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Tonoplast Vesicles of Opposite Sidedness from Soybean Hypocotyls by Preparative Free-Flow Electrophoresis[1]

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ABSTRACT

Tonoplast vesicles were purified from a microsomal fraction isolated from etiolated soybean hypocotyls (*Glycine max* L.) by preparative free-flow electrophoresis. Marker enzyme determinations and immunoblot analysis against the vacuolar-ATPase confirmed the nature and the purity of the isolated membranes. A purified tonoplast fraction also was obtained by consecutive sucrose and glycerol centrifugation which was further resolved into two different populations of vesicles (T_A and T_B) by free-flow electrophoresis. The determination of the sidedness of these different vesicles included concanavalin A binding as an imposed label, NADH-ferricyanide oxidoreductase cytochemistry, and ATPase latency. The tonoplast fractions, obtained by consecutive sucrose and glycerol gradient centrifugations, were found to consist of a mixture of two populations of vesicles of opposite sidedness. The least electronegative fraction obtained by free-flow electrophoresis (T_B) consisted predominantly of cytoplasmic side out tonoplast vesicles while a fraction of greater electronegativity (T_A) contained the cytoplasmic side in tonoplast vesicles. The relative amounts of each type of vesicle varied with the method of homogenization. Razor blade chopping, Polytron, and Waring Blendor homogenization gave predominantly cytoplasmic side out vesicles, whereas mashing with a mortar and pestle gave nearly equal amounts of the two populations of membrane vesicles of different orientation.

Recently, methods for large-scale isolation of intact vacuoles (1, 8, 29) have permitted a direct study of transport processes through the tonoplast, but this work is still impaired by the low yield of the vacuolar membrane (3). In addition, little is known about the protein or lipid composition of the tonoplast, or its enzymatic pattern. Progress in these fields requires large amounts of highly purified membranes. Moreover, transport studies with isolated vesicles of tonoplast, as well as topographic assignment studies to probe the organization of constituents within the membrane would be aided by preparations of purified membranes of known absolute orientations.

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In this work, we have used the preparative free-flow electrophoresis to isolate tonoplast vesicles (24). Two different populations of tonoplast vesicles were resolved. ATPase latency and Con A binding have already been used as tools to determine the sidedness of the plasma membrane vesicles (9). Here, we used similar approaches with the addition of ferricyanide oxidoreductase cytochemistry (20) to demonstrate that the two different populations of tonoplast vesicles resolved by preparative free-flow electrophoresis are cytoplasmic side out and cytoplasmic side in vesicles respectively. In addition, we showed that the tonoplast vesicles of each orientation were sealed and that their relative amounts varied with the method of tissue homogenization.

MATERIALS AND METHODS

Plant Material

Etiolated soybean hypocotyls (*Glycine max* [L.] Merr. var Williams) were obtained as previously described (9). Radiolabeled membranes were prepared by incubating overnight (15 h) 70 hypocotyl segments in 2 mL water containing 2 mCi of ^{32}P phosphate (New England Nuclear).

Membrane Isolations

Membrane preparation and free-flow electrophoresis were performed exactly as described earlier (9).

Consecutive Sucrose and Glycerol Gradient Centrifugation

The procedure was that of Scherer and Fisher (25). Membranes were prepared by homogenization of hypocotyl segments (100 g) in 100 mL buffer containing 8% (v/v) ethanolamine, 20 mM EDTA, 0.4 M sodium β-glycerophosphate, and 2 mM of DTT titrated with HCl to pH 7.5. The homogenate was filtered through one layer of Miracloth and the filtrate centrifuged for 10 min at $6{,}000g_{max}$. The supernatant was loaded into sucrose step gradients of the following steps: 4 mL 1.0 M, 6 mL 0.5 M, and 6 mL 0.3 M sucrose in homogenization medium. The sucrose gradients were centrifuged for 30 min at $110{,}000g_{max}$ (Beckman, SW-28 rotor, large buckets). The membranes collected at the interface 0.3 M sucrose/homogenization buffer were recentrifuged on a glycerol gradient consisting of a bottom layer of 1.2 M sucrose, followed

by 0.5 mL 50%, 1.5 mL 40%, 1.5 mL 30%, and 1.5 mL 20% glycerol, all in homogenization buffer. Residual space was filled with homogenization medium. The glycerol gradient was centrifuged for 2 h at $110{,}000g_{max}$ (Beckman, SW-28 rotor, slim buckets). The tonoplast-enriched membrane occurred at the upper interface (homogenization medium/loaded material).

