Biochimica et Biophysica Acta, 728 (1983) 267–280
Elsevier Biomedical Press

BBA 71542

CHARACTERIZATION AND SOLUBILIZATION OF NUCLEOTIDE-SPECIFIC, Mg^{2+}-ATPase AND Mg^{2+}-PYROPHOSPHATASE OF TONOPLAST *

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(Received August 30th, 1982)

Key words: Tonoplast; Mg^{2+}-ATPase; Pyrophosphatase; Proton transport; (Tulipa petal vacuole)

Nucleotide-specific, Mg^{2+}-dependent ATPase and Mg^{2+}-dependent pyrophosphatase were recovered with purified tonoplast obtained from isolated *Tulipa* petal vacuoles. Relative Mg^{2+}-dependent hydrolysis of ATP, GTP and pyrophosphate, the only substrates hydrolyzed to a substantial degree, was 1.0, 0.3, and 0.6, respectively. Tonoplast ATPase required Mg^{2+}, and essentially no Mg^{2+}-dependent or Mg^{2+}-independent *p*-nitrophenylphosphatase (which was associated with intact vacuoles) occurred with the membrane. Tonoplast ATPase was stimulated 10 to 30% by KCl, but was little effected by other cations (other than NH_4^+) or anions. No activity was observed with CaATP as substrate. The enzyme was cold stable and was inhibited by DCCD and Dio-9, but not by oligomycin. Its pH optimum was 7.0 and its specific activity was about 50 μmol P_i/mg protein per h at 37°C. Properties of membrane-bound and Polidocanol (polyoxyethylene ether, 9 lauryl ether) solubilized enzyme were similar. Reduced activity of solubilized enzyme was partially restored with phospholipids. Tonoplast ATPase appears to be an integral membrane component which requires phospholipids for maximal activity. Tonoplast Mg^{2+}-pyrophosphatase had a pH optimum of ≥ 8.5, was stimulated 2.5-fold by 50 mM KCl, and was largely lost upon detergent treatment. Properties of tonoplast ATPase observed are consistent with the characteristics of proton transport exhibited by isolated, intact *Tulipa* vacuoles (Wagner, G.J. and Lin, W. (1982) Biochim. Biophys. Acta 689, 261–266). These observations suggest that tonoplast ATPase functions in proton transport.

Introduction

The storage, sequestration and osmotic functions of higher plant vacuoles suggest the presence of an energy-transducing ATPase in the membrane enclosing the vacuolar compartment. Solute transport across this membrane, the tonoplast, may be initiated by transport ATPase like $(Na^+ + K^+)$-ATPase, by group translocation transport systems or it may be energized by a protonmotive force maintained by a proton translocating ATPase. In most mature plant cells, the cytoplasm-to-vacuole pH gradient is thought to be about 2 pH units [1], although in certain acid accumulators it is considerably greater [2]. Since electron transport chain components have not been found in association with tonoplast, the pH gradient which exists between cytoplasm and vacuole is probably maintained by a proton pump which translocates protons to the vacuole sap at the expense of ATP. The energy of this electrochemical gradient may be

* Research carried out at Brookhaven National Laboratory under the auspices of the United States Department of Energy.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(ethanesulfonic acid).

0005-2736/83/0000–0000/$03.00

used to energize solute transport via proton symports, antiports or uniports.

Evidence has been presented which supports the existence of ATPase in tonoplast from *Tulipa* and *Hippeastrum* petals [3,4], from storage roots of *Beta vulgaris* [5–7] and from lutoids of *Hevea brasiliensis* [8,9]. Tonoplast ATPase has also been observed in a number of plant tissues by cytochemical localization (Ref. 10 and references therein). Recently Marin et al. [11] demonstrated Mg^{2+}-dependent, electrogenic proton transport in *Hevea* lutoids and Wagner and Lin [12,13] observed proton transport in isolated petal vacuoles. ATP-dependent transport of sucrose [14], D-glucose, and its analog 3-*O*-methyl glucose [15] in red beet vacuoles and of citrate and basic amino acids (see Ref. 16) in lutoids has been reported.

In the present study we examined the properties of *Tulipa* tonoplast ATPase, paying particular attention to possible interference from nonspecific phosphatase and other phosphohydrolases. Tonoplast ATPase was solubilized and further characterized. Tonoplast Mg^{2+}-dependent, alkaline pyrophosphatase was also studied.

Materials and Methods

Preparation of protoplasts, vacuoles and tonoplast

Protoplasts and vacuoles of *Tulipa* cv. Aladdin were prepared essentially as previously described [17]. Tissue was digested for 4 to 5 h at 28°C to prepare protoplasts. Protoplasts were sedimented from digestion medium at $500 \times g$ and washed three consecutive times with 0.7 M mannitol, 20 mM Mes/KOH, pH 5.5 (protoplast-to-wash volume, 1 to 160) to remove digestion medium [18]. Vacuoles were prepared by gentle osmotic shock of washed protoplasts in 0.2 M KH_2PO_4/K_2HPO_4 (pH 8.0), 2 mM dithiothreitol. Sedimented vacuoles were resuspended in washing solution containing 0.7 M mannitol, 20 mM Hepes/KOH (pH 8), 2 mM dithiothreitol and 0.3 mM $MgCl_2$ (vacuole-to-wash volume, 1 to 30), were sedimented from this solution, and were finally lysed on resuspension in the above washing solution less mannitol [18]. Tonoplast was partially purified by sedimentation into step gradients consisting of 22% and 38% (w/w) sucrose steps containing 20 mM Hepes/KOH(pH 8), 2 mM dithiothreitol and 0.3 mM $MgCl_2$. Membrane (without color) equilibrated at the 22–38% sucrose interface after centrifugation at $115\,000 \times g$ for 30 min at 13°C. Linear sucrose gradients containing buffer, dithiothreitol and $MgCl_2$ in the same concentrations as the step gradients were centrifuged at $80\,000 \times g$ for 16 h at 13°C.

Phosphohydrolase assay

For all substrates (as Na^+ salts), reaction mixtures (0.5 ml) contained (unless otherwise specified), 50 mM Pipes/KOH (pH 7.0), 50 mM KCl, 13.6 mM Na_2EDTA, 3 mM substrate, sample and 18 mM $MgCl_2$. Reactions were initiated by addition of buffer, KCl, Na_2EDTA and substrate to sample with or without $MgCl_2$. Reaction mixtures were incubated at 37C for 0.5 to 1.0 h and terminated by addition of 0.5 ml of 10% trichloroacetic acid. Reaction mixtures containing boiled samples were used to monitor chemical hydrolysis of substrates and background inorganic phosphate levels.

