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Nitrate storage and retrieval in *Beta vulgaris*: Effects of nitrate and chloride on proton gradients in tonoplast vesicles

(transport/vacuoles/fluorescence quenching/nitrate/proton symport/chloride)

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ABSTRACT The fluorescent probes acridine orange and oxonol-V were used as indicators of pH gradients (ΔpH) and membrane potential differences (ΔΨ), respectively, in membrane vesicles believed to be derived from the tonoplast of *Beta vulgaris* L. Low concentrations of nitrate (1–5 mM) caused a partial dissipation of both ΔpH and ΔΨ at vesicle transport sites distinct from the H^+-ATPase. In contrast, chloride dissipated only ΔΨ. A model is proposed in which nitrate and chloride enter the plant cell vacuole in response to a potential generated by the tonoplast H^+-ATPase. Nitrate but not chloride may then be retrieved for metabolic use by the operation of a nitrate/proton symport at the tonoplast.

Nitrate, a major plant nutrient, is accumulated and stored in plant cell vacuoles, from which it may be transported under appropriate conditions (ref. 1 and refs. therein). However, virtually nothing is known about nitrate transport across the tonoplast. Studies of isolated vacuoles from *Beta* (2) and from *Neurospora* (3) as well as studies of membrane vesicles believed to be derived from the tonoplast in a variety of species (4–8) have shown that the tonoplast contains a characteristic proton-transporting ATPase which is inhibited by high concentrations of nitrate. Here we investigate the effects of nitrate on ATPase and on proton transport in a membrane-vesicle preparation for which there is *prima facie* evidence of a predominantly tonoplast origin, and we propose a model for nitrate movement into and out of the vacuole.

MATERIALS AND METHODS

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

Isolation of Membrane Vesicles. Vesicles identified as tonoplast (see *Discussion*) were isolated as described (8) except that the compositions of the homogenization and suspension media were based on the recommendations of Scherer and Morré (9, 10). The homogenization medium consisted of 10 mM DL-α-glycerophosphate, 0.65 mM ethanolamine (adjusted to pH 8.0 with conc. H_2SO_4), 0.28 M choline chloride, 26 mM potassium metabisulfite, 2 mM salicylhydroxamic acid, 0.2% (wt/vol) BSA (fraction V; essentially fatty-acid free), 10% (wt/vol) insoluble polyvinylpyrrolidone, 5 mM dithiothreitol, 0.5 mM butylated hydroxytoluene, and 1 mM Nupercaine, buffered to pH 8.0 with 70 mM Tris/Mes. The suspension medium consisted of 1.1 M glycerol and 5 mM dithiothreitol, buffered to pH 8.0 with 5 mM Tris/Mes. Choline and ethanolamine were included in the homogenization medium to minimize membrane degradation by phospholipase D (9), and Nupercaine and glycerophosphate or glycerol were added to inhibit phospholipase A and phosphatidic acid phosphatase activity, respectively (10). The KI solution, employed to diminish contamination of the vesicles with endogenous acid phosphatase (11), and the sucrose gradients were prepared in suspension medium. For the preparation of vesicles, the 0.25 M KI-treated pellet from 300–350 g of beet was resuspended in 12 ml of suspension medium, and 3-ml volumes were layered onto each of four step gradients consisting of 5 ml of 10% (wt/wt) and 5 ml of 23% (wt/wt) sucrose. After centrifugation at 80,000 × *g* for 2 hr, the membranes at the 10/23% sucrose interface were removed with a Pasteur pipette. The membranes were diluted 10- to 20-fold with the desired experimental solution, sedimented at 80,000 × *g*, and resuspended in 1–2 ml of the same solution.