ATPase Assays and Measurements of Structure Linked Latency

ATPase activity used an iso-osmotic medium containing 0.25 M sucrose, 50 mM KCl, 0.1 mM Na_2MoO_4, 3 mM $MgSO_4$, 3 mM ATP, 10 mM Hepes/Tris (pH 7.5), and 20 to 40 μg protein in a total volume of 0.5 mL (13). The assays were for 30 or 60 min at 30°C. Release of inorganic phosphate was estimated by a modified Fiske and Subbarow procedure (10). Measurements of structure-linked latency compared activities in the presence and absence of Triton X-100 (0.002–0.1%).

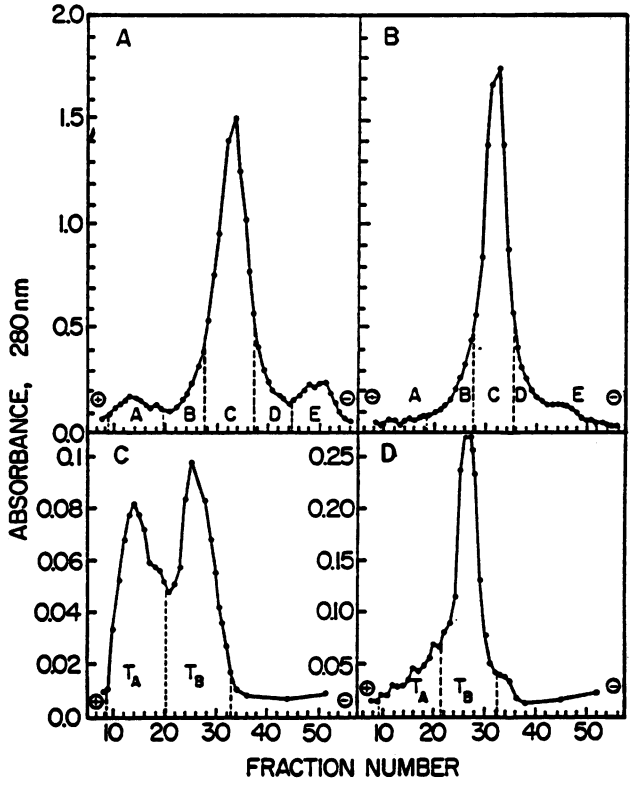

Figure 1. Distribution of membranes in free-flow electrophoresis separations monitored at 280 nm. A, Free-flow electrophoretic separation of total microsomes, the plant material was homogenized by mashing the soybean hypocotyls with a mortar and pestle. A to E, pooled fractions; the composition of these fractions based on biochemical markers and ATPase activities is summarized in Table I. B, Free-flow electrophoresis separation of total microsomes, the soybean hypocotyls were homogenized by razor blade chopping. A to E, pooled fractions. Note that this method of tissue homogenization gives less amount of membranes in fraction A. C, Consecutive sucrose and glycerol gradient centrifugation. The plant material was homogenized by mashing the soybean hypocotyls with a mortar and pestle. The tonoplast vesicles were resolved into two populations, T_A and T_B. D, Free-flow electrophoretic separation of tonoplast enriched membranes, obtained as before except that the soybean-hypocotyls were homogenized by razor blade chopping. The tonoplast vesicles were found primarily in fraction T_B.

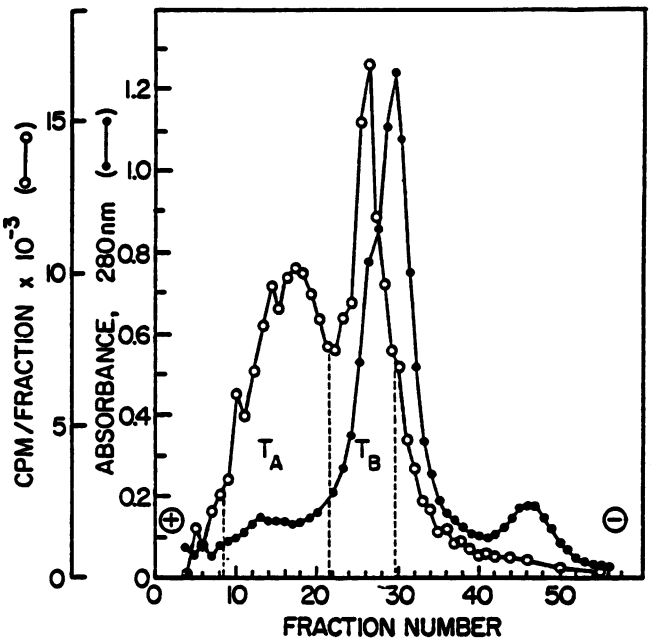

Figure 2. Coelectrophoresis of tonoplast vesicles obtained by consecutive sucrose and glycerol gradient centrifugation with total microsomes. Free-flow electrophoresis separation of total microsomes (absorbance 280 nm, (●) to which a small amount of ^{32}P-labeled purified tonoplast (radioactivity, (○) was added (see "Materials and Methods"). The results show two populations of tonoplast vesicles with distinct electrophoretic mobilities.