A modification of the procedure of Martin and Doty [19] was used to monitor inorganic phosphate released from substrates. Samples were extracted with 2 ml of isobutanol-toluene (1 : 1, v/v) followed by mixing with 0.5 ml silicotungstate reagent and 0.5 ml of ammonium molybdate reagent. One ml of the resulting organic phase was mixed with 2 ml of 2% H_2SO_4 in ethanol (v/v) and 0.5 ml of 222 μM $SnCl_2$ reagent. Under these conditions, A_{660} was linearly proportional to inorganic phosphate concentration over the range of 30 to 1200 nmol P_i. Extraction with isobutanol-toluene completely removed anthocyanin, when present. Protein was assayed according to the procedure of Bradford [20]. Cytochrome *c* oxidase and cytochrome *c* reductase were assayed essentially as summarized by Hodges and Leonard [21]. The following reagents were obtained from Sigma Chem. Co.: ATP(A-5394), ADP(Grade X), AMP(Type III), GTP(Type III), GDP(Type I), CTP(Type V), CDP(Type III), UTP(Type III), UDP(Type I), *p*-nitrophenyl phosphate (PNPP, Sigma 104), antimycin A. All other chemicals were reagent grade or better.

Solubilization of ATPase

Step-gradient-purified tonoplast was stirred with

1% (w/v) Polidocanol (polyoxyethylene ether, 9 lauryl ether; Sigma Chem. Co.) in 20 mM Tris-HCl (pH 7.4), 5 mM dithiothreitol for 60 min at 4°C. Subsequently, residual membrane was separated by centrifugation at 190000 × *g* for 30 min. For ATPase assay, the sediment was resuspended in 12% (w/v) sucrose, 20 mM Tris/HCl, pH 7.4 and the supernatant was used directly. Phospholipid dispersions were prepared from azolectin (Associated Concentrates, 32 61st St., Woodside, NY, 11377), phosphatidylserine, phosphatidylcholine and phosphatidylinositol (Sigma Chem. Co.) by sonicating N_2-dried lipids with 12% (w/v) sucrose, 20 mM Tris/HCl, pH 7.4 for 30 min (maximum temperature was 37°C) using an ultrasonic cleaner. Lipids were added to ATPase assays to a final concentration of 1 μM.

Results

The nucleotide specificity and Mg^{2+} dependence of phosphohydrolases of tulip vacuoles and tonoplast were investigated with use of various nucleotide phosphate esters, pyrophosphate, and several substrates of nonspecific phosphohydrolase. Results (Table I) indicated that tulip vacuoles hydrolyzed ATP most efficiently under the conditions tested and that 59% of vacuolar ATPase was Mg^{2+}-dependent. Hydrolysis of *p*-nitrophenyl phosphate and of pyrophosphate by tulip vacuoles was not Mg^{2+}-dependent and was about 36 and 67% that of ATP, respectively. Tulip tonoplast purified by centrifugation in a step gradient contained ATPase which was almost entirely (95%) dependent on the presence of Mg^{2+}. Membranes having density of 1.09 to 1.17 g/cm^3 were recovered from this gradient. GTPase and pyrophosphatase represented 32 and 60% of the ATPase level, respectively, and the Mg^{2+} dependency of these two activities was 77 and 90%, respectively. Hydrolysis of all other substrates by tonoplast was less than 30% that of ATP.

The influence of pH on three important activities (ATPase, *p*-nitrophenylphosphatase, and pyrophosphatase) associated with tulip vacuoles and tonoplast (step-gradient-purified) was measured (Fig. 1). ATPase of vacuoles was largely Mg^{2+}-dependent (Fig. 1A), and *p*-nitrophenylphosphatase was largly Mg^{2+} independent (Fig. 1B) over the entire pH range tested. Pyrophosphatase of vacuole sap (Fig. 1C) was little affected by Mg^{2+} below pH 6.5 (which was the approximate pH optimum of vacuolar ATPase) but was stimulated by Mg at higher pH. The pH optimum of *p*-nitrophenylphosphatase and pyrophosphatase of whole vacuoles was less than 6.5. The data shown in Fig. 1C were obtained with vacuolar sap (separate experiment) and those in Figs. 1A and 1B with sap plus membrane. Tonoplast ATPase (Fig. 1D) was clearly Mg^{2+}-dependent, and both Mg^{2+}-dependent and Mg^{2+}-independent *p*-nitrophenylphosphatase (Fig. 1E) were virtually absent from tonoplast preparations. Substantial pyrophosphatase was found to be associated with tonoplast (Fig. 1F). Tonoplast ATPase was optimal at pH 7 and pyrophosphatase at pH 8.5 or higher. In all the experiments described by Figs. 1

TABLE I

RELATIVE PHOSPHOHYDROLASE ACTIVITIES OF *TULIPA* VACUOLES AND TONOPLAST

Vacuoles were assayed at pH 6, and tonoplast at pH 7. Specific activities of Mg^{2+}-ATPase of vacuoles and tonoplast were 10.5 and 50.1 μmol P_i released/mg protein per h at 37°C, respectively. n.d., not determined. PNPP, *p*-nitrophenyl phosphate; PP_i, pyrophosphate; *β*-Gly-*P*, *β*-glycerol phosphate; Glc-6-*P*, glucose 6-phosphate.

Substrate	Percent of Mg^{2+}-ATPase activity			
	Tulipa vacuoles		*Tulipa* tonoplast	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
ATP	41.1	100.0	5.4	100.0
CTP	51.2	38.5	n.d. [a]	n.d. [a]
GTP	23.5	20.7	7.3	32.2
ITP	28.4	29.5	6.0	19.3
UTP	50.6	35.9	n.d.	n.d.
ADP	42.2	38.9	4.8	17.9
CDP	31.8	12.0	n.d.	n.d.
GDP	20.5	28.6	4.0	27.1
UDP	41.6	38.7	n.d.	n.d.
AMP	n.d.	n.d.	2.0	0.5
UMP	n.d.	n.d.	2.0	1.9
PNPP	46.8	36.4	4.5	6.1
PP_i	70.4	67.0	6.1	59.7
β-Gly-*P*	n.d.	n.d.	3.3	5.7
Glucose-6-*P*	n.d.	n.d.	2.8	3.5

[a] See Fig. 3.

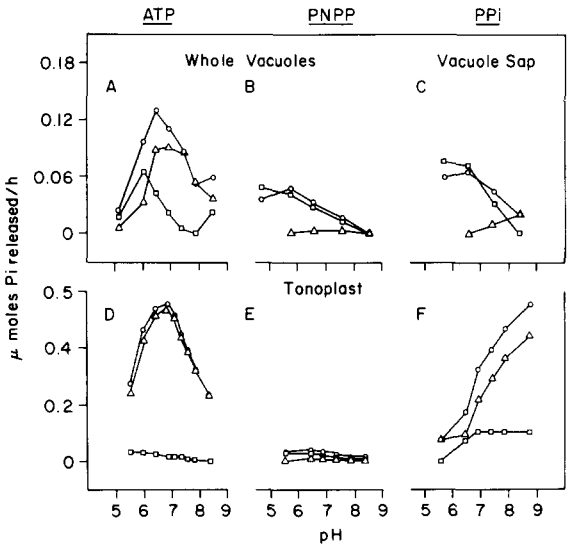

Fig. 1. Effect of pH on *Tulipa* vacuole ATPase and *p*-nitrophenylphosphatase, vacuole sap pyrophosphatase, and tonoplast ATPase, *p*-nitrophenylphosphatase, and pyrophosphatase. 1A: vacuole ATPase, 1B: vacuole *p*-nitrophenylphosphatase; 1C: vacuole-sap pyrophosphatase; 1D: tonoplast ATPase; 1E: tonoplast *p*-nitrophenylphosphatase; 1F: tonoplast pyrophosphatase. □———□, Minus Mg^{2+}; ○———○, plus Mg^{2+}; △———△, Mg^{2+}-dependent.

and 2 the actual pH of reactions was monitored during incubation.

