpH generated by ATP was partially vanadate-sensitive (Fig. 9). At steady-state fluorescence, the rate of H⁺ pump into the vesicles is equal to the rate of H⁺ leak. The increase in fluorescence caused by vanadate indicated that H⁺ leak was faster than H⁺ pumping, i.e. the H⁺ pumping was inhibited. Addition of Na₂HPO₄ (200 μM) instead of vanadate had no effect. In one experiment, the distribution of H⁺ pumping as measured by quinacrine quenching showed a similar distribution in the dextran gradient as the Mg,KCl-ATPase activity. We conclude that plant membranes have two types of H⁺-pumping ATPase: one vanadate-sensitive and one insensitive to vanadate.

DISCUSSION

MeA Distribution as ΔpH Probe. Using MeA distribution as a ΔpH probe, we have shown that nonmitochondrial membranes

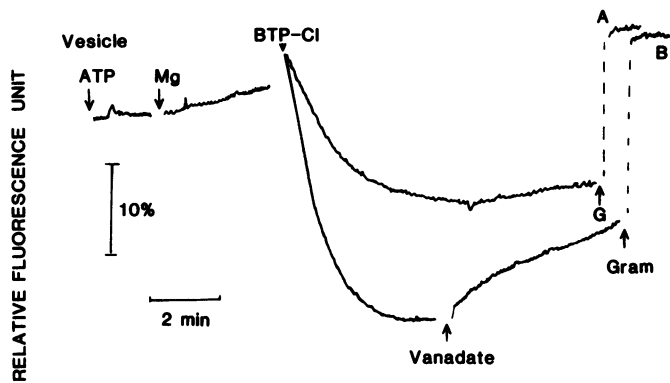


FIG. 9. Vanadate inhibition of ATP-dependent quinacrine fluorescence quench in sealed microsomal vesicles from oat roots. The final reaction mixture contained 10 mM Hepes-BTP (pH 7.5), 0.33 mM EGTA, 2.5 μ M quinacrine, 190 mM mannitol, 1.5 mM $MgSO_4$, 1.5 mM ATP, and 75 μ g (trace A) or 150 μ g (trace B) protein. Cl-BTP (50 mM) was added to initiate the reaction. To reaction B, 7.5 μ l of 20 mM Na-orthovanadate was added at the arrow to obtain a final concentration of 100 μ M. Final gramicidin (Gram or G) and ethanol concentration were 2.5 μ g/ml and 0.5%, respectively.

from oat roots have H^+ -pumping ATPases. This conclusion is supported by the specific requirement for ATP and Mg to generate a Δ pH (inside acid) in vesicles (Table II) and inhibition of Δ pH by DCCD (Table III). MeA distribution was a valid Δ pH probe by at least three criteria (12). (a) Ionophores that release proton gradients (nigericin or CCCP plus valinomycin [in the presence of KCl]) decreased ATP-dependent MeA uptake (Fig. 2). (b) High concentrations of MeA dissipated the Δ pH, because it acted as an uncoupler (Fig. 1b). Similarly, NH_4Cl (10 mM) prevented generation of a pH gradient (Fig. 7). (c) ATP-dependent MeA uptake was not dependent on a specific ammonia transporter since potentially competitive concentrations of NH_4Cl (40 μ M) did not decrease MeA uptake (Table III). However, MeA distribution is a useful Δ pH probe only in membranes with few or no specific ammonium porters as shown below.

Properties and Origin of the H^+ Pumps. We found that plant membranes have at least two types of H^+ -pumping ATPases: one vanadate-sensitive and one relatively insensitive to vanadate (Figs. 8 and 9). These results are supported by the presence of at least two types of ATPases in oat roots (Fig. 8b), tobacco callus (31), and partial vanadate inhibition of a membrane potential (29, 32). Quinacrine quench represents Δ pH generated by both types of H^+ -pumping ATPases (Fig. 9).