Concanavalin Test for Membrane Sidedness Determinations

Electron microscopy of Con A peroxidase labeled membranes were as described (9) with embedding in Epon (16). Thin sections were observed unstained.

Immunoblot Analysis

The procedure was as described previously (30). Polyclonal antibodies (a gift from Mandala and Taiz) were prepared against the 72-kD subunit ATPase preparations from maize seedlings (18). Proteins from different membrane fractions were separated by SDS-PAGE according to Laemmli (14) using 10% acrylamide. Proteins were transferred to nitrocellulose by electroblotting (12) and then analyzed for immunoreactivity with a biotinylated secondary antibody and avidin-peroxidase and 4-chloro-1-naphthol as colorimetric substrate. Mol wt markers were stained separately with 0.1% amido black.

Dextran and Sucrose Linear Gradient Centrifugations

Tonoplast fractions were resuspended in homogenization medium and layered over a 10% (w/w) dextran (Sigma: average mol wt 81,500) cushion prepared in the same medium (26, 27). After centrifugation for 2 h at 70,000*g* (Beckman, SW-50-1 rotor), the band at the interface was collected.

Other Procedures

Marker enzymes were tested with the procedures previously described (24). Plasma membranes were stained with phosphotungstic acid at low pH as reported (23). Proteins were determined according to Bensadoun and Weinstein (6) using bovine serum albumin as the standard.

RESULTS

When a microsomal fraction from soybean hypocotyls was resolved by preparative free-flow electrophoresis (Fig. 1A), fraction A, shown previously to be highly enriched in tonoplast vesicles, was separated well to the left of the bulk of the membrane materials in the most electronegative-fractions. Free-flow electrophoretic separations of total microsomes and tonoplast-enriched membranes from consecutive sucrose and glycerol gradient centrifugation of homogenates prepared by mashing with mortar and pestle and a Waring blender were compared (Fig. 1). The mashing procedure for tissue homogenization yielded large A fractions (Fig. 1A), whereas with razor blade chopping or polytron homogenization (data not shown) fewer tonoplast vesicles were obtained (Fig. 1B). With tonoplast fractions prepurified by consecutive sucrose and glycerol gradient (Fig. 1C), two fractions were obtained. These were designated T_A corresponding to electrophoresis fraction A from total microsomes (Fig. 1, A and B) and T_B corresponding to free-flow electrophoresis fraction B (Fig. 1, A and B). On the other hand, if instead of mortar and pestle homogenization, razor blade chopping was used, the sucrose/glycerol gradient procedure yielded dominantly a fraction T_B with very little fraction T_A (Fig. 1D) consistent with the analysis of total microsomes (Fig. 1B). Thus, free-flow electrophoresis resolved two distinct fractions of putative tonoplast vesicles of different electrophoretic mobility, and the relative amounts of the two populations could be varied by the conditions of the homogenization.

Comigration of tonoplast fractions prepared by the sucrose/glycerol gradient procedure with free-flow electrophoretic fractions A and B was demonstrated by mixing experiments in which total microsomes were combined with a small amount of ^{32}P-labeled purified tonoplast by the sucrose/glycerol gradient procedure prior to electrophoresis (Fig. 2).

Table I shows the distribution of biochemical markers and ATPase activities among the free-flow electrophoretic fractions. The bulk of the mitochondria (succinate-INT reductase and Cyt *c* oxidase activities) and ER (NADPH Cyt *c* reductase activity) was concentrated in the center of the electrophoretic separation (fraction C). These enzyme activities also were recovered partly in fractions B and D. The Golgi apparatus (fucosyltransferase activity) was present in similar amounts both in fractions B and C. Most of the plasma membrane vesicles (PTA-reactive membrane) were found in fraction E.

The measurements of ATPase activities and their responses to inhibitors also identified the different membranes among the free-flow electrophoresis fractions (Table I). Fraction E contained a vanadate-inhibited ATPase activity insensitive to nitrate and azide, and fraction A contained a nitrate-inhibited ATPase activity insensitive to vanadate and azide. Only a

Table I. *Specific Activities and Distribution of Total Activities of Marker Enzymes among Free-Flow Fractions*