The occurrence and recovery of ATPase, pyrophosphatase, and *p*-nitrophenylphosphatase in vacuoles and vacuole fractions from a single preparation of *Tulipa* are presented in Table II. Vacuole sap accounted for nearly all of the Mg^{2+}-independent ATPase, and tonoplast accounted for all of the Mg^{2+}-dependent ATPase of whole vacuoles. Similar results were observed for pyrophosphatase. Tonoplast specific activities for Mg^{2+}-dependent ATPase and Mg^{2+}-dependent pyrophosphatase were 4- to 5-fold that of whole vacuoles while *p*-nitrophenylphosphatase specific activity was 1/5 that of whole vacuoles. From Table II and Fig. 1 it is clear that *p*-nitrophenylphosphatase is restricted to the vacuole sap and is largely Mg^{2+}-independent. The specific activities of tonoplast ATPase and pyrophosphatase were 50 and 36 μmol P_i released/mg protein per h, respectively (Table II). No Mg^{2+}-independent pyrophosphatase was observed in the experiments described by Table II. In other experiments (Table I, Fig. 1) a low level of this activity was observed. Mg^{2+}-independent ATPase of tonoplast was absent or at a very low level in all cases (Fig. 1, Tables I and II).

Further definition of step-gradient-prepared tulip tonoplast on a 6 to 40% (w/w) sucrose gradient produced a single peak of Mg^{2+}-dependent ATPase which was coincident with the major protein peak observed (Fig. 2). When membrane sedimented from lysed vacuoles was applied directly to an identical linear gradient, a similar profile was produced (not shown). The step-gradient procedure was routinely performed so that all

TABLE II

OCCURRENCE OF PHOSPHOHYDROLASE IN *TULIPA* VACUOLES AND VACUOLE FRACTIONS AT pH 7

The protein contents of aliquots used to assay phosphohydrolases were 38.2, 28.6, and 9.5 μg for vacuoles, vacuole sap, and tonoplast, respectively. Activities as μmol P_i released/h at 37°C. S/A, specific activities as μmol P_i released/mg protein per h.

Fraction	ATPase				Pyrophosphatase				*p*-Nitrophenylphosphatase			
	$-Mg^{2+}$	Mg^{2+}	Mg^{2+}-dependent		$-Mg^{2+}$	$+Mg^{2+}$	Mg^{2+}-dependent		$-Mg^{2+}$	$+Mg^{2+}$	Mg^{2+}-dependent	
			Activity	S/A			Activity	S/A			Activity	S/A
Vacuole	0.37	0.79	0.42	11.0	0.75	1.09	0.34	8.9	0.28	0.45	0.18	4.8
Vacuole sap	0.33	0.31	0	–	0.71	0.51	0	–	0.22	0.30	0.08	2.8
Tonoplast	0.0	0.47	0.47	49.4	0.0	0.34	0.34	35.5	0.02	0.03	0.01	1.05

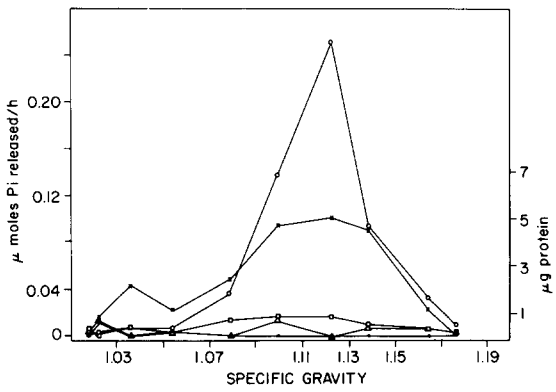

Fig. 2. Isopycnic separation of step-gradient-prepared *Tulipa* tonoplast in a 6 to 40% (w/w) sucrose gradient. ○——○, Mg^{2+}-ATPase; △——△, Mg^{2+}-ADPase; □——□, Mg^{2+}-CTPase; ●——●, *p*-nitrophenylphosphatase; and ×——×, protein.

the results presented could be compared directly. No Mg^{2+}-independent activity was found in the gradient shown in Fig. 2 and ATPase was maximal at about 1.12 g/cm^3. Hydrolysis of ADP, *p*-nitrophenyl phosphate, and CTP (the last not previously measured, see Table I) were at a low level. Results obtained with a 22 to 38% (w/w) sucrose gradient are shown in Fig. 3. All three of the principal Mg^{2+}-dependent phosphohydrolase activities found to be associated with tulip tonoplast, (ATPase, GTPase, and pyrophosphatase) were maximal at 1.115 to 1.135 g/cm^3 in this gradient.

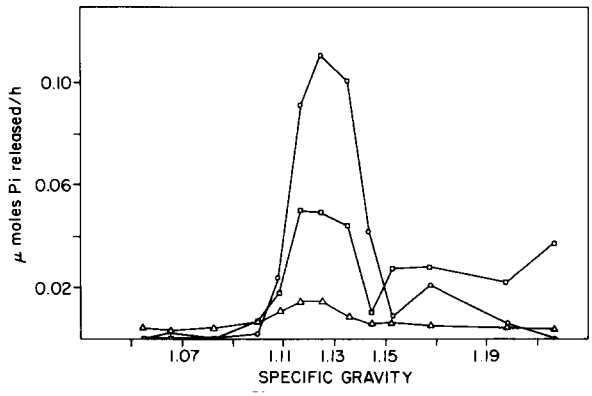

Fig. 3. Phosphohydrolase activities associated with linear-gradient (22 to 38% w/w sucrose) purified *Tulipa* tonoplast. ○——○, Mg^{2+}-ATPase; □——□, Mg^{2+}-pyrophosphatase; △——△, Mg^{2+}-GTPase.

No Mg^{2+}-independent ATPase, GTPase, or pyrophosphatase were observed (not shown). Mg^{2+}-GTPase activity at 1.115 g/cm^3 (Fig. 3) was about 13% that of Mg^{2+}-ATPase. In a similar experiment a value of 16% was obtained. Yet, Mg^{2+}-GTPase activity of step-gradient-purified tonoplast (Table I and two similar experiments) was 31 to 37% that of Mg^{2+}-ATPase. The reason for this phenomenon is not understood and is being further investigated.