MeA uptake (Fig. 8a) is a valuable indicator of H^+ -pumping activity from one type of ATPase (vanadate-insensitive) (Fig. 8b). One explanation for the difference between MeA and quinacrine could be that MeA, frequently used as an analog for studying ammonium transport, diffuses rapidly out of vesicles with ammonium porters, whereas quinacrine does not. This would explain why MeA could not be used for measuring H^+ pumping in plasma membrane vesicles of *Neurospora* (28). Lack of MeA accumulation by vanadate-sensitive ATPase supports the idea that plasma membrane vesicles of higher plants are enriched in specific ammonium porters (24) as in algae and fungi (12). Vesicles showing MeA uptake probably have few or no ammonium porters as in the tonoplast of higher plants (24).

Several other properties of the H^+ pump as measured by MeA distribution were different from those of a plasma membrane ATPase and may be those of a vacuolar ATPase. (a) MeA uptake (Fig. 8a) was enriched in vesicles lighter in density than markers of the ER (NADH-Cyt *c* reductase), Golgi (UDPase) (not shown), or plasma membrane (vanadate-sensitive ATPase) (Fig. 8b), similar to results of Mettler *et al.* (21). The relative distribution was

similar to that of sucrose gradients where intact tonoplast or tonoplast vesicles have an isopycnic density of about 1.10 to 1.12 g/cc which is lighter than ER, Golgi, or plasma membrane (23). (b) H^+ -pumping ATPase was vanadate-insensitive, similar to an ATPase associated with the vacuolar membranes from higher plants (35) and fungi (11). However, a vanadate-sensitive ATPase may also exist on vacuolar membranes (7). (c) As discussed later, the H^+ -translocating ATPase was anion-sensitive, specifically stimulated by Cl^- . This property is similar to that of a vacuolar ATPase from *Hevea* (1), red beet root (35), a microsomal ATPase of oat roots (4) and turnip (27). (d) H^+ pumping measured by MeA uptake had a K_m for Mg,ATP of about 0.1 mM (Fig. 3) which is lower than the K_m (0.4 mM) of plasma membrane ATPase from oat roots (14). Vacuolar ATPases of red beet root (35) and yeast (11) also show low K_m (about 0.2 mM) for ATP. (e) PPI generated a Δ pH (Table II) consistent with the presence of pyrophosphatase activity in tonoplast of higher plants (36).

Proton pumping activity as measured by MeA uptake could be associated with right-side-out vesicles of tonoplast (30, 31) either derived from resealed vacuolar membranes or small intact vacuoles. A similar conclusion was reached by several studies using sealed microsomal vesicles of corn coleoptiles (10, 21) or roots (9). These studies demonstrated only a vanadate-insensitive, H^+ -pumping ATPase with properties similar to the H^+ pump (MeA uptake) reported in this paper. Direct evidence supporting this idea will depend on the properties of vacuolar membranes. ATP-dependent H^+ pumping has been detected in isolated vacuoles (34) or vacuo-lysosomes (19, 20) in the presence of $MgCl_2$ (6). Though the properties of the vacuolar ATPase have not been well-characterized, a vanadate-insensitive H^+ pump (19) and ATPase activity (35) have been detected in higher plant vacuoles. The possibility of H^+ pumps on secretory vesicles, ER, Golgi (10), and proplastid membranes cannot be excluded. Since these membranes originated from the ER, it would not be surprising to find a continuum of ATPase types perhaps varying with cell type and developmental stage.