Marker Enzymes	Activity	Free-Flow Electrophoresis Fractions				
		A	B	C	D	E
Succinate-INT reductase	OD/h/mg protein	0.25	1.02	2.36	0.61	0.03
	%	1.0	10.7	86.1	2.2	
Cyt *c* oxidase	OD/min/mg protein	2.08	6.59	14.76	5.54	1.16
	%	1.3	10.0	78.4	9.0	1.3
NADPH Cyt *c* reductase	OD/min/mg protein	0.16	0.48	0.68	0.44	0.32
	%	1.8	13.4	65.4	13.0	6.4
Fucosyltransferase	cpm/h/mg protein	ND[a]	2740	1380	380	ND
	%		55	43	2	
PTA staining	% PTA-reactive	1 ± 1	9 ± 2	19 ± 4	33 ± 8	97 ± 1
	%		5	12	21	62
ATPase	nmoles Pi/min/mg protein	48.7	46.2	67.5	37.0	15.8
	%	4.9	17.8	66.8	7.9	2.6
$-KNO_3$ inhibited[b]		20 ± 5	44 ± 5	66 ± 3	50 ± 2	None
$-NaN_3$ inhibited		None	49 ± 4	83 ± 5	66 ± 5	None
$-V_2O_5$ inhibited		None	None	None	None	42 ± 3

[a] Not detected. [b] Inhibition as percentage of initial ATPase activity, by nitrate (50 mM), azide (1 mM), or vanadate (100 μM): vanadate was prepared according to Gallagher and Leonard (12).

slight inhibition by nitrate was observed for membranes of fraction A. The other fractions exhibited ATPase activity strongly inhibited both by both nitrate and azide.

Finally, the identification of tonoplast was checked by immunological probes. Immunoblot analysis revealed that the sites antigenic to the ATPase antibody were associated essentially with fraction A as evidenced by strong reactivity of peroxidase-linked anti-rabbit IgG (Fig. 3). Reactivity also was seen in fraction B, but not with electrophoretically purified plasma membrane (fraction E) and the membranes of fractions C and D.

To determine the basis for the two distinct populations of tonoplast vesicles obtained by electrophoretic separation of the prepurified tonoplast fraction, the possibility of differences in absolute membrane orientation was investigated using structure-linked enzyme latency with ATPase as the marker. With purified tonoplast vesicles by consecutive sucrose/glycerol gradient centrifugation (Fig. 4A) or from free-flow electrophoresis fraction A (Fig. 4B), the pH dependence of ATPase activity was similar both in the presence and in the absence of low concentrations of Triton X-100. More interesting, a difference in latency was observed comparing the purified

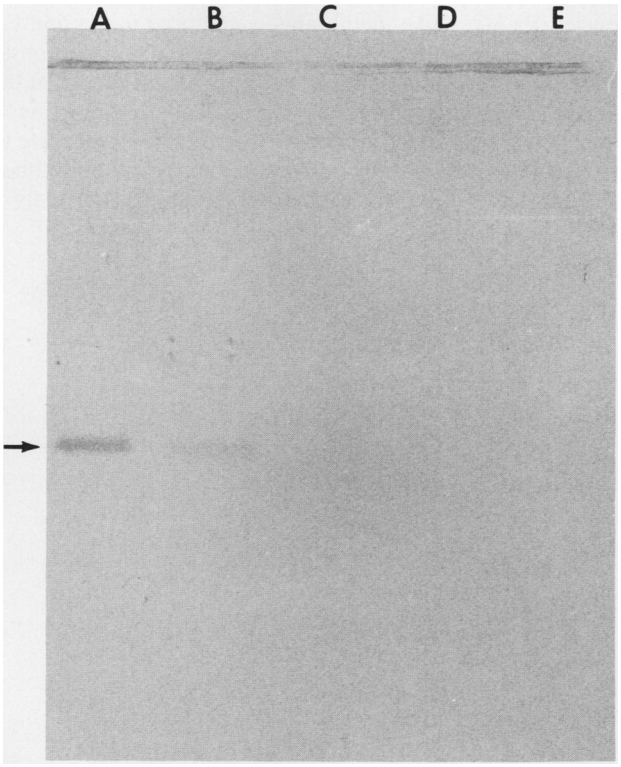

Figure 3. Immunoblot analysis of reactivity of tonoplast ATPase (72 kD subunit) antibody to membrane fractions obtained by free-flow electrophoretic separation of total microsomes. The plant material was homogenized by mashing the soybean hypocotyls with a mortar and pestle. Proteins from membrane fractions (50 μg protein/lane) loaded in the different lanes were fractions A–E. Arrow designates the position of the 72 kD subunit ATPase.