Enzyme Assay. ATPase activity was measured as the rate of ADP-dependent NADH oxidation in a coupled system containing NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase ($ATP \rightarrow ADP + P_i$; $ADP + PEP \rightarrow ATP + pyruvate$; $pyruvate + NADH \rightarrow lactate + NAD$). The rate of ADP formation was measured in a 1-ml reaction volume containing 20–30 μg of membrane protein, 50 mM KCl, 0.6 mM Tris ATP, 3 mM $MgSO_4$, 30 mM Tris/Mes (pH 8.0), 3 mM phosphoenolpyruvate, 0.184 mM NADH, 1 unit of pyruvate kinase (EC 2.7.1.40 from rabbit muscle), and 1 unit of lactate dehydrogenase (EC 1.1.1.27 from hog muscle). The rate of ADP-dependent NADH oxidation was measured as the decrease in absorbance at 340 nm with time.

Fluorescence Assays. The formation of inside-acid pH gradients across the vesicle membranes was measured as the rate of fluorescence quenching of the permeant amine dye acridine orange (8, 12). Membrane vesicles (30–35 μg of membrane protein), 3 mM Tris/Mes buffer (pH 8.0), 3 mM Tris ATP, 50 mM KCl or the appropriate salt as indicated in the figure legends, 250 mM sorbitol, and 5 μM acridine orange were added to the sample cell to give a final volume of 1.0 ml. Proton translocation was initiated by the addition of 3 mM $MgSO_4$, and the fluorescence decrease with time was measured at 25°C with a Perkin Elmer spectrofluorimeter model LS-5 at excitation and emission wavelengths of 495 and 540 nm, respectively, and a slit-width of 5 nm for both emission and excitation. The formation of an inside-positive membrane potential across the membranes of the tonoplast vesicles was measured as the rate of fluorescence quenching of the oxonol dye, Oxonol-V [bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol] (13). Assay conditions were as above, except that 2 μM Oxonol-V was used and the decrease in fluorescence with time was measured at excitation and emission wavelengths of 610 and 640 nm, respectively, and a slit-width of 5 nm for excitation and 10 nm for emission.

Protein. Protein was measured by a modification of the dye-binding method (14), in which membrane protein was partially solubilized with 0.5% (wt/wt) Triton X-100 before the addition of the dye reagent concentrate (Bio-Rad). Bo-

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Abbreviations: ΔpH, pH gradient; ΔΨ, membrane potential gradient.

vine serum albumin was employed as the protein standard. This method gives membrane protein values more than twice as large as the Lowry method and correspondingly lower specific activities.

Chemicals. General laboratory reagents were obtained from BDH and Sigma. Pyruvate kinase and lactate dehydrogenase were purchased from Boehringer Mannheim. Acridine orange was purchased from Sigma, and Oxonol-V, from Molecular Probes, Junction City, OR. Na_2ATP was purchased from Boehringer Mannheim and converted to Tris ATP by cation-exchange with Dowex 50W (Tris form; 100–200 mesh).

RESULTS

Calibration of Fluorescent Probes in the Vesicle Preparation. Quenching of the fluorescence of acridine orange and of Oxonol-V was used to indicate gradients of pH (ΔpH) and membrane potential (ΔΨ) across the vesicle membranes. Fig. 1 shows the response of these probes over a range of membrane protein concentrations. As illustrated in Fig. 2, acridine orange fluorescence responds in a reproducible, if not ideal, way to imposed gradients of pH in the vesicle preparation (cf. refs. 12 and 15). Calibration of Oxonol-V response (Fig. 3) was made in two ways. A range of membrane potentials was created by imposing proton gradients in the presence of gramicidin or by imposing potassium gradients in the presence of valinomycin. The slight difference in the response with the two methods may indicate some permeability to counter-ions. In any case, the oxonol fluorescence responds satisfactorily to membrane potential.

