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Isolation and Partial Characterization of Vacuoles from Tobacco Protoplasts[1]

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ABSTRACT

Protoplasts from suspension-cultured cells of *Nicotiana glutinosa* L. were lysed in 0.3 molar sorbitol in 2 millimolar ethylenediaminetetraacetate-tris(hydroxymethyl) aminomethane (pH 7.5) to release intact vacuoles. The vacuoles were purified by centrifugation in a Ficoll step gradient. About 11% of the vacuoles and 13% of the acid phosphatase activity was recovered in the purified vacuole fraction, suggesting that the vacuole is the major site for acid phosphatase in these cells. NADH-cytochrome *c* reductase, malate dehydrogenase, and cytochrome *c* oxidase activities were reduced during vacuole purification. The majority of the adenosine 5'-triphosphate (ATP) hydrolytic activity of purified vacuoles was associated with nonspecific acid phosphatase and not with a transport ATPase. As judged by acid phosphatase distribution and electron microscopy, the effective density of vacuoles in a sucrose gradient was low (less than 1.1 grams per cubic centimeter), although an unequivocal estimate of the vacuole or tonoplast density was not possible from the experiments conducted.

The role of the vacuole in the ionic relations of plant cells is not well understood. Major transport functions have been proposed for the vacuole (8, 17), but it has been difficult to investigate the ion transport capabilities of the tonoplast because of the confounding effects of the plasma membrane. It would be desirable to isolate intact vacuoles from plant cells to investigate the transport properties of this cellular compartment.

Recently, various methods have been developed for the isolation of mature vacuoles (2, 9, 16, 18, 27). These techniques have been used in studies on the storage of malic acid in *Bryophyllum* leaf cells (3) and of the cyanogenic glucoside of *Sorghum* (25), the localization of acid hydrolases in *Hippeastrum* flower petal protoplasts (4), and of proteinase inhibitors in tomato leaf cells (28), the biosynthesis of anthocyanin (7), the hydrolytic and storage function of the vacuole (2, 23), and the enzymic properties of the tonoplast (2, 10, 15).

Here, we report the isolation and partial characterization of vacuoles from protoplasts of tobacco suspension cells. We also describe attempts to determine the effective density of vacuoles in a sucrose gradient.

MATERIALS AND METHODS

Protoplast Isolation. Protoplasts were isolated from suspension cultures of tobacco (*Nicotiana glutinosa* L.) as described previously (20). Briefly, suspension-cultured cells were incubated in 1% Cellulysin, 0.2% Macerase, and 0.7 M mannitol (pH 5.8) for 4 h at 27 C to digest the cell walls. The suspension was filtered and protoplasts collected and washed by repeated centrifugations at 150*g* in 0.7 M mannitol. The final protoplast pellet (2–3 ml packed volume from 20 to 30 g fresh weight of cells) was suspended in 5 volumes of 0.7 M mannitol.

Vacuole Isolation. Vacuoles were released by addition of the protoplast suspension to 10 volumes of 0.3 M sorbitol, 2 mM EDTA-Tris (pH 7.5), and 2.5 mM DTT. The mixture was stirred gently for 10 min. The reduced osmotic strength of the sorbitol buffer and the chelating effects of EDTA led to rapid lysis of the protoplasts and release of intact vacuoles (Fig. 1). Much of the remaining protoplasm formed a large aggregate which was removed by filtration through four layers of cheesecloth. Large, mature vacuoles were collected by centrifugation at 500*g* for 10 min (Fig. 2). The pellet was suspended by very gentle agitation in 0.5 ml of 0.5 M sorbitol, 0.25 mM EDTA-Tris (pH 7.2), and 1.0 mM DTT (suspension buffer). This 500*g* vacuole fraction was layered on a Ficoll step gradient consisting of 3 ml of 7.5% (w/v) Ficoll in suspension buffer (bottom layer) and 5 ml of suspension buffer (top layer), and centrifuged at 1,000*g* for 30 min. After centrifugation, the vacuoles remained at the Ficoll interface (Fig. 2).

