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Membrane Transport in Isolated Vesicles from Sugarbeet Taproot[1]

I. ISOLATION AND CHARACTERIZATION OF ENERGY-DEPENDENT, H^+-TRANSPORTING VESICLES

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DONALD P. BRISKIN*, W. ROBERT THORNLEY, AND ROGER E. WYSE
Plant Biochemistry and Bioregulation Laboratory, United States Department of Agriculture, Agricultural Research Service and Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322

ABSTRACT

Sealed membrane vesicles were isolated from homogenates of sugarbeet (*Beta vulgaris* L.) taproot by a combination of differential centrifugation, extraction with KI, and dextran gradient centrifugation. Relative to the KI-extracted microsomes, the content of plasma membranes, mitochondrial membranes, and Golgi membranes was much reduced in the final vesicle fraction. A component of ATPase activity that was inhibited by nitrate co-enriched with the capacity of the vesicles to form a steady state pH gradient during the purification procedure. This suggests that the nitrate-sensitive ATPase may be involved in driving H^+-transport, and this is consistent with the observation that H^+-transport, in the final vesicle fraction was inhibited by nitrate. Proton transport in the sugarbeet vesicles was substrate specific for ATP, insensitive to sodium vanadate and oligomycin but was inhibited by diethylstilbestrol and *N,N'*-dicyclohexylcarbodiimide. The formation of a pH gradient in the vesicles was enhanced by halide ions in the sequence $I^- > Br^- > Cl^-$ while F^- was inhibitory. These stimulatory effects occur from both a direct stimulation of the ATPase by anions and a reduction in the vesicle membrane potential. In the presence of Cl^-, alkali cations reduce the pH gradient relative to that observed with bis-tris-propane, possibly by H^+/alkali cation exchange. Based upon the properties of the H^+-transporting vesicles, it is proposed that they are most likely derived from the tonoplast so that this vesicle preparation would represent a convenient system for studying the mechanism of transport at this membrane boundary.

Energy dependent, primary proton transport appears to be a ubiquitous property of higher plant cells (21 and references therein). The energy conserved in the electrochemical potential gradient of protons, the proton motive force, can then provide the driving force for the transport of other solutes such as sugars according to the chemiosmotic hypothesis (18). The latter process has been termed 'secondary' transport since the coupling to metabolic energy is indirect via an ion gradient (11 and references therein). Studies with intact plant cells have suggested that primary proton transport can occur both at the plasma membrane (cytoplasm to cell exterior) and at the tonoplast (cytoplasm to vacuole lumen) (27 and references therein). When membrane fractions enriched with plasma membrane (16) or tonoplast (15) are prepared, they are found to contain ATP hydrolytic activity which has been postulated to reflect the presence of ATP-fueled proton pumps responsible for carrying out these primary transport events (16, 21, 27).

A significant advance to the study of membrane transport in higher plants came with the development of the methodology to isolate sealed membrane vesicles by Sze (25). The sealed vesicle system allowed the demonstration that these membrane-associated ATPases isolated from higher plant cells could directly transport protons (27). Several laboratories have since characterized ATPase-mediated proton transport in sealed vesicles thought to be derived from the plasma membrane (27 and references therein) and from the tonoplast (22 and references therein). These two types of proton-transporting vesicles can be distinguished by differences with respect to ion stimulation of proton transport (and ATPase activity), inhibitor sensitivity, and intrinsic membrane density (7, 17, 26, 27).

A major goal of this laboratory is to understand the mechanism and energization of sucrose transport at the plasma membrane and tonoplast of plant cells within the context of photosynthate partitioning. Achieving this goal will require a thorough knowledge of the driving forces for sucrose transport and the properties of the membrane carriers responsible for coupling this driving force to the movement of sucrose. Although work up to the present time with sealed vesicles has emphasized ATP-dependent primary transport, this system can be extremely useful when utilized to investigate a secondary process such as sucrose transport. Indeed, sealed membrane vesicle systems have proven useful when applied to secondary transport systems present in bacterial (12 and references therein), fungal (9), and animal (28 and references therein) cell membranes.

The two papers presented in this series describe the development of a sealed vesicle system isolated from sugarbeet taproot, useful for studying the properties of sucrose transport across the tonoplast. This paper describes the isolation and characterization of the sugarbeet vesicles while in a subsequent paper (6), the sugarbeet vesicle system is used to examine the mechanism of sucrose transport.