Response of Probes to ATPase Activity and to Chloride. The experiment of Fig. 4 illustrates a typical response of the two fluorescent probes during ATPase activity. When the ATPase was activated by addition of Mg^{2+}, there was quenching of the fluorescence of both acridine orange and Oxonol-V, indicating acidification of the vesicle contents and formation of an inside-positive electrical potential difference. Both effects were reversed on addition of gramicidin, which collapses proton and cation gradients. In the presence of chloride, the potential was smaller and the pH gradient was increased. This type of response has been observed in other species (15, 16). Chloride stimulates the ATPase in the presence or absence of ionophores or detergents (8, 15, 16), but uptake of chloride (in membranes from corn) is abolished by the proton ionophore carbonylcyanide *m*-chlorophenylhydrazone (17). The effect of chloride in increasing the pH gradient (Fig. 4) is therefore attributable mainly to direct stimulation of the ATPase, whereas its dissipating effect on the potential may result from its accumulation in response to the electrical potential generated by the H^+-ATPase. In support of this interpretation is the fact that when time is allowed for chloride distribution to come to equilibrium with the membrane potential across the membrane, there is no longer any dissipation of the potential by this ion (unpublished observation). Although chloride is observed to dissipate the potential, there is no indication that it has any tendency to dissipate the pH gradient.

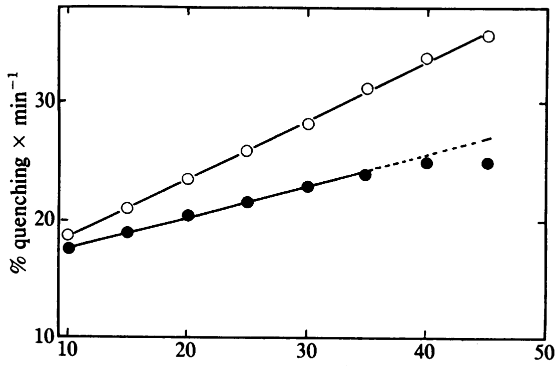

FIG. 1. Effect of membrane protein concentration on the response of acridine orange and Oxonol-V. Membrane vesicles were incubated in 1 ml of 3 mM Tris/Mes, pH 8.0/3 mM Tris ATP/50 mM KCl/250 mM sorbitol containing either 5 μM acridine orange (○) or 2 μM Oxonol-V (●). For Oxonol-V experiments, 25 mM K_2SO_4 was used instead of 50 mM KCl. For both probes, the reaction was started by adding 3 mM $MgSO_4$.

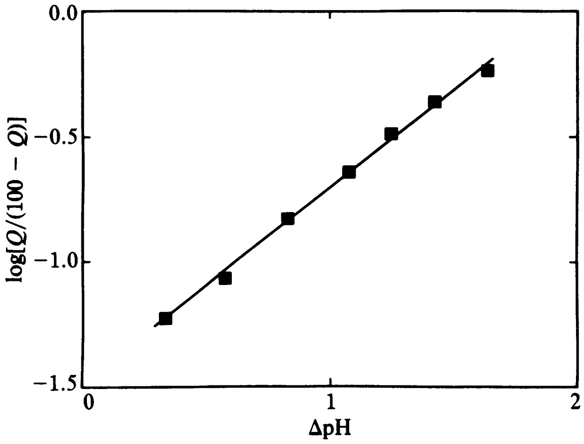

FIG. 2. Calibration of acridine-orange fluorescence quenching. Membrane vesicles containing 30 μg of protein were incubated in 1 ml of 3 mM Tris/Mes, pH 7.0/250 mM sorbitol/5 μM acridine orange. Acid-interior pH gradients were imposed by additions of aliquots of 2 M NaOH. Measurements of pH in the cuvette before and after addition of NaOH were used to calculate ΔpH. *Q*, percent quenching of fluorescence.

Chloride and Nitrate Effects on ΔpH and ΔΨ Generated by H^+-ATPase. The effects of chloride and nitrate on ΔpH were compared (Fig. 5) by adding various concentrations of these