Sucrose Gradient Centrifugation. Purified vacuoles or other membrane preparations from the vacuole lysate (see figure legends) were layered on 36-ml linear gradients ranging from 10 to 45 or 50% (w/w) sucrose in 1 mM Tris-Mes (pH 7.2) and 0.5 mM DTT. The gradients were centrifuged at 80,000*g* for 15 h at 4 C in a Beckman SW 27 rotor and fractionated (1.5-ml fraction) as previously described (14). Per cent sucrose was determined by refractometry.

Enzyme Assays. Phosphatases were assayed at 38 C in a 1-ml reaction volume containing 3 mM substrate, 30 mM Tris-Mes (at desired pH), 3 mM $MgSO_4$, and 50 mM KCl. Other mono- or divalent ions were substituted as indicated. Orthophosphate released was determined by the formation of molybdenum blue (6). NADH-Cyt *c* reductase, Cyt *c* oxidase, and malate dehydrogenase were assayed spectrophotometrically at room temperature (22 C) as described (6, 11, 22). Protein was determined with the Folin phenol reagent (6).

Microscopy. For electron microscopy, vacuole or other membrane fractions were fixed in 2% glutaraldehyde in 100 mM K-phosphate (pH 7.2) and 0.25 M sucrose for 20 min and then pelleted by centrifugation at 80,000*g* for 30 min. The pellets were washed with phosphate buffer and postfixed in 1% OsO_4 for 2 h. Samples were dehydrated in a graded acetone series and embedded in Spurr's resin (20). Thin sections were either stained in uranyl acetate and lead citrate or in a PACP[3] stain which has been used to specifically stain the plasma membrane (14, 22).

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[3] Abbreviation: PACP: periodic acid-phosphotungstic acid-chromic acid.

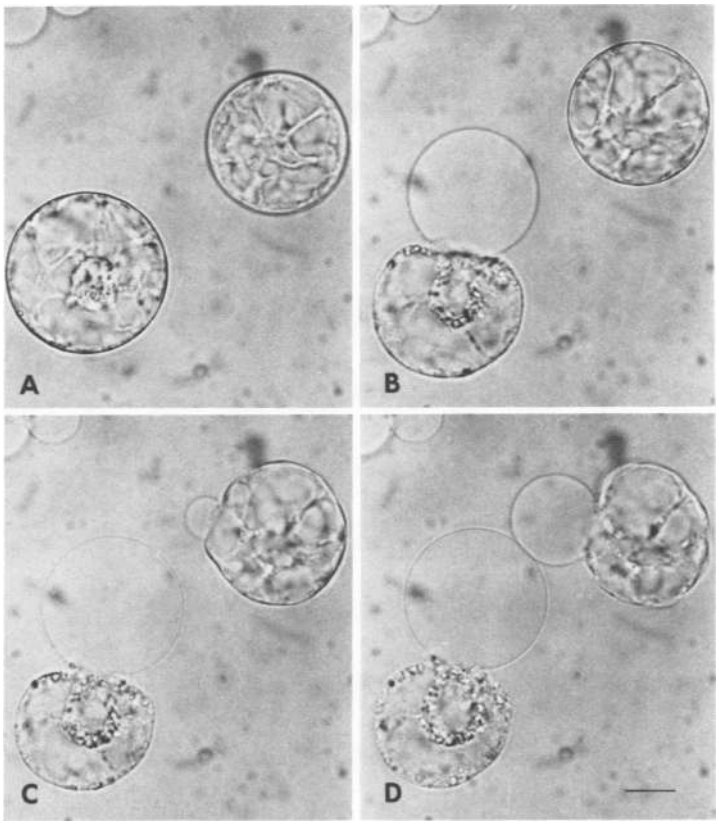

FIG. 1. Light micrographs showing release of vacuole by osmotic lysis of tobacco protoplasts. Lysis was induced by addition of H_2O to the protoplast suspension. A: protoplasts immediately after addition of H_2O; B: 30 s after addition of H_2O; C: 45 s; D: 60 s. Bar represents 10 μm.