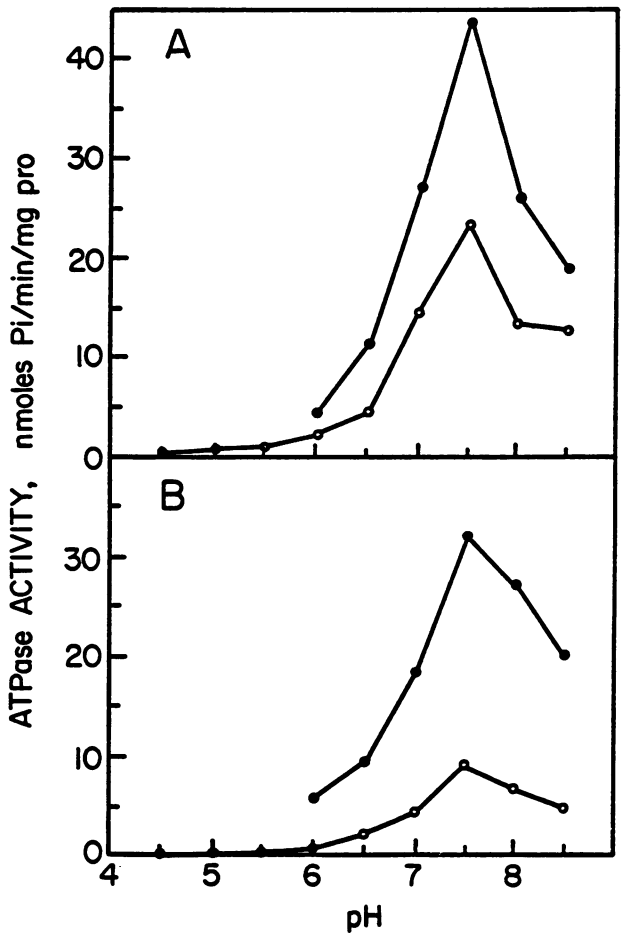

Figure 4. pH dependency of ATPase activity of tonoplast vesicles isolated by consecutive sucrose and glycerol gradient centrifugation (A) or from free-flow electrophoresis-fraction A (B). The plant material was homogenized by mashing the soybean hypocotyls with a mortar and pestle. The ATPase activity was determined before treatment with 0.02% Triton X-100 (○) or after treatment with 0.02% Triton X-100 (●). The vesicles obtained from free-flow electrophoresis fraction A exhibited a greater latent ATPase activity than those by consecutive sucrose and glycerol gradient centrifugation.

tonoplast vesicles from the two different sources. With fraction A from free-flow electrophoresis, Triton stimulation was about 4-fold (Fig. 4B), whereas with tonoplast vesicles from consecutive sucrose and glycerol gradient centrifugation, the Triton stimulation was about 2-fold (Fig. 4A; Table II). Moreover, when the membranes isolated by consecutive sucrose and glycerol gradient centrifugation were subfractionated by free-flow electrophoresis, a similar stimulation of ATPase activity by detergent treatment (4-fold) was observed with vesicles of fraction T_A, whereas with vesicles of fraction T_B, almost no stimulation occurred (1.1-fold) (Table II; Fig. 5). With both tonoplast populations, maximum latency was attained with a detergent protein ratio of about 2.0 (1.7–2.4).

Table III shows the effects of gramicidin and various inhibitors on ATPase activity of fractions T_A and T_B. Gramicidin

Table II. *Latency of ATPase Activity of Isolated Tonoplast Vesicles from Soybean Hypocotyl Microsomes by Gradient Centrifugations and Free-Flow Electrophoresis in Series*

The ATPase specific activities reported with Triton X-100 were determined from Triton X-100 dose response curves as illustrated in Figure 5.

Fraction	ATPase Specific Activity		
	− Triton X-100	+ Triton X-100	Ratio
Free-flow fraction A	9.0	31.8	3.5
Sucrose/glycerol gradient	23.6	43.7	1.8
Free-flow fraction T_A	4.5	18.2	4.0
Free-flow fraction T_B	15.6	16.5	1.1

did not stimulate significantly the ATPase activity, and the stimulation of Triton was largely above that of gramicidin ruling out the possibility that Triton collapses ion gradients just like ionophores and thus stimulates the ATPase activity of cytoplasmic side out vesicles. In addition, the tonoplast fractions T_A and T_B were found to be similar based on inhibition of ATPase activity (Table III) and protein patterns of SDS-gels (Fig. 6).

To confirm that the differences observed in latency were for fractions of opposite orientations, direct electron microscope observations were employed using Con A-peroxidase label to mark the surface of tonoplast vesicles. The imposed electron dense label marked both populations of tonoplast vesicles (Fig. 7), *i.e.* isolated membranes by consecutive sucrose and glycerol gradient centrifugation, then subfractionated by free-flow electrophoresis. However, the tonoplast vesicles from fraction T_A exhibited greater electron density (Fig. 7A) than did the tonoplast vesicles from fraction T_B (Fig. 7B).

To demonstrate that the differences in ATPase latency and in reactivity of the imposed electron dense markers were not due to differences in degree of vesicle sealedness, vesicles of all fraction were analyzed by the dextran gradient distribution method for vesicle integrity. Either by free-flow electrophoresis or consecutive sucrose and glycerol gradient centrifugation, and for different methods of tissue homogenization, in average more than 80% of the tonoplast vesicles banded at the interface (Table IV). No differences were apparent between the different procedures used for the isolation and the purification of tonoplast vesicles except for Polytron homogenization which yielded the lowest proportion of membranes banding at the interface (Table IV).