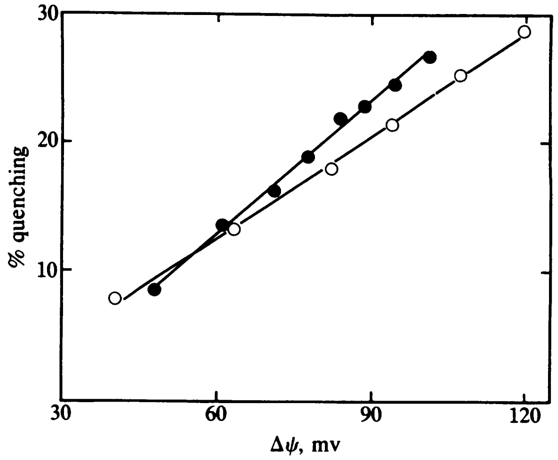

FIG. 3. Calibration curve for Oxonol-V response to positive-interior membrane potential. For H^+-diffusion potentials (○), membrane vesicles containing 30 μg of protein were incubated in 2 ml of 2 mM Tris/Mes, pH 7.0/250 mM sorbitol/2 μM gramicidin/2 μM Oxonol-V. Changes in fluorescence were initiated by the addition of aliquots of 0.5 M H_2SO_4. The imposed ΔpH was calculated by measuring the pH in the cuvette before and after addition of H_2SO_4. Results were corrected for decay of ΔpH by extrapolation of % quenching back to time of addition of H_2SO_4. For K^+-diffusion potentials (●), membrane vesicles were loaded by incubation with 2 mM K^+/Mes (pH 7.0) during 3 hr at 4°C. Thirty micrograms of tonoplast vesicle protein loaded with 2 mM K^+/Mes were incubated in 2 ml of 2 mM Tris/Mes, pH 7.0/250 mM sorbitol/2 μM valinomycin/2 μM Oxonol-V and changes in fluorescence were initiated by the addition of aliquots of 0.5 M K_2SO_4.

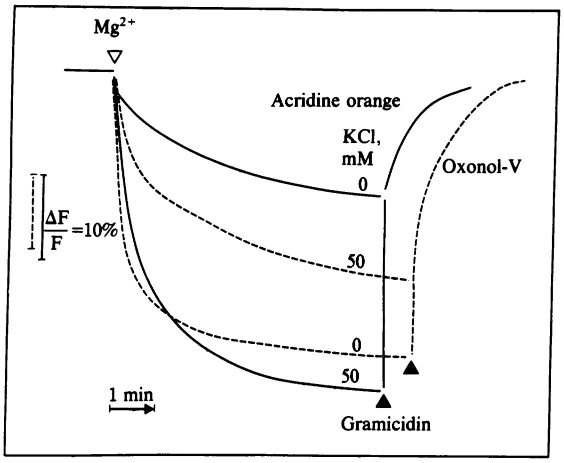

FIG. 4. Response of acridine orange and Oxonol-V during ATPase activity. Membrane vesicles containing 30 μg of protein were incubated in 1 ml of 3 mM Tris/Mes, pH 8.0/3 mM Tris ATP/250 mM sorbitol containing 5 μM acridine orange or 2 μM Oxonol-V. ATPase activity was initiated by addition of 3 mM $MgSO_4$ as indicated. Gramicidin (▲) was added to a final concentration of 2 μM at the indicated times; 50 mM KCl was present as indicated. In the absence of KCl, 25 mM K_2SO_4 was included. F, fluorescence.

ions after a pH gradient had been generated by the ATPase. Chloride increased ΔpH as expected, but nitrate had a biphasic effect, causing first an increase and later a decrease in ΔpH. Although nitrate is known to inhibit the ATPase, the concentrations used here were too low to have a significant inhibitory effect (see below). Moreover, the dissipation of ΔpH by nitrate in Fig. 5 was faster than that resulting from removal of ATP by addition of hexokinase plus glucose, again demonstrating that this effect of nitrate cannot be due to inhibition of the ATPase.

The results shown in Fig. 5 suggest that nitrate enters the vesicles, thus dissipating ΔΨ in the same manner as chloride does. The dissipation of ΔΨ should result in an increase in H^+-ATPase activity, which is known to be limited by the membrane potential in the absence of permeant counter-ions (18). This increase in ATPase activity could account for the initial effect of nitrate in increasing ΔpH. The subsequent dissipation of ΔpH could result from the efflux of nitrate together with protons via a nitrate/proton symport mechanism.