MATERIALS AND METHODS

Plant Material. Sugarbeets (*Beta vulgaris* L. var GWD-2) were greenhouse grown in 3.5-gallon pots. Nutrient solution (Mon-

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santo)[2] was applied twice a week and natural light was supplemented with fluorescent lamps. Following harvest, the tops of the plants were removed and the storage roots were maintained at 4°C until use.

Isolation of Membrane Vesicles. Sealed membrane vesicles were isolated by a modification of the method of Sze (25, 26). The storage roots were cut into sections and homogenized with an Oster vegetable juice extractor in a medium containing 250 mM sorbitol, 2 mM EGTA, 15 mM β-mercaptoethanol, 0.5% (w/v) BSA (fraction V powder), 5% (w/v) polyvinylpyrrolidone (PVP-40), 70 mM Tris-HCl (pH 8.0), and 4 mM DTE. The homogenate was filtered through four layers of cheesecloth and centrifuged at 7,700*g* (8,000 rpm) for 15 min in Sorvall SS-34 rotor. The pellets were discarded, and the supernatant was centrifuged at 80,000*g* (32,500 rpm) for 30 min in an IEC A-237 rotor to obtain a microsomal pellet. The microsomal pellet was suspended in 250 mM sorbitol, 1 mM bis-tris-propane/Mes (pH 7.2), 1 mM DTE (vesicle suspension media) to a protein concentration of about 1.5 mg/ml and treated with 0.25 M KI in vesicle suspension buffer to reduce nonspecific phosphatase (5). Following incubation for 20 min on ice, the KI-treated membranes were centrifuged at 80,000*g* (32,500 rpm) for 30 min in an IEC A-237 rotor. The KI-extracted membrane pellet was suspended in 4.0 ml of vesicle suspension media and layered over a cushion of 6% (w/w) dextran in vesicle suspension media. The dextran step gradient was centrifuged at 115,000*g* (26,000 rpm) for 2 h in an IEC SB-283 rotor. Following centrifugation, the sealed vesicles present at the 6% dextran interface were removed with a pasteur pipette.

Enzyme Assays. Phosphate hydrolyzing activity was measured in a 1.0-ml reaction volume containing 0.1 ml of membrane suspension and the released Pi was determined by the method of Ames (1). The reactions were carried out for 15 to 20 min at either 38°C (Table I) or 25°C (Table VI). For the determination of ATPase activity, the standard assay contained 3 mM ATP (tris-salt, pH 7.2), 3 mM $MgSO_4$, 50 mM KCl, and 30 mM Tris-Mes (pH 7.2). Inhibitor sensitive components of ATPase represented the differences in activity observed in the absence and presence of each inhibitor. For the determination of Triton-stimulated UDPase activity (19), the reaction contained 3 mM UDP (tris-salt, pH 6.5), 3 mM $MnSO_4$, 30 mM Tris-Mes (pH 6.5), and 0.03% (v/v) Triton X-100 (when present). To remove Triton interference with the Ames assay procedure (1), 1.5% (w/v) SDS (phosphate free) was present in the acid-molybdate-ascorbate color reagent. Triton-stimulated UDPase represented the difference in activity measured in the presence or absence of the detergent.

Optical Measurement of the Vesicle pH Gradient. Proton transport in membrane vesicles was measured by the quenching of quinacrine fluorescence (3, 13, 26). The standard assay contained 250 mM sorbitol, 5 mM ATP (bis-tris-propane salt, pH 7.2), 5 mM $MgSO_4$, 5 μM quinacrine, 50 mM KCl, 25 mM bis-tris-propane/Mes (pH 7.2), and about 50 μg membrane protein. Any variation in the reaction conditions are indicated in "Results." The fluorescence measurements were made at room temperature (25°C) with a Perkin-Elmer LS-5 Spectrofluorimeter with the excitation monochrometer set at 430 nm and the emission monochrometer set at 500 nm.