DISCUSSION

In a previous paper (9), we reported the absolute orientations of plasma membrane vesicles obtained in highly purified fractions by preparative free-flow electrophoresis and by aqueous two-phase partition. The plasma membrane was identified by the use of cytochemical and biochemical probes, *i.e.* PTA[2] staining, NPA binding, sterol glucoside synthetase,

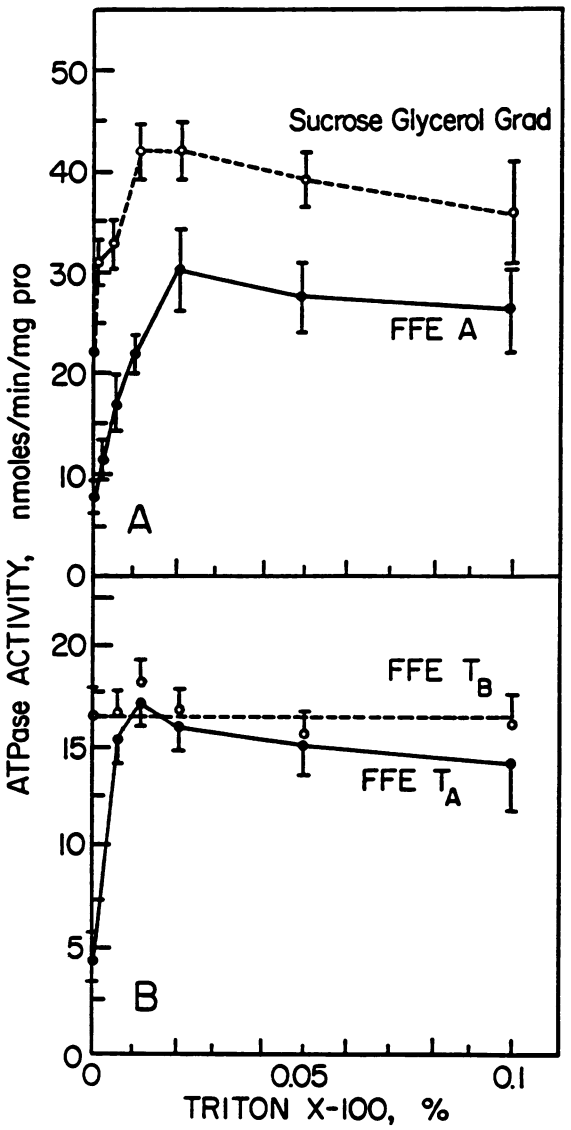

Figure 5. Latency of ATPase activity of isolated tonoplast vesicles. ATPase activity of tonoplast vesicles as a function of detergent (Triton X-100) concentration, obtained by consecutive sucrose and glycerol gradient centrifugation (upper curve, A) or from free-flow electrophoresis fraction A (lower curve, A). In B, the latency is shown for two electrophoretic fractions obtained by free-flow electrophoresis of tonoplast vesicles first obtained by consecutive sucrose and glycerol gradient centrifugation. One fraction, comigrating in the position of fraction B, had a very low latency of ATPase activity (1.1-fold), whereas the second fraction comigrating in the position of fraction A had a much higher latency (4.0-fold).

[2] Abbreviations: PTA, phosphotungstic acid; NPA, *N*-1-naphthylphthalamic acid.

Table III. *Effect of Azide, Nitrate, Vanadate, and Gramicidin on ATPase Activity of Tonoplast Vesicles Isolated by Gradient Centrifugations and Free-Flow Electrophoresis in Series*

	Activity			
	Tonoplast fraction T_A		Tonoplast fraction T_B	
	nmolPi/min/mg	*% of control*	*nmolPi/min/mg*	*% of control*
Control (−Triton X-100)	4.4	100	25.3	100
Gramicidin	4.8	109	25.3	100
Control (+Triton X-100)[a]	22.4	100	26.1	100
Azide	22.9	102	25.1	96
Vanadate	21.8	98	24.2	93
Nitrate	16.7	75	17.7	68

[a] ATPase activity was measured in the presence of 0.02% Triton X-100, the level of inhibitors was as for Table I.

glucan synthetase II (25). A similar spectrum of markers is not available for the vacuolar membranes of plant cells (3).

The identification of the tonoplast through the different fractions obtained by free-flow electrophoresis of microsomal membranes was previously achieved by membrane thickness measurements and nitrate sensitive ATPase activity (25). The most electronegative fraction (fraction A) was found to consist of highly pure tonoplast vesicles. In this work, the activity of several markers of intracellular membranes, ATPase activities and immunoreactivity with a tonoplast ATPase antibody were checked for the different pooled fractions. Less than 2% of the marker activities were recovered in fraction A which strongly reacted with the tonoplast ATPase antibody and was the only fraction containing a nitrate inhibited ATPase resistant to azide, confirming the purity and the identity of tonoplast vesicles obtained by preparative free-flow electrophoresis.