Further evidence that nitrate dissipates both ΔpH and ΔΨ is shown in Fig. 6. The vesicles were preequilibrated with nitrate before activation of the ATPase with Mg^{2+}; under these conditions there is no evidence of a biphasic effect on ΔpH. The initial rate of fluorescence quenching of both acridine orange and Oxonol-V was decreased by nitrate (see ref. 15 for a justification that the initial rate of quench of acridine dyes is proportional to the rate of H^+ influx). Only 1 or 2 mM nitrate was sufficient to give 50% inhibition of the fluorescence response, whereas 50% inhibition of ATPase activity in this preparation requires about 20 mM nitrate (8). Although the initial rates of quenching were greatly affected by the presence of KCl (Fig. 4), the percentage inhibition by nitrate was not very different (Fig. 6).

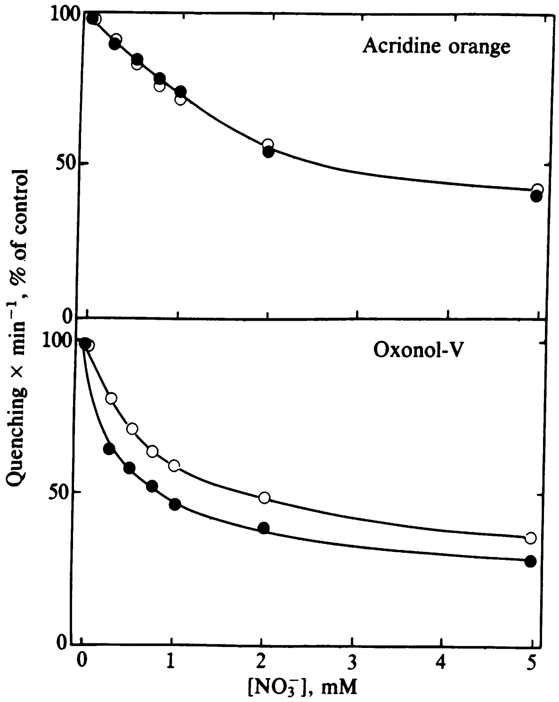

FIG. 6. Effect of nitrate on the rate of fluorescence quenching of acridine orange and Oxonol-V. Conditions as described in the legend to Fig. 1, except for the absence of K_2SO_4 and the presence (●) or absence (○) of 50 mM KCl.

Effects of Nitrate on ATPase Activity. Measurements of ATPase activity in the presence of gramicidin (Fig. 7) confirmed that direct inhibition of the ATPase by nitrate cannot account for its effects on proton gradients. On the contrary,