The distribution of four marker enzymes in protoplast and vacuole fractions was monitored to assess the purity of vacuoles and step-gradient-purified tonoplast and to determine whether NADH-cytochrome *c* reductase is associated with tulip tonoplast. The marker enzymes [22] assayed were cytochrome *c* oxidase (mitochondrial inner membrane), antimycin A-insensitive NADH-cytochrome *c* reductase (ER, mitochondrial outer envelope, and nuclear envelope [23]), antimycin A-insensitive NADPH-cytochrome *c* reductase (ER) and latent IDPase (Golgi). The results (Table III) indicated that about 1.6% of the total NADH reductase recovered in protoplast fractions accompanied step-gradient-purified tonoplast. No cytochrome *c* oxidase, NADPH-cytochrome *c* reductase, or latent IDPase were detected in the tonoplast preparation (Table III). NADH-cytochrome *c* reductase activities of particulate, cytosol, vacuole, and tonoplast fractions in the presence of antimycin A (1 μg/ml) were 77, 69, 73, and 92%, respectively, of those in the absence of the inhibitor. The fraction (cytosol) enriched in cytosol (KH_2PO_4/K_2HPO_4 solution medium into which vacuoles emerge from protoplasts, which remained after filterable particulate materials (particulate fraction) and intact vacuoles were removed) was separated into 100 000 × *g* soluble and insoluble fractions. The 100 000 × *g* insoluble material is expected to be enriched in ER, mitochondria, and other membranes and organelles. As expected, most of the cytochrome oxidase and reductase activity associated with the cytosol fraction was sedimentable. Using Method 1 to prepare protoplast fractions (Table III) recovery of activities was good. Similar amounts of protein and activities were lost when protoplast fractions were prepared using Method 2 (Table III). It is assumed that losses occurred during concentration

TABLE III

DISTRIBUTION OF MARKER ENZYMES IN *TULIPA* PROTOPLAST FRACTIONS

Protoplast fractions were prepared from approximately $4 \cdot 10^6$ protoplasts. Tonoplast and vacuole sap fractions were from $1 \cdot 10^6$ vacuoles. Two methods were used to fractionate protoplasts, Methods 1 and 2. In Method 1 bovine serum albumin (0.3 mg/ml) was added during protoplast lysis, while in Method 2 no additions were made. In both methods, isolated fractions were centrifuged at $100000 \times g$ for 30 min. The supernatants were removed, concentrated by ultrafiltration [25], diluted 10-fold with 10 mM Hepes/Tris (pH 8) and reconcentrated, dialysis step. The ultrafiltration membrane used for Method 2 samples was pretreated with 1 mg/ml bovine serum albumin. Concentration and dialysis removed anthocyanin and other interfering substances. The $100000 \times g$ sediment of each fraction and its corresponding supernatant (concentrated and dialyzed) were recombined before assay. Cytosol soluble and insoluble fractions were assayed separately. Step-gradient prepared tonoplast was obtained as described in the Materials and Methods. NADH-cytochrome *c* reductase was assayed in the presence and absence of antimycin A (1 μg/ml) and data obtained without inhibitor are presented (see text for effect of inhibitor). From Method 1 data, 84, 90 and 111% of the cytochrome *c* oxidase, NADH-cytochrome *c* reductase and latent IDPase, respectively, in protoplasts were recovered in protoplast fractions. Recovery of protein (now shown) was 95%. With Method 2 (where, with the exception of latent IDPase, similar amounts of protein and activities were lost during concentration and dialysis and/or due to proteolysis) 57, 51, 51, 73 and 98% of the protein, cytochrome *c* oxidase, NADH-cytochrome *c* reductase, NADPH-cytochrome *c* reductase (data not shown, see results), and latent IDPase, respectively, in protoplasts were recovered in protoplast fractions.

Fraction	Cytochrome *c* oxidase					NADH-cytochrome *c* reductase					Latent IDPase				
	Method 1		Method 2			Method 1		Method 2			Method 1		Method 2		
	Act.	% [a]	Act.	S/A [b]	%	Act.	%	Act.	S/A	%	Act.	%	Act.	S/A	%
Protoplast	46.7	–	33.2	5.6	–	112	–	80.0	14.4	–	6.51	–	7.1	1.9	–
Cytosol															
(a) soluble	3.1	8	1.1	0.68	6.3	4.0	4.1	1.2	0.9	2.8	5.20	72	5.1	5.3	71
(b) $100000 \times g$ sediment	19.8	51	8.0	16.7	48	43.3	43	16.7	34.8	41	0.58	8	0.78	2.4	11
Particulate	16.3	42	7.8	5.8	46	50.4	50	21.9	16.4	54	0.87	12	1.03	1.1	14.5
Vacuole sap	0	0	0	0	0	1.3	1.3	0.5	4.6	1.2	0.58	8	0.15	1.9	2.1
Tonoplast	0	0	0	0	0	1.6	1.6	0.64	22.0	1.6	0	0	0	0	0

[a] Percent of the sum of the activity recovered in protoplast fractions.

[b] S/A, specific activities as: A_{550}/min per mg protein for oxidase and reductase and μmol P_i/h per mg protein for latent IDPase.

and dialysis of fractions. Fractions were also assayed for NADPH-cytochrome *c* reductase and activity observed with protoplasts was about 2% of that observed with NADH as substrate (data not shown). No significant amount of NADPH-cytochrome *c* reductase was observed in step-gradient-prepared or linear-gradient-purified tonoplast preparations. Earlier observations [17] concerning the purity of vacuole preparations isolated by the method used here are consistent with the data presented here and indicate that contamination of vacuoles and tonoplast with other cellular membranes and organelles is minimal. The activities of protoplast and tonoplast Mg^{2+}-dependent ATPase were found to be about 13 and 50 mol P_i/mg protein per h, respectively. At least 20% of protoplast ATPase (measured as described) was attributed to tonoplast.

Step-gradient-prepared, linear-gradient-separated tonoplast was assayed for Mg^{2+}-ATPase, antimycin A-insensitive NADH-cytochrome *c* reductase, NADPH-cytochrome *c* reductase and light scattering (membrane) and the results are presented in Fig. 4 (data are the mean of three experiments). A single major peak of NADH reductase occurred at about 1.117 g/cm^3, and Mg^{2+}-ATPase peaked at 1.124 g/cm^3 in this gradient. No Mg^{2+}-independent ATPase was observed in the gradient (data not shown). Membrane (light scattering) distribution in the gradient was similar to that of protein (Fig. 2). No significant NADPH-cytochrome *c* reductase activity was observed. Latent IDPase was absent (data not shown).

Since many (40 to 70%) vacuoles burst after emerging from protoplasts, the KH_2PO_4/K_2HPO_4 solution used to prepare vacuoles is expected to be enriched in tonoplast. However, as already indicated (Table III and Ref. 17) this suspension also contains numerous markers which indicate the presence of other particulate constituents that are not aggregated and removed as filterable particulate material during vacuole preparation. Sedimentable ($100\,000 \times g$) material from the fraction (cytosol) (Table III) was separated on a 22 to 45% (w/w) linear sucrose gradient to determine whether tonoplast could be recovered from this fraction. Mg^{2+}-ATPase occurred in the gradient as two peaks, the major one at about 1.115 to 1.125 g/cm^3 and the minor one at 1.09 g/cm^3 (Fig. 5). No Mg^{2+}-independent activity was observed. Two peaks of antimycin A-insensitive NADH-cytochrome *c* reductase were observed, one of which was coincident with the highest level of protein in the gradient at about 1.13 g/cm^3 and the other at 1.115 g/cm^3. These data are consistent with the equilibration of *Tulipa* smooth ER at about 1.115 g/cm^3, tonoplast at 1.12 g/cm^3, and rough ER at about 1.14 g/cm^3 (results of ER-EDTA shift experiments substantiate this conclusion, Wagner and Hrazdina, unpublished data) and suggest that tonoplast in this fraction is extensively contaminated with membranes which have similar density.