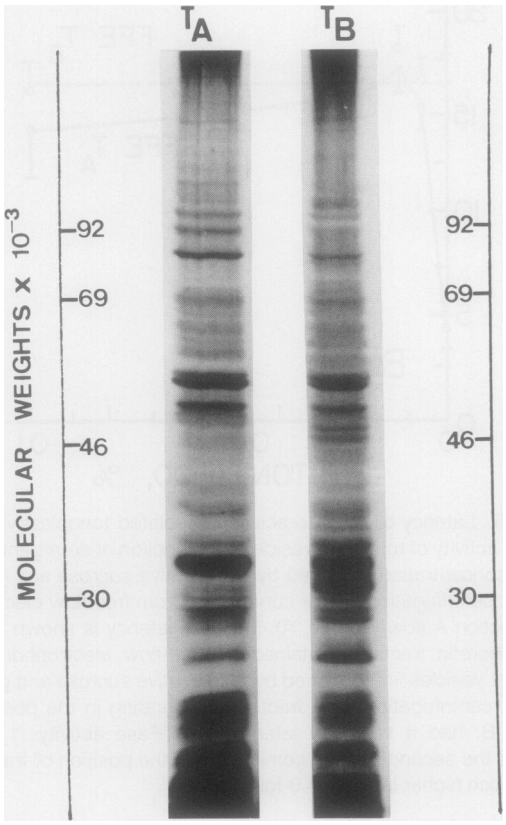

Figure 6. Comparison of polypeptide composition of tonoplast fractions T_A and T_B isolated from soybean hypocotyls by gradient centrifugations and free-flow electrophoresis in series. Equal amounts of protein were loaded in each well. The 10% SDS-polyacrylamide gels were silver stained. The apparent mol wt were estimated by comparison with standards.

Such isolated tonoplast vesicles can provide useful models to study transport (29), especially if the vesicles are sealed and defined in their absolute orientations. Since membranes are asymmetric in their organization, breakage could result in closed membrane vesicles that expose either the original external side or the original internal side to the new external medium to constitute either cytoplasmic side out or cytoplasmic side in vesicles. Our findings show that as tonoplast membranes vesiculate, they give either mixed populations of vesicles of both orientations or vesicles predominantly orientated in one direction only, depending upon the method of tissue homogenization. Using gradient centrifugations and free-flow electrophoresis in series, highly purified fractions of the two kinds of vesicles exhibiting similar protein patterns and characteristics for ATPase activity were obtained.

We have used both morphological and biochemical methods to establish orientation of the isolated tonoplast vesicles in these studies. The ATPase of the tonoplast is among the best characterized of the enzyme associated with this cell compartment (5, 17, 19, 23, 29). The active site has been located at the cytoplasmic surface of the tonoplast such that vesicles where the cytoplasmic side is out should not exhibit a strong detergent-linked latency for ATPase whereas oriented cytoplasmic side in should exhibit maximal ATPase activity only after the vesicles are ruptured with detergent. Vesicles of fraction T_A for which Triton stimulation was much greater than for fraction T_B are considered to be predominantly cytoplasmic side in, while tonoplast vesicles of fraction T_B are predominantly cytoplasmic side out. Similar increases in ATPase activity, upon treatment of vesicles with detergent, have been used previously by Larsson *et al.* (15) to provide indications of the sidedness of vesicles of plasma membrane obtained by two-phase partition.

As a further indication of sidedness and to demonstrate homogeneity of the final purified fractions, binding of Con A was used as an extrinsic marker. This lectin binds specifically to α-D-mannopyranosyl and α-D-glycosylyl residues. By com-