Optical Measurement of the Vesicle Membrane Potential. The production of a vesicle membrane potential was monitored by the quenching of Oxonol V fluorescence (24, 29). Membrane potential measurements were carried out in the presence of 250 mM sorbitol, 5 mM (bis-tris-propane salt, pH 7.2), 5 mM $MgSO_4$, 15 μM Oxonol V, 25 mM bis-tris-propane, Mes (pH 7.2), 50 mM monovalent ions (when present), and about 50 μg membrane protein. The fluorescence was measured at room temperature (25°C) with the excitation monochrometer set at 590 nm and the emission monochrometer set at 650 nm. Further details on the conditions of membrane potential measurement are given in Figure 2.

Protein Assay. Protein was determined by the method of Bradford (4) using BSA as a standard. The protein content of the homogenate was estimated by noting the volume increase in the homogenization media due to the presence of the homogenized tissue and determining the protein in fresh homogenizing media diluted to the same extent. This value for the protein contributed by the BSA present in the homogenization media was then subtracted from the total protein content of the homogenate to estimate the amount of protein contributed by the plant tissue.

RESULTS AND DISCUSSION

Isolation of Sealed Vesicles from Sugarbeet Taproot. The method used to isolate sealed membrane vesicles was based upon the published procedure of Sze (25, 26) modified for the use of sugarbeet storage tissue. A mechanized homogenization method was required in order to disrupt the storage tissue and the best results were obtained using an Oster vegetable juice extractor. Since our goal was to use the sealed vesicle system for the study of sucrose transport, it was desirable to isolate the vesicles in the absence of sucrose. For this reason, sorbitol replaced sucrose as the osmoticum in both the homogenization and suspension media. In addition the presence of 15 mM β-mercaptoethanol, 0.5% (w/v) BSA, 5% (w/v) polyvinylpyrrolidone (PVP-40), and 4 mM DTE as protectants in the homogenization media were essential to the recovery of sealed vesicles competent in H^+-transport.

In preliminary experiments, the sugarbeet root homogenate was centrifuged at 13,000*g* prior to collection of microsomal membranes by centrifugation at 80,000*g* and following KI extraction of the microsomes (to diminish nonspecific phosphatase, see Briskin and Poole [5]), the sealed vesicles were recovered at a 10% (w/w) dextran gradient interface (25, 26). It was found, however, that maximal enrichment of H^+-transporting activity on a protein basis relative to a reduction in contaminant marker enzyme activities (see below) occurred when the low speed centrifugation was reduced to 7,700*g* and the density of the dextran at the gradient interface was reduced to 6% (w/w). The distribution of phosphohydrolase activity, H^+-transport, and protein during the isolation of sealed vesicles by this method is shown in Table I.

It is apparent that as the sugarbeet membranes are carried through the isolation procedure, the proportion of the control ATPase activity in each fraction that is sensitive to nitrate is progressively increased. In contrast, the ATPase activity that is sensitive to azide, an inhibitor of mitochondrial F_1-ATPase (25 and references therein), is progressively decreased during the isolation procedure. Vanadate-sensitive ATPase activity, representative of the plasma membrane ATPase (and possibly nonspecific phosphatase) (10, 19) and Triton X-100 stimulated UDPase, representative of Golgi membranes were first enriched to the KI-extracted microsomal pellet and then substantially decreased when the membranes were centrifuged on a 6% dextran cushion. Comparisons between the properties of ATPase activity associated with isolated vacuoles (14 and references therein, 15) and the properties of ATPase and H^+-transport activity in sealed vesicle fractions isolated from microsomes (2, 3, 17, 20, 22) have strongly suggested that this ATPase activity and H^+-transport that is nitrate sensitive but insensitive to vanadate, azide, or oligomycin is associated with the tonoplast. Therefore, it would appear that the final vesicle fraction is

[2] The mention of firm names or trade products does not imply that they endorsed or were recommended by the United States Department of Agriculture over other firms or similar products not mentioned.