The sensitivity of step-gradient-purified tonoplast to anions and cations was tested and results are presented in Table IV. Mg^{2+}-ATPase was stimulated 10–30% by KCl (results from eight

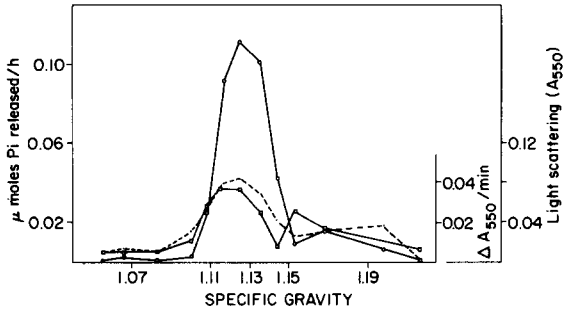

Fig. 4. Occurrence of ATPase, and NADH-cytochrome *c* reductase after separation of *Tulipa* tonoplast in a 22 to 38% (w/w) sucrose gradient. O——O, Mg^{2+}-ATPase; □——□, NADH-cytochrome *c* reductase; ------, light scattering (A_{550}).

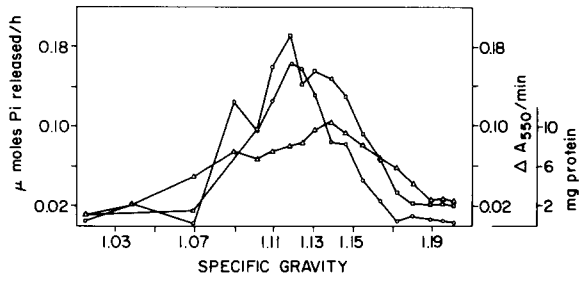

Fig. 5. Isopycnic separation of particulate material recovered from the cytosol fraction obtained from *Tulipa* protoplasts. See results for description of sample. O——O, Mg^{2+}-ATPase; □——□, NADH-cytochrome *c* reductase; △——△, protein.

TABLE IV

ION STIMULATION OF *TULIPA* TONOPLAST ATPase AT pH 7.0

All reaction mixtures were titrated with Tris and ATP was converted to its Tris salt as described by Hodges and Leonard [21]. The free acid of EDTA was used in all cases. Concentration of added salt was 50 mM except for experiments containing malic and maleic acids. Titration of these acids with appropriate base to pH 7.0 brought added salt to about 100 mM. Specific activity of ATPase in the presence of KCl was 47 μmol P_i/mg protein per h. Data are presented as percent of Mg^{2+}-dependent ATPase activity measured without added salt. Similar results were obtained when EDTA was excluded from ATPase assay and 3 mM $MgCl_2$ was used (see Methods).

Anion	Cation		
	K^+	Na^+	NH_4^+
No salt	100	100	100
Chloride	125	114	152
Bicarbonate	85	71	–
Malate	94	87	–
Maleate	105	94	135
Sulfate [a]	105	94	131
Nitrate	90	81	–

[a] Similar results were obtained when 3 mM $MgSO_4$ was substituted for 3 mM $MgCl_2$ as the source of divalent cation.

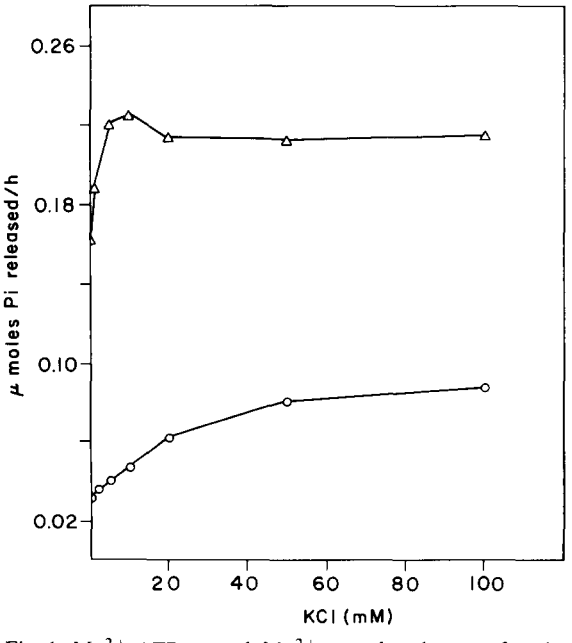

Fig. 6. Mg^{2+}-ATPase and Mg^{2+}-pyrophosphatase of *Tulipa* tonoplast as a function of KCl concentration. △——△, Mg^{2+}-ATPase; ○——○, Mg^{2+}-pyrophosphatase.

TABLE V

EFFECT OF VARIOUS INHIBITORS AND UNCOUPLERS ON *TULIPA* TONOPLAST ATPase AT pH 7

Specific activity of Mg^{2+}-ATPase without additions was 41 μmol P_i/mg protein per h. All reactions contained 50 mM KCl.

Addition	Mg^{2+}-dependent ATPase	
	μmol P_i/h	%
None	0.377	100.0
Dio-9 (20 μg/ml)	0	0
Fusicoccin (100 μM)	0.342	90.7
KF (18 mM)	0.102	27.1
Ethanol (1.5%)	0.381	101.1
CCCP (10 μM), 1.5% ethanol	0.410	108.9
DCCD (2 μM), 1.5% ethanol	0.222	59.0
DCCD (25 μM), 1.5% ethanol	0.062	16.5
Oligomycin (5 μg/ml)	0.363	96.2
Ammonium molybdate (100 μM)	0.415	110.1

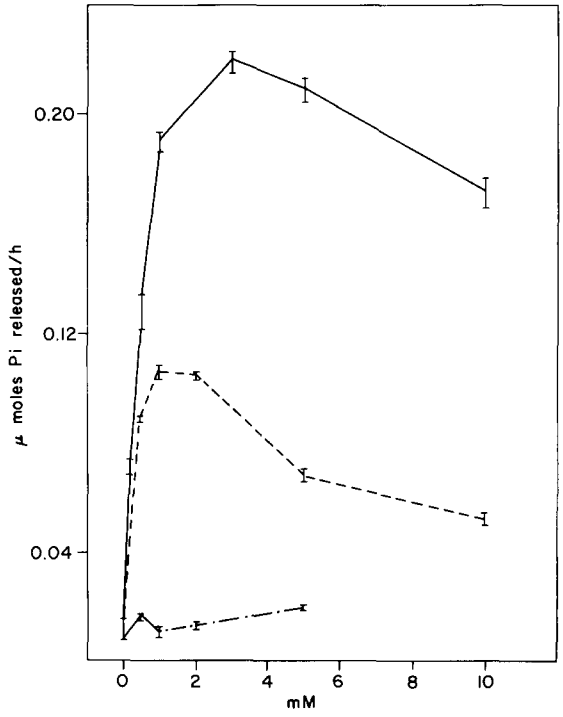

Fig. 7. Effect of divalent cation concentration on *Tulipa* tonoplast ATPase (measured with 50 mM KCl). Magnesium was excluded during tonoplast purification and no EDTA was used. ———, $MgCl_2$; — — —, $MnCl_2$; ·—·—·, $CaCl_2$.