Salt Effects on Proton Pumping. A partial explanation for Cl^- stimulation of the proton pump can be attributed to dissipation of the electrical potential. A H^+ -pumping ATPase generates an electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) made up of two entities (a proton concentration gradient [Δ pH] and an electrical gradient [$\Delta\psi$]) that at steady-state are related by the following expression (at 25°C):

$$\Delta\bar{\mu}_{H^+} = \Delta\psi - 59 \Delta\text{pH}$$

We have expressed $\Delta\psi$ and Δ pH in relative units. The electrical and chemical components are interconvertible while $\Delta\bar{\mu}_{H^+}$ remains constant. Thus, permeant anions, like Cl^- , stimulated Δ pH generation at the expense of $\Delta\psi$ (Table IV; Ref. 32). Impermeant anions, like IDA, benzene sulfonate, or sulfate, did not dissipate $\Delta\psi$ and could not stimulate Δ pH formation (Table IV).

In addition to dissipation of the membrane potential, Cl^- specifically stimulated a proton-pumping ATPase activity based on several properties: (a) 10 mM BTP-Cl only partially (62%) dissipated the electrical potential (Table IV) but stimulated maximum proton gradient generation (Fig. 6); (b) Cl^- stimulated ATPase activity in the absence of electrochemical gradients in oat roots (not shown) as in tobacco callus (31); (c) nitrate inhibited Cl^- -stimulated Δ pH formation (Fig. 5) and ATPase activities (31); and (d) DIDS, which blocks anion transport in red blood cells and corn root protoplasts (15), inhibited Cl^- -stimulated proton pumping in our system (Table III). The mode of action of NO_3^- is not understood. It appeared to behave in part as a permeant anion, by stimulating Δ pH (Fig. 5), and in part as an inhibitor of the Cl^- -stimulated H^+ -pumping ATPase (Fig. 5), perhaps by blocking a Cl^- channel. DIDS is a specific anion transport inhibitor in intact corn root protoplasts, but we found that DIDS (5 μ M) also inhibited ATPase activity and proton pumping in the

absence of Cl⁻ (not shown). The four observations listed above clearly show that a H⁺-pumping ATPase is anion-sensitive, specifically stimulated by Cl⁻.

Cl⁻-stimulation of a H⁺-pumping ATPase could be caused by one or a combination of several possibilities: (a) Cl⁻ stimulates the H⁺-pumping ATPase and is not transported itself; (b) Cl⁻ is cotransported with H⁺ into vesicles either directly by the ATPase or indirectly via a specific anion channel (3); or (c) H⁺/Cl⁻ are cotransported out of the vesicles driven by a Cl⁻ or H⁺ gradient or both. Though we have not separated these alternatives, ATP increased 36-Cl⁻ uptake from 16 to 84 nmol/mg protein in vesicles from tobacco callus (data not shown).

In general, cations or anions that dissipated either $\Delta\Psi$ or ΔpH or both, stimulated ATPase activity (Table IV) consistent with the idea that ATPase activity is regulated by the electrochemical potential (30). Thus, NH₄Cl stimulated ATPase activity similar to gramicidin or nigericin, because Cl⁻ dissipated $\Delta\Psi$ (in addition to specific anion stimulation) and NH₄⁺ dissipated ΔpH (Table IV; Ref. 31). K⁺ dissipated a Cl⁻-dependent ΔpH , whereas BTP did not, probably because K⁺ is a relatively permeant cation (Fig. 7). This conclusion is supported by the observations that K⁺ stimulated ATPase activity more than BTP (Table IV), Tris, or choline (31). K⁺ dissipation of a pH gradient could be caused by a H⁺/K⁺ exchange mechanism that permits H⁺ efflux and K⁺ uptake into the vesicles even in the absence of ATP. So far we have seen no evidence of specificity among inorganic alkali cations.

We concluded from these results that plant membranes have at least two types of H⁺-pumping ATPases. One is vanadate-sensitive and probably enriched in the plasma membrane. One is vanadate-resistant, anion-sensitive (specifically stimulated by Cl⁻) and could be enriched in vacuolar-type membranes. These results are consistent with the idea of electrogenic H⁺ pumps at the plasma membrane (H⁺ extrusion) and tonoplast (H⁺ transported from cytosol to vacuole) of higher plant cells.

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