Table I. *Distribution of Phosphohydrolase Activities, H+-Transport, and Protein during the Isolation of Membrane Vesicles from Sugarbeet Taproot*

	Fraction			
	Homogenate	Microsomal	KI-extracted	Vesicles
ATPase activities[a] (μmol Pi/h·mg)				
Control	26.1 (100)	29.0 (100)	33.5 (100)	34.1 (100)
ΔKNO_3	4.2 (16.1)	7.4 (25.5)	10.6 (31.6)	19.3 (56.6)
ΔNaN_3	10.6 (40.6)	6.9 (23.8)	3.9 (11.6)	2.4 (7.0)
ΔN_3VO_4	14.7 (56.3)	22.4 (77.2)	25.6 (76.4)	8.8 (25.6)
Triton stimulated UDPase (μmol Pi/h·mg)	21.5	30.0	28.9	3.5
H+-Transport[b] (% quench/mg protein)	47.0	122.8	161.6	310.0
Protein (mg)	290.0[c]	31.2	21.8	2.76

[a] Control ATPase assay contained 3 mM ATP (Tris salt, pH 7.2), 3 mM $MgSO_4$, 30 mM Tris Mes (pH 7.2), 50 mM KCl, and 20 to 40 μg protein. Inhibitor-sensitive components of ATPase represent the difference between the control activity and the activity in the presence of 50 mM KNO_3 (ΔKNO_3) or 10 mM NaN_3 (ΔNaN_3), or 50 μM (ΔNa_3VO_4). When the effect of NO_3^- was examined, KNO_3 replaced KCl in the assay. Values in parentheses represent the percent of the control activity. [b] Assayed in the presence of 5 mM ATP (bis-tris-propane salt, pH 7.2), 5 mM $MgSO_4$, 50 mM KCl, 2 μM Quinacrine, 250 mM sorbitol, and 25 mM bis-tris-propane/Mes (pH 7.2). The data are expressed as the ionophore reversible quench per mg protein (see Fig. 1). [c] Estimated as described in "Materials and Methods."

enriched with membranes derived from the tonoplast and that the amount of membranes derived from the plasma membrane, Golgi apparatus, and mitochondria is much reduced.

In the purification of the vesicles from the microsomal pellet to the final dextran gradient fraction, a close correspondence between the enrichment in nitrate-sensitive ATPase activity and ATP-dependent H+-transport was observed (Table I). This suggests that the nitrate-sensitive component of ATPase may be involved in driving H+-transport. Consistent with this proposal is the observation that H+-transport, in the final dextran gradient fraction, is inhibited by nitrate (Fig. 1). The lack of a correspondence between the fold enrichment of nitrate-sensitive ATPase and H+-transport from the homogenate to the microsomal pellet may result because nitrate sensitivity is not absolutely specific for the putative tonoplast ATPase. The mitochondrial ATPase is also sensitive to nitrate (22 and references therein) and much of this activity would be removed by the 7,700*g* centrifugation.

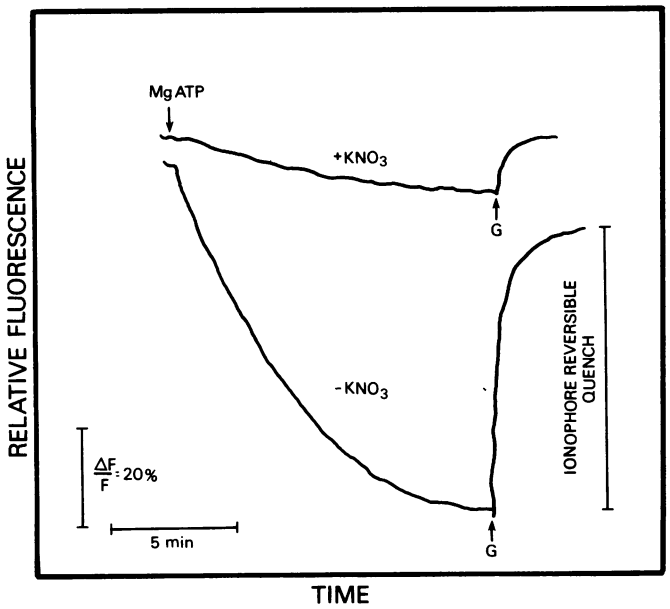

FIG. 1. Quenching of quinacrine fluorescence by sugarbeet vesicles. Proton transport was measured in the presence of 250 mM sorbitol, 5 mM ATP, 5 mM $MgSO_4$, 50 mM KCl, 5 μm quinacrine, and 25 mM bis-tris-propane/Mes (pH 7.2). Fluorescence was measured at 25°C with the excitation at 430 nm and emission at 500 nm. Nitrate (KNO_3), when present, was at 50 mM. Fluorescence quenching was reversed by the addition of 5 μM gramicidin D (G).