experiments) and about 50% by NH_4^+ whereas, Li^+ and choline$^+$ had little effect (activities with LiCl and choline chloride were 90 and 95%, respectively, of that without added salt). Some stimulation by Cl^- was apparent. The effect of K^+ concentration on tonoplast Mg^{2+}-ATPase and Mg^{2+}-pyrophosphatase measured with 3 mM $MgCl_2$ is shown in Fig. 6. In these experiments ionic strength was not maintained at a constant level but maximal ATPase was observed with 10 mM K^+ and higher levels of KCl were not inhibitory. Mg^{2+}-pyrophosphatase was stimulated 2.5-fold by 50 mM KCl.

Effects of various inhibitors and uncouplers on tonoplast Mg^{2+}-ATPase prepared on a step gradient are shown in Table V. The inhibitors DCCD, Dio-9, and KF substantially inhibited whereas fusicoccin and oligomycin did not. Ammonium molybdate (100 μM) stimulated *Tulipa* tonoplast ATPase 10%.

Divalent cation activation of tonoplast ATPase is described in Fig. 7. The K_m for Mg^{2+} and Mn^{2+} was about 0.3 mM. Essentially no activity was observed in the presence of Ca^{2+} alone and maximal activity was obtained with 3 mM Mg. In the presence of 50 mM KCl, somewhat higher activity occurred when $MgSO_4$ was substituted for $MgCl_2$.

TABLE VI

CHARACTERISTICS OF TONOPLAST-BOUND AND POLIDOCANOL-SOLUBILIZED ATPase AND PYROPHOSPHATASE

Percentages are based on ATPase activity observed in the presence of 4.6 mM $MgCl_2$, 50 mM KCl and without DCCD. Measurements of detergent soluble fractions (except in lipid stimulation experiments as indicated) were made with 1 μM/assay of added azolectin. Values in parenthesis indicate the percent of stimulation (K^+ and lipid stimulation) and percent of inhibition (DCCD). Data are representative of two or more experiments. Specific activity of membrane bound Mg^{2+}-ATPase (+50 mM KCl) was 49 μmol P_i/mg protein per h.

Characteristic		ATPase		Pyrophosphatase	
		Membrane-bound (%)	Solubilized (%)	Membrane-bound (%)	Solubilized (%)
Lipid stimulation	no addition	100.0 (6%)	13.4 (349%)	37.6 (24%)	6.4 (23%)
	+ azolectin	106.0	60.1	46.9	7.9
	+ phosphatidylserine	–	60.8	–	–
	+ phosphatidylserine	–	31.3	–	–
	+ phosphatidylinositol	–	63.3	–	–
K^+ stimulation	$-K^+$	76.4 (31%)	28.3 (112%)	15.5 (143%)	–
	$+K^+$	100.0	60.1	37.6	–
Nucleotide specificity	ATP	100.0	100	–	–
	GTP	37.0	66	–	–
	UTP	–	17	–	–
	ADP	18.0	7	–	–
Divalent ion stimulation	$-Mg^{2+}$	2.3	6.6	2.1	2.0
	$+Mg^{2+}$	100.0	60.1	37.6	6.4
	$+Ca^{2+}$	4.0	5.8	–	–
DCCD inhibition	– DCCD	100.0 (76%)	60.0 (36%)	37.6 (24%)	–
(7.5 μM)	+ DCCD	24.5	38.3	28.4	–
Stability	control	100.0	60.1	37.6	–
	18 h room temp.	40.7	4.2	6.9	–
	18 h 4°C	44.8	21.6	20.7	–

Treatment of step-gradient-prepared tonoplast with Polidocanol resulted in a loss of about 85% of tonoplast ATPase and pyrophosphatase (Table VI). Addition of azolectin, phosphatidylserine or phosphatidylinositol stimulated solubilized ATPase about 350% (to a level which was 60% that of membrane-bound enzyme). Solubilized pyrophosphatase was stimulated only 24% by azolectin. Phosphatidylcholine was less effective in restoring activity to solubilized ATPase. Potassium stimulation of solubilized ATPase (measured with lipid present) was somewhat higher than that of bound enzyme.

Discussion

The presence in *Tulipa* tonoplast of an Mg^{2+}-dependent, nucleotide specific ATPase has been directly demonstrated in this study without resorting to the use of inhibitors to selectively suppress nonspecific phosphohydrolase such as that which apparently accompanies tonoplast from *Hevea* [8] and *Beta vulgaris* [5,6,16].

In an earlier study Lin et al. [3] found Mg^{2+}-dependent ATPase to be associated with vacuoles and partially purified tonoplast obtained from petal vacuoles of *Hippeastrum* and *Tulipa* (cv. Red Shine). Preliminary analysis of pH dependence and potassium stimulation indicated that Mg^{2+}-ATPase of *Tulipa* vacuoles was higher at pH 6 than at pH 9 and that activity at pH 6 appeared to be stimulated 40 to 50% by potassium. In that study, two measures of nonspecific phosphohydrolase were examined with whole vacuole samples. In the first, ATP was found to be a better substrate than GTP, CTP, or UTP by a factor of 2 to 3 and in the second, *p*-nitrophenyl phosphate was observed to be a poor substrate compared with ATP. Briskin and Leonard [24] criticized that work primarily because we found little evidence for *p*-nitrophenylphosphatase. They correctly point out that in a later study [25] we observed *p*-nitrophenylphosphatase activity in whole *Hippeastrum* vacuoles.

Clearly, it is important that nonspecific phosphohydrolase be carefully monitored during study of vacuolar ATPase. A number of investigators have verified the presence of acid phosphatase (generally measured as *p*-nitrophenylphosphatase) in vacuoles isolated from a variety of tissues and species. Indeed, we were the first to observe this activity directly in isolated vacuoles of *Hippeastrum* [25]. Further, we showed in that study that the level of vacuolar *p*-nitrophenylphosphatase varied with the developmental stage of *Hippeastrum* petals. Anthesis stage petals contained low levels of acid *p*-nitrophenylphosphatase compared with early-bud and senescence stage petals. This was not a surprising finding since the level of acid phosphatase in plants is known to be influenced by nutrient state, stress, developmental stage and other factors and to vary from species to species. For these reasons, it is not surprising that anthesis-stage *Tulipa* petals contain relatively little *p*-nitrophenylphosphatase.