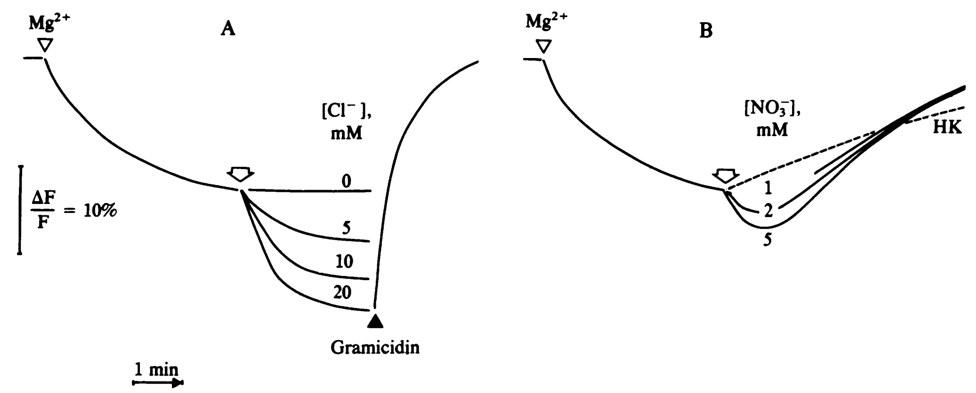

FIG. 5. Response of acridine orange during ATPase activity. Membrane vesicles containing 30 μg of protein were incubated in 2 ml of 30 mM Tris/Mes, pH 8.0/3 mM Tris ATP/250 mM sorbitol/5 μM acridine orange and stirred continuously. ATPase activity was initiated by addition of 3 mM $MgSO_4$ as indicated. Curves A: At the indicated time (⇦), aliquots of $(CH_3)_4N^+$ Cl^- were added at the final concentrations indicated. Curves B: At the indicated time (⇦), aliquots of $(CH_3)_4NNO_3$ were added at the final concentrations indicated or hexokinase (HK; EC 2.7.1.1, from yeast) and D-glucose were added to give final concentrations of 10 units/ml and 2 mM, respectively. ΔF/F, relative fluorescence.

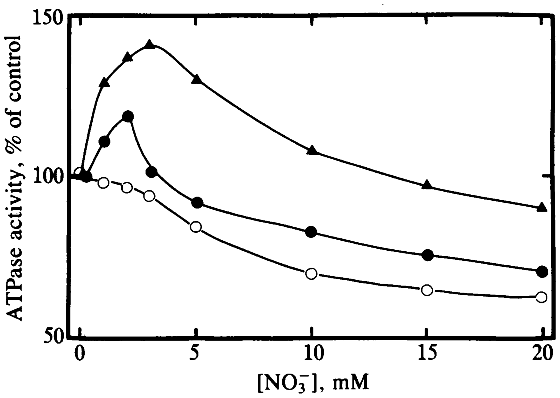

FIG. 7. Effect of nitrate on ATPase activity. Membrane vesicles containing 30 μg of protein were incubated in assay medium for ATPase activity (see *Materials and Methods*) in the presence of 50 mM KCl and 1 μM gramicidin (○); in the presence of 50 mM KCl (●); and in the absence of KCl (▲). $(CH_3)_4NNO_3$ was added to give the final concentrations indicated. Control (100%) activity was 56, 30, and 5.6 μmol of ATP/mg of protein per hr with KCl plus gramicidin, KCl without gramicidin, and no KCl, respectively.

ATPase activity in the absence of gramicidin was stimulated by nitrate, indicating a dissipation of $\Delta\Psi$ or ΔpH, or both, at transport sites distinct from the ATPase. Maximal stimulation of ATPase activity was observed when nitrate was added in the absence of other permeable ions. The counter-ion in this case was tetramethylammonium, which we have found to have no effect on $\Delta\Psi$ or ΔpH (results not shown). In the presence of KCl, the stimulation of ATPase activity by nitrate was smaller, presumably because the ATPase was already approaching its maximal rate and because dissipation of the potential by chloride allowed less nitrate to enter the vesicles. The time course of ATPase activity (not shown) revealed an immediate increase in rate on addition of nitrate, attributable to the dissipation of $\Delta\Psi$ and accounting for the transient increase in ΔpH seen in Fig. 4. There was an additional smaller increase in ATPase activity after 2–3 min, accompanying the dissipation of ΔpH (see Fig. 4). The data of Fig. 7 indicate the final rates of ATPase activity, which remained constant for at least 15 min.

Chloride and Nitrate Effects on ΔpH Generated by H^+-Pyrophosphatase. Comparison of chloride and nitrate effects in Figs. 4–6 indicates that both chloride and nitrate dissipate $\Delta\Psi$, but that nitrate, unlike chloride, also dissipates ΔpH. The low concentrations of nitrate required to dissipate ΔpH (Fig. 6), the rate of collapse of ΔpH (Fig. 5), and the stimulation of ATPase activity by nitrate (Fig. 7) all indicate that this effect cannot be due to inhibition of the ATPase. Nevertheless, it would be more convincing to compare the effects of chloride and nitrate on ΔpH in a situation in which neither anion can affect the proton pump. Such an opportunity is afforded by the recent demonstration (18) that a pyrophosphatase in this vesicle preparation also catalyzes H^+ transport. The pyrophosphatase is sensitive to univalent cations, being activated by K^+ but not by Na^+, and is insensitive to both chloride and nitrate (2, 18). Fig. 8 (reproduced from ref. 18) shows quenching of acridine orange fluorescence after activation of pyrophosphatase activity. The proton gradient formed in the presence of KNO_3 was less than that formed in the presence of KCl or K_2SO_4, showing once more that nitrate diminishes ΔpH by a mechanism independent of the proton pump. The apparently less complete dissipation of ΔpH in this case can be attributed to the fact that the pyrophosphatase is less active than the ATPase (a higher concentration of vesicles was used for this experiment); thus the membrane potential would not permit as large an influx of nitrate into the vesicles.