In quantitating H+-transport measured optically by the quenching of quinacrine fluorescence (3, 13, 26), we have expressed the data in terms of the steady state pH gradient established across the vesicle membrane. This is taken as the fluorescence (%) restored from the steady state level of fluorescence by the addition of gramicidin D, a channel-forming ionophore (Ionophore Reversible Quench, Fig. 1). In contrast, other laboratories (3, 22) have often used the initial rate of fluorescence quenching (% quench min^{-1}) for quantitation of H+-transport since this would correspond to the activity level of H+-ATPase (3). Since it is our interest in subsequent studies to use the H+-ATPase to produce the driving force for sucrose transport, steady state pH gradient formation is a more important factor in our case. However, it was found that a correspondence between the initial rate of fluorescence quenching and the magnitude of the steady state pH gradient generally occurred. Therefore, the properties of the H+-transporting vesicles presented in terms of steady state pH gradient formation in this study are comparable to those properties expressed in terms of the initial rate of fluorescence quench in other studies.

Substrate Specificity of H+-Transport. ATP was the most effective substrate for the establishment of a steady state pH gradient in sugarbeet vesicles (Table II). GTP and UTP could only partially substitute for ATP; however, no measurable transport was detected with ADP or *p*-nitrophenol phosphate. Churchill and Sze (7), Bennett *et al.* (2), and more recently Rea and Poole (23) have demonstrated that pyrophosphate can drive H+-transport in tonoplast vesicle preparations. The vesicles isolated from sugarbeet taproot in this study could also transport H+ using pyrophosphate but always to a lesser extent than the H+-transport found with ATP (Table II). In the studies carried out by Rea and Poole (23), kinetic analysis suggested that ATP and pyrophosphate driven H+-transport were the result of two separate enzymes. This would be consistent with the different deter-

Table II. *Substrate Specificity of H⁺-Transport in Membrane Vesicles Isolated from Sugarbeet Taproot*

Substrate[a]	H⁺-Transport
	% ionophore reversible quench
ATP	57
GTP	7
UTP	2
ADP	0
p-nitrophenyl-P	0
PPi	30.1

[a] Phosphate compounds with the exception of *p*-nitrophenyl phosphate were present as bis-tris-propane salts (pH 7.2).

Table III. *Effect of Various Inhibitors on H⁺-Transport in Membrane Vesicles from Sugarbeet Taproot*

Inhibitor[a]	H⁺-Transport
	% ionophore reversible quench
Control	38
50 mM KNO_3	16
100 mM KNO_3	9
50 μM Na_3VO_4	37
10 μg/ml Oligomycin	36
10 μM DES	7
10 μM DCCD	27

[a] Control assay was carried out in the presence of 5 mM ATP, 5 mM $MgSO_4$, 5 μm quinacrine, 50 mM KCl, 25 mM bis-tris-propane/Mes (pH 7.2), and 250 mM sorbitol. When the effect of NO_3^- was examined, KNO_3 replaced KCl in the assay. Oligomycin, DES, and DCCD were present in 0.5% ethanol.

gent solubilities of the ATPase and pyrophosphatase activities associated with tulip petal vacuoles (30).

Inhibitor Sensitivity of H⁺-Transport. As previously stated, the H⁺-transport in the final vesicle fraction was inhibited by nitrate (Fig. 1). Fifty mM nitrate resulted in about a 68% reduction in the steady state pH gradient while in the presence of 100 mM, a 89% reduction was observed (Table III). The production of a pH gradient by the sugarbeet vesicles, however, was unaffected by vanadate, an inhibitor of plasma membrane ATPase (10, 20) and oligomycin, an inhibitor of mitochondrial ATPase (22 and references therein). This indicates that although residual plasma membrane (ΔNa_3VO_4, Table I) and mitochondrial ATPase (ΔNaN_3, Table I) activities are present in the final vesicle fraction, these transport enzymes cannot account for the observed H⁺-transport. DES[3] and DCCD were also inhibitors of H⁺-transport in the sugarbeet vesicle system (Table III). These two agents have been shown to inhibit the ATPase associated with isolated vacuoles (14, 15) and the nitrate sensitive H⁺-ATPase present in vesicle fractions isolated from several species (22 and references therein).