In addition to the studies of Lin et al. [3], other recent studies have contributed to direct evidence supporting the existence of tonoplast ATPase. D'Auzac [8,9] and, Leigh and Walker [6,7] have detected ATPase and acid phosphatase in membrane recovered in a sediment from osmotically lysed vacuoles. In these studies ammonium molybdate was used to inhibit the acid phosphatase component. D'Auzac found ATPase of *Hevea* to have maximal activity at pH 7.5 to 8 when assayed in the presence of Mg^{2+} and KCl [8,9]. At this pH, ammonium molybdate inhibited ATPase 16% and *p*-nitrophenylphosphatase 96%. Leigh and Walker [6] showed that beet root vacuoles contain at least two distinct phosphohydrolases. One, which has a pH optimum of 5.5, is sensitive to ammonium molybdate, lacks substrate specificity, and is restricted to the vacuole sap. The other has optimal activity at pH 7.5 to 8, is apparently insensitive to ammonium molybdate (but is greatly stimulated by NH_4^+ [7]), has nucleotide specificity, and is recovered in equal proportions with membrane and sap. With *Hippeastrum* we observed only a small amount of *p*-nitrophenylphosphatase in association with tonoplast purified on a linear sucrose gradient [4]. Interestingly, sonication of vacuoles to prepare *Hippeastrum* tonoplast vesicles of uniform size increased the amount of *p*-nitrophenylphosphatase associated with the membrane vesicles [4].

Briskin and Leonard [24] examined the nucleotide specificity, potassium stimulation, and membrane-to-sap distribution of vacuolar phos-

phohydrolase of tobacco. Vacuoles were isolated from protoplasts prepared from tobacco cultured cells. Except for UTP and AMP, all of the nucleoside di- and tri-phosphate substrates tested and *p*-nitrophenylphosphate were hydrolyzed equally well by tonoplast separated on a step gradient of sucrose. Equilibration of tonoplast in a linear sucrose gradient (after sonication of vacuoles) indicated that the density of the membrane 1.12 g/cm^3. Little ATPase or *p*-nitrophenylphosphatase was observed in this region of the gradient; however, more ATPase than *p*-nitrophenylphosphatase was apparent.

In the present study we have examined the properties of ATPase and other phosphohydrolases in *Tulipa* (cv. Aladdin) vacuoles and have extended this work to the study of purified tonoplast and detergent solubilized tonoplast ATPase. The variety Aladdin was chosen because it has superior cold storage characteristics and fresh petals can be made available for 11 months of the year. Also, tonoplast isolated from this variety bears little *p*-nitrophenylphosphatase.

The nucleotide specificity of *Tulipa* tonoplast enzyme at pH 7 (Table I) bears striking resemblance to that reported by Leigh and Walker [6] for beet vacuoles measured at pH 8 and in the presence of 0.1 mM ammonium molybdate. In both *Tulipa* tonoplast and beet vacuoles hydrolysis of GTP and GDP was about 30% that of ATP. The data of Leigh and Walker [6] reflect only activity in the presence of Mg^{2+}. In this study activity observed in the presence and absence of Mg^{2+} is reported so that ATPase and nonspecific hydrolase can best be monitored. Also, the method of assay used (addition of 13 mM EDTA to sample followed by addition of 18 mM $MgCl_2$) provides control of endogenous divalent cation. However, similar results were obtained when tonoplast was assayed without EDTA and with 3 mM $MgCl_2$. It is clear from the data shown in Table I that hydrolysis by tonoplast of all substrates was primarily Mg^{2+}-dependent whereas that by vacuoles of most substrates except ATP was Mg^{2+}-independent. Leigh and Walker [6] found that hydrolysis of pyrophosphate by beet vacuoles was about 66% that of ATP. We observed similar results with *Tulipa* tonoplast.

Comparison of pH profiles for ATP, *p*-nitrophenylphosphate, and pyrophosphate hydrolysis by whole vacuoles or vacuole sap and tonoplast provides convincing evidence that *Tulipa* tonoplast bears Mg^{2+}-dependent ATPase, and alkaline pyrophosphatase but virtually no *p*-nitrophenylphosphatase (Fig. 1). Again, activity in the presence and absence of Mg^{2+} is reported to allow comparison of ATPase and nonspecific phosphohydrolase. Leigh and Walker [6] observed a similar pH dependence for Mg^{2+}-dependent ATPase of beet vacuoles, measured in the presence of ammonium molybdate. With 3 mM $MgCl_2$ and 50 mM KCl, Mg^{2+}-dependent ATPase of beet vacuoles [6] and *Hevea* lutoids [8,9] had pH optima of about pH 7.5 and 7.5 to 8, respectively. The optimum of *Tulipa* tonoplast Mg^{2+}-ATPase was about 7 (Fig. 1D).

Tulipa tonoplast pyrophosphatase has an alkaline pH optimum (Fig. 1F) like that reported for beet vacuoles [26] and particulate pyrophosphatase from roots and cotyledons of sugar beet [27]. It is interesting to speculate on the role of pyrophosphatase of tonoplast. Largely Mg^{2+}-independent pyrophosphatase of vacuole sap (Table I) has an acid pH optimum (Fig. 1C) whereas the activity associated with tonoplast has a distinct alkaline pH optimum. These two components may represent enzymes with different functions. Like true inorganic pyrophosphatases [28] the *Tulipa* tonoplast component requires Mg^{2+} for activity (Fig. 1F). This component may have a regulatory function as has been suggested for pyrophosphatase in animals or it may be involved in Ca metabolism [28]. Walker and Leigh [26], making analogy to Mg^{2+}-dependent, K^+-stimulated, pyrophosphate-energized proton transport in *Rhodospirillum rubrum*, have suggested that tonoplast pyrophosphatase may energize vacuolar transport reactions.

It is clear from the data in Table II that Mg^{2+}-dependent ATPase is absent from the vacuole sap of *Tulipa*. All of this activity present in vacuoles is recovered with tonoplast. About 80% of the ATPase activity of *Tulipa* cv. Red Shine vacuoles was recovered with tonoplast in an earlier study [3]. This is in contrast to the recovery of only 50% of beet vacuole ATPase with the membrane [5–7]. Similarly, all of the alkaline pyrophosphatase of *Tulipa* vacuoles was recovered with the membrane.

Tulipa tonoplast has a density of about 1.12 g/cm^3 as determined from its behavior in a 6 to 40% sucrose gradient (Fig. 3) and a 22 to 38% gradient (Fig. 4). The majority of the protein and light scattering material (membrane) associated with step-gradient-prepared tonoplast was coincident with ATPase in a linear gradient (Figs. 2 and 4, respectively). The difference in the shape of the ATPase profile from that of the protein and light-scattering profiles may be due to the presence of a biochemically heterogeneous population of membrane vesicles and/or fragments with ATPase-enriched components equilibrating at 1.12 g/cm^3. Alternatively, protein and membrane at 1.08 to 1.11 g/cm^3 and 1.13 to 1.15 g/cm^3 may in part represent contamination with other cellular membranes. Prominant cellular membranes having densities similar to that of tonoplast are Golgi (1.12 to 1.15 g/cm^3), smooth ER (1.11 to 1.12 g/cm^3), rough ER (1.15 to 1.18 g/cm^3), and mitochondrial and chloroplast inner membranes (1.14 to 1.18 g/cm^3) [22]. Step-gradient and linear-gradient prepared tonoplast were examined for the presence of certain of these membranes. Of the marker enzymes tested, only antimycin A-insensitive NADH-cytochrome *c* reductase was associated with linear-gradient-purified tonoplast (Table III, Fig. 4.). The presence of this activity in *Tulipa* tonoplast preparations is not surprising since tonoplast is thought to be derived from the endoplasmic reticulum [29]. Leigh and Branton (see Ref. 29) reported an increase in the specific activity of vacuolar NADH-cytochrome *c* reductase during purification of vacuoles and others have reported the occurrence of this activity with isolated vacuoles (see Ref. 30).