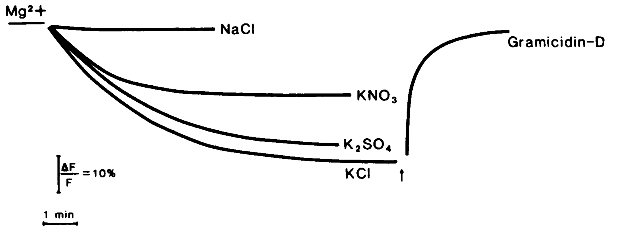

FIG. 8. Pyrophosphate-dependent quenching of acridine orange fluorescence (reproduced from ref. 18). Membrane vesicles containing 125 μg of protein were incubated in 1 ml of 30 mM Tris/Mes (pH 8.0) containing 3 mM Tris PP$_i$; KCl (50 mM), K_2SO_4 (25 mM), NaCl (50 mM), KNO_3 (50 mM), or gramicidin D (5 μM) were added as indicated. Pyrophosphatase activity was initiated by the addition of Mg^{2+}. $\Delta F/F$, relative fluorescence.

DISCUSSION

Identification of the Membrane Fraction as Tonoplast. The material used in this study consists of membranes collected from a 10%/23% sucrose interface after sucrose density gradient centrifugation of microsomal membranes from *B. vulgaris* (8). Identification of this material as tonoplast is based on the following evidence: (*i*) Membranes from lysed intact vacuoles band at this position on sucrose density gradient centrifugation (7). (*ii*) Marker enzyme activities indicate only small amounts of other identifiable membranes at this density. Thus glucan synthetase II activity is essentially zero (7), vanadate-sensitive ATPase is typically zero (8) or 4–8% of total ATPase (7), although it can be as high as 13% in some preparations (18). ATPase sensitive to azide, oligomycin, or both is 4% (7) or 9% (8) of total ATPase, cytochrome *c* oxidase activity is essentially zero (7), and latent IDPase is very low (8). Some NADH-cytochrome *c* reductase activity is present but is also found in intact vacuole preparations (8). (*iii*) The ATPase activity of this fraction has been well characterized (7, 8), is essentially identical in every respect to that of isolated intact vacuoles (2), and has a comparable specific activity. (*iv*) The pyrophosphatase activity of this fraction, due to a membrane-bound enzyme distinct from the ATPase (18), likewise is identical in properties with that of isolated intact vacuoles (19) and is of comparable specific activity. (*v*) Membranes from lysed intact vacuoles show ATPase and also proton transport properties very similar to those of the material used in the work reported here (7).

If these observations are not conclusive, they certainly represent strong *prima facie* evidence that the dominant component of the present membrane fraction is tonoplast. Furthermore, the present study is concerned largely with the effects of nitrate on pH and potential gradients generated by the tonoplast-type H^+-ATPase and by the H^+-transporting pyrophosphatase. Thus, the nitrate effects are seen in the same vesicles that contain the two enzymes identified in isolated vacuoles.