Ion Effects upon H⁺-Transport and ATPase Activity. The effect of various monovalent cations (present as Cl^- salts) upon the production of a vesicle pH gradient was examined (Table IV). Proton transport was enhanced in the presence of the monovalent ions and nearly similar steady state pH gradients were found with K^+, Rb^+, Cs^+, Na^+, and Li^+. A larger pH gradient and higher initial quench rate was consistently observed when bis-tris-propane replaced alkali cations in the transport assay. This was also observed by Sze (26) and may reflect a reduction in the pH gradient with monovalent cations possibly by a H^+/monovalent cation exchange process.

When the effect of various anions (present as K^+ salts) upon

[3] Abbreviations: DES, diethylstilbestrol; DCCD, *N,N'*-dicyclohexylcarbodiimide; IDA, iminodiacetate.

Table IV. *Effect of Various Cations on H⁺-Transport in Membrane Vesicles from Sugarbeet Taproot*

Ions[a]	H⁺-Transport
	% ionophore reversible quench
Control	14
KCl	33
RbCl	31
CsCl	34
NaCl	30
LiCl	31
BTP/Cl	39

[a] Control assay was carried out in the presence of 5 mM ATP, 5 mM $MgSO_4$, 5 μM quinacrine, 25 mM bis-tris-propane/Mes (pH 7.2), and 250 mM sorbitol. Monovalent salts were present at 50 mM.

Table V. *Effect of Various Anions on H⁺-Transport in Membrane Vesicles from Sugarbeet Taproot*

Ions[a]	H⁺-Transport
	% ionophore reversible quench
Control	14
KCl	33
KBr	35
KI	56
KF	10
KNO_3	5
K_2SO_4	15
KIDA	16

[a] Control assay was carried out in the presence of 5 mM ATP, 5 mM $MgSO_4$, 25 mM bis-tris-propane/Mes (pH 7.2), 5 μM quinacrine, and 250 mM sorbitol. Anions were present at 50 mM.

the production of a vesicle pH gradient was tested (Table V), I^- was found to have the greatest effect. The relative order by which anions stimulated pH gradient production was found to be: $I^- > Br > Cl^- > SO_4^{2-} > IDA^-$ while NO_3^- and F^- were inhibitory. The large stimulation by I^- was consistently observed and most pronounced when the KI stock solution was freshly prepared. If the solution was stored for extended periods of time, I^- stimulation was diminished to a level below that of Cl^- or Br^-. This most likely reflects decomposition of the KI stock by oxidation to iodine and iodate.

Based upon the results presented in Table IV and Table V, it is apparent that the production of a pH gradient in sugarbeet vesicles is most affected by the anion present in the assay. Alkali cations present as Cl^- salts gave approximately the same pH gradient (although slightly less than bis-tris-propane) while K^+ present with IDA gave little stimulation above the level found in the absence of monovalent ions. Anions could stimulate pH gradient formation by two separate mechanisms or both could occur simultaneously. One possibility is that anions could increase the pH gradient by directly stimulating the ATPase as an enzyme activator. The other possibility would be related to a reduction in the vesicle membrane potential. Since the total energy available in the electrochemical gradient of protons ($\Delta\mu H^+$ in mv) is composed of both a membrane potential component ($\Delta\psi$, in mv) and a pH gradient component (ΔpH, in pH units) which are interconvertible:

$$\Delta\mu H^+ = \Delta\psi + 59\ \Delta\text{pH (at 25°C)}$$

then if the anion is permeant and can reduce $\Delta\psi$, it will increase ΔpH.

The effect of various anions on the ATPase activity associated with the sugarbeet vesicles was then tested (Table VI). The activity was assayed in the presence of 50 μM vanadate and 10 μg/ml oligomycin which did not affect pH gradient formation

Table VI. *Effect of Various Anions on ATPase Activity in Membrane Vesicles from Sugarbeet Taproot*

Ions	ATPase Activity[a]		
	− Gramicidin	+ Gramicidin	Gramicidin stimulation
	μmol Pi/h·mg		*Difference*
Control	15.1	29.2	14.1
KCl	22.6	44.1	21.5
KBr	23.3	44.3	21.0
KI	28.2	42.8	14.6
KF	5.9	17.7	11.8
KIDA	15.6	32.3	16.7
K_2SO_4	15.8	31.9	16.1