The results shown in Fig. 5 suggest that in *Tulipa* the density of smooth ER is about 1.11 to 1.12 g/cm^3 and that of rough ER is 1.13 to 1.15 g/cm^3, and tonoplast has an intermediate density of about 1.12 g/cm^3. The sample for this gradient consisted of particulate materials that were not aggregated and therefore were not filtered from the KH_2PO_4/K_2HPO_4 solution used to prepare vacuoles. This material is expected to contain tonoplast originating from broken vacuoles, membranes that escape with cytosol during vacuole and cytosol emergence from protoplasts and organelles that were not aggregated and filtered. The major peak of Mg^{2+}-dependent ATPase observed in this gradient had maximum activity at about 1.12 g/cm^3. We attribute this activity to tonoplast. Virtually no Mg^{2+}-independent ATPase was observed in this gradient. The profiles obtained for NADH-cytochrome *c* reductase and protein imply the presence of smooth and rough ER at the densities indicated above. These results suggest that tonoplast in this fraction is severely contaminated with other light membrane components. However, there is little evidence to suggest that components other than tonoplast bear Mg^{2+}-ATPase. Therefore, this fraction may be useful for obtaining solubilized tonoplast ATPase. Also, there is little evidence to suggest the presence of plasma membrane in this fraction or in association with tonoplast as prepared. Plasma membrane, which bears Mg^{2+}-ATPase and has a characteristic density of 1.16 to 1.18 g/cm^3 [21] may be completely entrapped with aggregated material formed during vacuole emergence from protoplasts. This material is removed by filtration prior to recovery of vacuoles.

Specific ATPases occurring in plants are generally stimulated by potassium. In an earlier study, Lin et al. [3] observed that *Tulipa* cv. Red Shine ATPase was stimulated 40% by K^+. In this study a maximum of 30% stimulation by K^+ was observed at the pH optimum of the enzyme. Similar results were obtained with *Hevea* [9]. Examination of the concentration dependence of K^+ stimulation in *Tulipa* revealed that maximum activity occurred at about 10 mM K^+, but about the same level of activity was obtained between 5 and 100 mM K^+ (Fig. 7). Hydrolysis of pyrophosphate by tonoplast was more than doubled on addition of 100 mM KCl. The highest level of ATPase observed in these experiments was with NH_4^+ salts (Table IV). Comparison of K^+ and Na^+ salts and Cl^- and SO_4^{2-} salts suggests a low level of stimulation by Cl^- (Table IV). Our results are similar to those obtained with *Hevea* lutoids [9] except that we did not observe stimulation by organic anions. Walker and Leigh [7] have reported substantial stimulation of beet vacuole ATPase (measured with whole vacuoles) by nearly all of the many different substances tested.

In earlier studies we observed that *Tulipa* cv. Red Shine vacuole ATPase was insensitive to

oligomycin, was inhibited by Dio-9, and was stimulated slightly by CCCP [3]. Similar results were obtained in this study with *Tulipa* cv. Aladdin (Table V). Orthovanadate, an inhibitor of plasma membrane ATPase [31], also inhibited the tonoplast enzyme (Wagner, unpublished data). A marked inhibition of tonoplast ATPase by DCCD which was not observed in the earlier study [3], was found here. Sensitivity of *Tulipa* tonoplast and beet root vacuole [7] ATPase to various inhibitors appears to be similar.

D'Auzac examined the sensitivity of lutoid ATPase to Mg^{2+}, Mn^{2+}, and Ca^{2+} in the presence of ammonium molybdate [9]. They found that the highest activity was observed in the presence of 2 mM $MnCl_2$, and higher levels of Mn^{2+} were inhibitory. A lesser and constant level of activity was observed with 2 to 10 mM $MgCl_2$. Activity in the presence of 2 mM Ca^{2+} was one-third that seen with 2 mM Mg^{2+}. In this study we found that Mg^{2+}-ATPase of tonoplast was highest in the presence of 3 mM $MgCl_2$, (and $MgSO_4$) and higher levels of Mg^{2+} were inhibitory (Fig. 7). Activity in the presence of Mn^{2+} was about half that with the same concentration of Mg^{2+}, and Ca^{2+} could not substitute for Mg^{2+} or Mn^{2+}. Using sulfate salts, Walker and Leigh [7] found that the relative activities of beet vacuole ATPase in the presence of Mg^{2+}, Mn^{2+} and Ca^{2+} were 100, 105 and 46%, respectively.

We have attempted to determine if tonoplast ATPase is an intrinsic or extrinsic membrane ATPase by applying procedures used to dissociate coupling factor (extrinsic ATPase) from mitochondrial, chloroplast and bacterial membranes. Incubation of tonoplast with 5 mM EDTA in 20 mM Hepes/KOH (pH 8.0), 2 mM dithiothreitol, at 4°C for 30 min released no active ATPase from membrane and 89% of the control activity was recovered with membrane sedimented from the EDTA solution. The $CHCl_3$ extraction procedure used to remove CF_1 from chloroplast membranes [32], and treatment with 0.5% deoxycholate or 0.1% Triton X-100 resulted in loss of essentially all activity. No Ca^{2+}-stimulated ATPase was observed to result from these treatments. D'Auzac [9] used 2% deoxycholate to remove phosphatase activity from lutoid membranes, but did not report solubilization of active ATPase using this detergent. We found that a low level of active enzyme was released after solubilization of membrane with 30 mM octylglucoside, but the best results were obtained with Polidocanol (Table VI). The latter detergent was used by Okita et al. [33] to solubilize ATPase from *Nitzcschia alba.* Like $(Na^+ + K^+)$ ATPase of rabbit kidney cortex [34], detergent solubilized tonoplast ATPase was stimulated by phosphatidylserine, phosphatidylinositol and less so by phosphotidylcholine. Azolectin, a mixture of soybean phospholipids, also was effective in restoring activity lost during solubilization. Phospholipids were less effective in restoring pyrophosphatase activity (Table VI).

Results of solubilization experiments suggest that tonoplast ATPase is an intrinsic membrane protein which requires phospholipids for maximal activity. This result was expected since bacteria, mitochondria and chloroplasts (all of procaryotic origin, the last two considered as endosymbionts) are the only structures known to contain extrinsic (coupling factor type) ATPase and the ontogeny of tonoplast is not thought to be related to that of chloroplast or mitochondrial inner membranes.

Acknowledgments

The authors thank George Li for his assistance in part of this work, B. Rubinstein for the sample of fusicoccin and B. Volcani for a detergent sample.

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