Nitrate Effects. High concentrations of nitrate are known to inhibit the H^+-ATPase. The physiological significance of this is not clear. A nitrate-insensitive H^+-transporting pyrophosphatase (18), of lower activity than the ATPase, may play a role in maintaining vacuole function at high nitrate levels. Cytoplasmic nitrate concentrations of 1–5 mM have relatively small effects on the ATPase in this material (Fig. 7). We have presented various lines of evidence that low concentrations of nitrate (1–5 mM) cause a partial dissipation of both ΔpH and $\Delta\Psi$ at vesicle transport sites distinct from the H^+-ATPase. In contrast, chloride dissipates only $\Delta\Psi$. An investigation of nitrate effects in a vesicle preparation from oat roots (16) showed no sign of such transport effects of nitrate. The greater sensitivity of the ATPase to nitrate in that study may have obscured the effects of nitrate transport.

A simple model applicable to nitrate transport in membrane vesicles of *B. vulgaris* and, by extension, to nitrate accumulation in and retrieval from vacuoles *in vivo* is shown in Fig. 9. According to this model, nitrate enters the vacuole (or membrane vesicle) through a uniport pathway in response to the potential generated by the H^+-ATPase, thus causing a partial dissipation of $\Delta\Psi$. The accumulated nitrate may then exit via a proton-symport pathway, thereby dissipating ΔpH. The different results seen for chloride can be readily explained if there is no proton-symport efflux pathway for that ion. Then only dissipation of $\Delta\Psi$ would be observed, and this will eventually disappear when the vesicle is "filled" with chloride (i.e., when the chloride distribution comes to equilibrium with the potential).

It may be noted that the dissipation of membrane potential by nitrate, unlike that due to chloride, does not decrease with time (unpublished observation), suggesting that the distribution of nitrate does not come to equilibrium with the membrane potential. The partial dissipation of ΔpH also appears to continue indefinitely in the presence of nitrate. However, when the nitrate concentration is reduced by dilution, the pH gradient recovers, indicating that nitrate causes no permanent change in the membrane. In further contrast with the results for chloride, pH gradients generated by a proton-translocating pyrophosphatase (Fig. 8) were dissipated by nitrate, although the pyrophosphatase is not inhibited by this ion.

In addition to providing a simple explanation for the present results, the model of Fig. 9 makes sense in terms of physiology, since a means is provided for the retrieval of nitrate from the vacuole when required for metabolism, whereas there would seem to be little need for retrieval of chloride, which serves mainly as an osmoticum in the vacuole. The energy cost of the apparently futile cycle of nitrate in and out of the vacuole may be the price paid for the availability of the stored nitrate. However, it is also likely that the influx and efflux of nitrate are under regulatory control in the intact cell. Thus, Aslam *et al.* (20) have given evidence that transfer of nitrate from a storage pool to a metabolic pool in etiolated barley leaves is regulated by light. Such control could be achieved either by altering the balance between $\Delta\Psi$ and ΔpH through the action of other transport systems or by direct regulation of the activity of the putative nitrate/proton symporter.

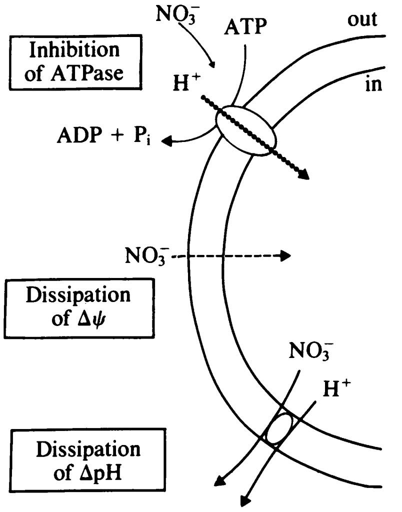

FIG. 9. Model relating experimental observations to proposed mechanisms for nitrate influx and efflux across the tonoplast membrane. For details see text.

We thank Dr. Phil Rea for the initial suggestion, based on his study of the H^+-pyrophosphatase (Fig. 8), that nitrate dissipates ΔpH in this material. Thanks are due to Dr. Heven Sze for suggesting the experiment of Fig. 5 and to Morris Manolson for helpful discussion. This research was supported by the Natural Sciences and Engineering Research Council of Canada and by the Department of Education of Quebec.

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