[a] ATPase was assayed at 25°C in the presence of 250 mM sorbitol, 5 mM ATP (bis-tris-propane salt, pH 7.2), 5 mM $MgSO_4$, 25 mM bis-tris-propane/Mes (pH 7.2), and 17 μg of membrane protein. Fifty μM Na_3VO_4 and 10 μg/ml oligomycin were present in all assays and anions (when added) were present at 50 mM. Gramicidin D was present at 5 μM.

but would reduce the level of plasma membrane and mitochondrial ATPase activity. In the absence of gramicidin D, which would collapse the proton electrochemical gradient, the relative order of anion stimulation of ATPase was similar to the relative order of anion stimulation of pH gradient formation. When gramicidin D was present all of the activities were increased indicating that the H^+-translocating ATPase is regulated by the proton electrochemical gradient. However, the relative order of anion stimulation of ATPase above the level with $MgSO_4$ alone changed so that I^-, Cl^-, and Br^- now showed a similar effect. In the presence or absence of gramicidin D, IDA^-, and SO_4^{2-} were similar to the control treatment while F^- was inhibitory. Since I^-, Cl^-, and Br^- stimulate ATPase activity above the control level under conditions where the proton electrochemical gradient is collapsed, these ions must have a direct effect upon the H^+-ATPase. Since the direct effect upon the H^+-pump is the same for these anions, the sequence: $I^- > Br^- > Cl^-$ for the stimulation of ATPase in the absence of gramicidin D and for pH gradient formation may be related to the ability of these anions to dissipate the vesicle membrane potential.

To test this proposal, the vesicle pH gradient and membrane potential were examined under various ionic conditions (Fig. 2). The vesicle membrane potential was monitored by the quenching of oxonol V fluorescence (24 and references therein). This probe, which responds to a positive interior vesicle membrane potential, demonstrates a fluorescence quench which is reversible by the addition of gramicidin D or CCCP (24, 29). When the assays were carried out in the absence of monovalent ions, a relatively small steady state pH gradient and relatively large membrane potential were found. When monovalent ions were included in the assay, it was found that the relative order of effectiveness for monovalent anions in stimulating pH gradient formation was also the order for decreasing the steady state membrane potential. When valinomycin was included with KI, in a separate experiment, the membrane potential was fully collapsed and the rapid attainment of a steady state pH gradient occurred. Since this assay was carried out with a different membrane preparation, the steady state pH gradient cannot be compared to the other treatments; however, it was generally found to be slightly larger than a KI treatment in the absence of valinomycin.

Taken together, the results of this section indicate that halide anions stimulate pH gradient formation both by a direct effect upon the ATPase and by reducing the membrane potential. The latter effect most likely accounts for the sequence: $I^- > Br^- > Cl^-$ observed for anions in stimulating pH gradient formation. This relative order of effectiveness of the halide anions is different than that observed by other workers (2, 17) for stimulating H^+-