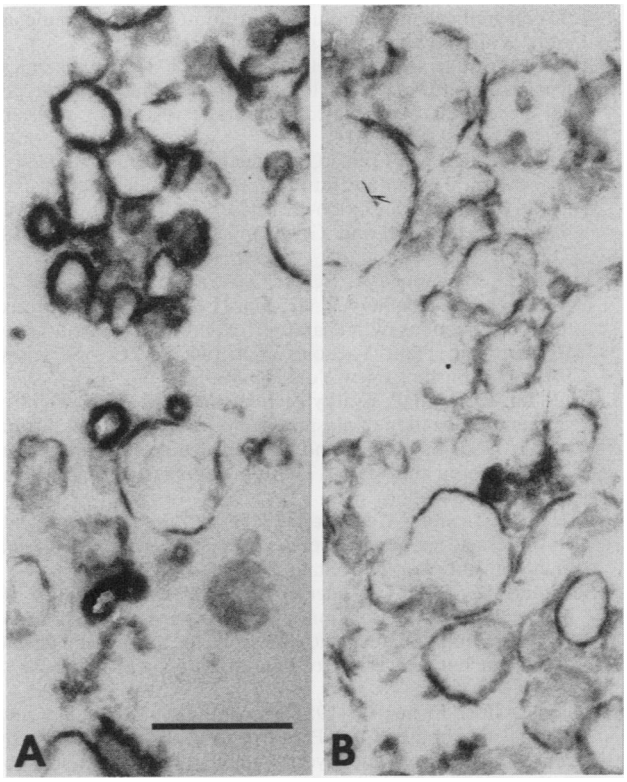

Figure 7. Con A-peroxidase labeling to show sidedness of tonoplast orientation in the isolated vesicles. A, Tonoplast vesicles of fraction T_A, with a cytoplasmic side in orientation, strongly bind the Con A; B, tonoplast vesicles of fraction T_B, with the cytoplasmic side out, bind Con A less strongly. These vesicles were separated as described for Figure 1C. Scale bar = 0.5 μm.

paring vesicles of tonoplast fraction T_A and tonoplast fraction T_B, marked differences in Con A reactivity were observed. Tonoplast vesicles of fraction T_A were uniformly strongly reactive (cytoplasmic side in), whereas vesicles of tonoplast fraction T_B were uniformly weakly reactive (cytoplasmic side out). Our results can be compared to those for yeast using Con A labeled with tritium or with a fluorescent dye (7). It was shown that isolated vacuoles of *Saccharomyces cerevisiae* did not bind Con A unless the vacuoles were rendered permeable and their inner membrane surface made accessible. The

Table IV. *Assay for Integrity of Tonoplast Vesicles Obtained by Gradient Centrifugations and Free-Flow Electrophoresis*

Fraction Loaded	Fraction Recovered	Total Protein Recovered		
		Hand chopped (razor blade)	Mechanical (Polytron)	Smash (mortar/pestle)
		%		
Fraction A	Interface	92	68	
Experiment 1	Pellet	8	32	
Fraction A	Interface	87	81	88
Experiment 2	Pellet	13	19	12
Sucr/Glycerol	Interface	100		100
Gradients	Pellet	ND[a]		ND

[a] Not detected (no pellet).

authors concluded that both plasma membrane and tonoplast carry Con A binding sites exclusively on the surface opposite to the cytoplasmic matrix. As cytoplasmic side in plasma membrane vesicles isolated from soybean strongly reacted with Con A (19), a similar asymmetric distribution of Con A binding sites on plasma membrane and tonoplast was indicated.

The difference in absolute orientations deduced from Con A binding and from ATPase latency was confirmed as well by enzyme cytochemistry (20). As a morphological indication of sidedness, we used the location of the reaction products deposited upon cytochemical incubation of NADH-ferricyanide oxidoreductase (so-called Hatchett's brown). This activity is present in purified tonoplast vesicles (4) at specific activities near those found in plasma membrane vesicles. NADH-ferricyanide oxidoreductase activity was seen in broken cell preparations only associated with the outer (cytoplasmic) tonoplast surface (20). With isolated vesicles, reaction product was seen only with tonoplast vesicles from fraction T_B marking them as cytoplasmic side out and not with tonoplast vesicles from electrophoretic fraction T_A.

That vesicles of fraction A were sealed is indicated by the high degree of ATPase latency. For fraction B, which does not show ATPase latency, it was necessary to utilize yet another approach. Here we have determined the sealedness by centrifugation in dextran gradients. This method has been used to separate sealed vesicles from leaky vesicles and is based on differences in buoyancies of the sealed and leaky vesicles in density gradients of high mol wt polymers. The rationale was developed with erythrocyte membranes (27, 28). When applied to the purified tonoplast vesicles, this method showed that vesicles were more than 80% sealed. Evidence for proton pumping in fraction B (22) is a further indication of vesicle sealedness.

The differences in membrane sidedness likely contribute to the differences in surface charge that account for the specific electrophoretic migration of cytoplasmic side in (greatest electronegativity) and cytoplasmic side out vesicles. By analogy with plasma membrane, it is expected that the external tonoplast surface will carry the greatest negative surface charge. However, the large difference in migrational ability on preparative free-flow electrophoresis between tonoplast (most electronegative) and plasma membrane (2, 25) was quite unexpected and remains unexplained. Surprisingly, both cytoplasmic side out and cytoplasmic side in tonoplast vesicles exhibit a greater electrophoretic mobility with free-flow electrophoresis under the conditions we use that do either of the corresponding (fractions C and E) plasma membrane fractions.

Finally, tonoplast vesicles of known orientations will represent useful tools for studying the relative topography of tonoplast constituents. These vesicles will be particularly useful for the study of carriers involved in transport processes (influx, efflux) across the membrane.

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