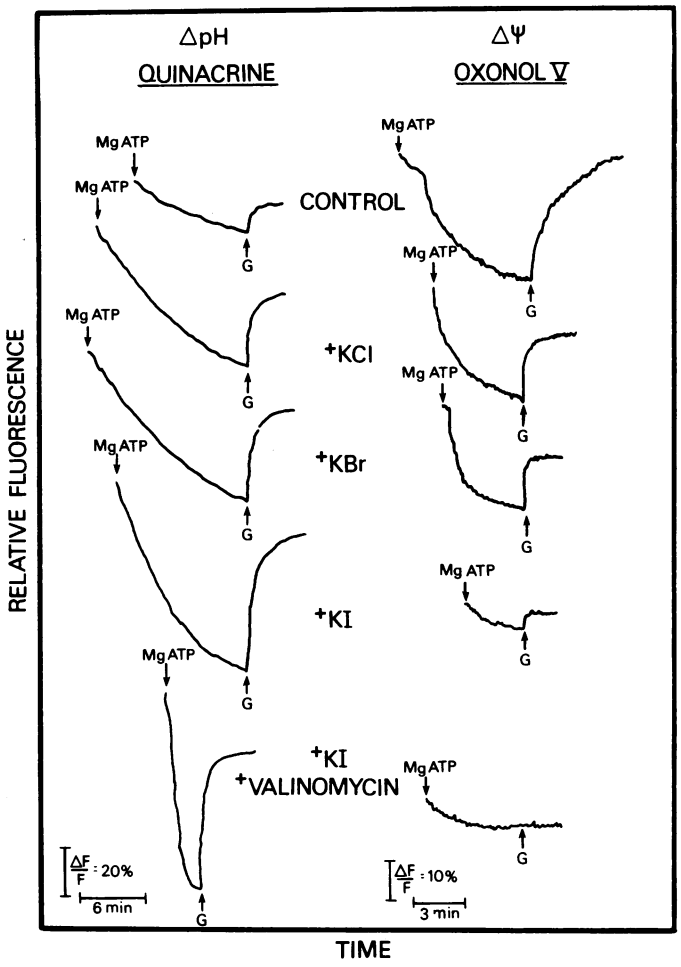

FIG. 2. Comparison of the vesicle pH gradient and membrane potential found under various ionic conditions. Measurement of H^+-transport was the same as in Figure 1 except that the control assay contained 250 mM sorbitol, 5 mM ATP, 5 mM $MgSO_4$, and 25 mM bis-tris-propane/Mes (pH 7.2). For the measurement of the pH gradient, 5 μM quinacrine was present while for the measurement of the vesicle membrane potential 15 μM oxonol V was present. When added, monovalent ions were present at 50 mM and valinomycin at 5 μM.

Table VII. *Effect of Refrigerator Storage on H^+-Transport in Membrane Vesicles from Sugarbeet Taproot*

Storage Time[a]	H^+-Transport[b]
h	*% ionophore reversible quench*
Initial	45
24	47
48	41
72	32

[a] Samples stored in refrigerator at 4°C. [b] Assayed in the presence of 5 mM ATP, 5 mM $MgSO_4$, 5 μM quinacrine, 50 mM KCl, 25 mM bis-tris-propane/Mes (pH 7.2), and 250 mM sorbitol.

transport where Cl^- and Br^- were generally found to have the greatest effect. This order of effectiveness, however, is similar to the order of increasing heats of hydration and the order of increasing ionic radius (8 and references therein). Thus the degree to which these halide anions can act as permeant-charged species to reduce the membrane potential (and increase the pH gradient) may be related to a physiochemical process such as the removal of associated water. Whether or not this is acomplished by an anion channel as proposed by Bennett and Spanswick (3) is

uncertain.

Stability of the Sugarbeet Vesicles. To test the stability of the sugarbeet vesicles for pH gradient formation, membrane preparations were stored for up to 72 h at 4°C and H^+-transport was assayed at 24-h time intervals (Table VII). The sugarbeet vesicle preparation was exceptionally stable and full transport capability could be observed after 24 h of storage. Transport began to decline after 48 h to a level that was 71% of the initial level at 72 h. If the vesicles were frozen under liquid N_2 and maintained at −80°C for 24 h, a 20% loss in H^+-transport capability occurred most likely from freezing damage.

CONCLUSIONS

By a modification of the method of Sze (25, 26), sealed membrane vesicles could be isolated from sugarbeet taproot which were capable of carrying out ATP-dependent, H^+-transport. Marker enzyme analysis revealed that the final vesicle preparation was enriched with membranes derived from the tonoplast and that the relative amount of membranes derived from the plasma membrane, Golgi apparatus, and mitochondria was much reduced (Table I). The use of sugarbeet as a source of tonoplast vesicles has the advantage that vesicle preparations can be prepared in bulk quantities from the large storage taproot and that the H^+-transport capacity of the vesicles is exceptionally stable over time (Table VI).

Similar to what has been observed in putative tonoplast vesicles isolated from other species (3, 7, 17, 20), the ATPase activity and proton transport in sugarbeet vesicles was nitrate sensitive (Tables I and III), substrate specific for ATP (Table II), and stimulated by anions (Tables V and VI). As previously observed by Bennett and Spanswick (3) and Churchill and Sze (7), this stimulation occurs both by direct effects upon ATPase and by a reduction in the membrane potential. The relative order of stimulation by anions ($I^- > Br^- > Cl^-$), however, was different in the sugarbeet preparation with iodide having the largest effect. This relative order for anions was accounted for by membrane potential reduction (Fig. 2), and this may reflect the localized environment that anions must pass in order to carry out this process.

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