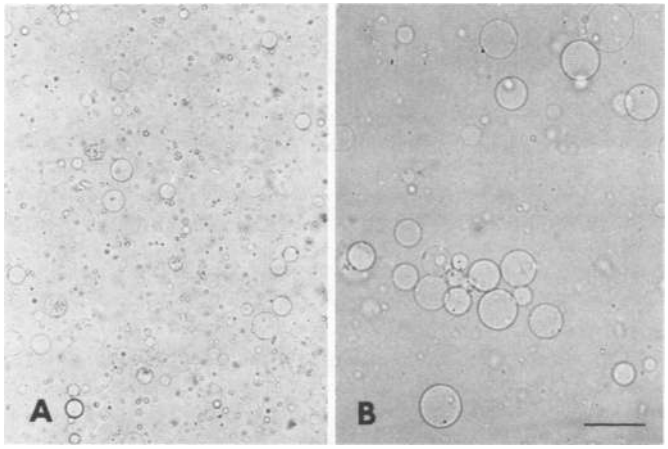

FIG. 2. Light micrographs of vacuoles purified from tobacco protoplasts. A: crude vacuole fraction obtained by centrifuging the lysed protoplasts at 500 *g* for 10 min. B: Purified vacuole preparation from the Ficoll-sorbitol gradient. Bar represents 50 μm.

RESULTS

Vacuole Isolation. The first large scale isolation of mature vacuoles from higher plant protoplasts employed osmotic shock in 200 mM K-phosphate (pH 8.0) and 3 mM $MgCl_2$ to release the vacuole (27). Attempts to use this procedure with tobacco protoplasts were not satisfactory because of incomplete protoplast lysis and excessive aggregation of vacuoles with other cellular components. Satisfactory cell lysis was obtained by addition of tobacco protoplasts to 0.3 M sorbitol in 2 mM EDTA-Tris (pH 7.5). Subsequent purification steps required the absence of ionic salts and the inclusion of 0.25 mM EDTA to avoid aggregation. DTT was also included to help preserve enzyme activities.

Of the enzymes tested (Table I), acid phosphatase showed the largest increase in specific activity during vacuole purification. About 13% of the total phosphatase activity was recovered in the vacuole fraction. Based on the assumption of one vacuole released per protoplast and the determination of total number of protoplasts and isolated vacuoles, approximately 11% of vacuoles were recovered in the vacuole fraction. This is similar to the recovery of acid phosphatase in the vacuole fraction and is consistent with the idea that the vacuole is the primary site for phosphatase in the protoplast.

It was determined that there were approximately 1.1×10^7 vacuoles per mg protein in the vacuole fraction. Assuming an average of one vacuole released per protoplast (Fig. 1) and 760,000 protoplasts per mg protein (20), it can be calculated that at a minimum of about 7% of the protoplast protein is associated with the vacuole. The expected maximum increase in specific activity of a vacuolar enzyme would then be 14- to 15-fold upon complete purification of intact vacuoles. The increase in acid phosphatase was about 26-fold in the vacuole fraction compared to the protoplast preparation (Table I). Part of this increase may be due to an increase in measurable acid phosphatase induced by lysis of the protoplast. A similar response has been described by Lin *et al.* (15).

In contrast to reports of the association of NADH-Cyt *c* reductase with the tonoplast (9, 10), the activity of this enzyme was markedly decreased in the vacuole preparation (Table I). Malate dehydrogenase and Cyt *c* oxidase were also reduced during vacuole purification with less than 1% of the total activity recovered in the final vacuole preparation (Table I).

Microscopy. As shown by light microscopy, the vacuole preparation was substantially free of intact protoplasts (Fig. 2). The majority of the isolated vacuoles were from 20 to 30 μm in diameter, as compared to an average diameter for protoplasts of about 28 μm (20).

Table I. *Enzyme Activities and Total Protein in Various Fractions Obtained during the Vacuole Isolation Procedure*

Fraction	Protein	Acid Phosphatase (pH 5.5)	IDP Hydrolysis (pH 7.5)	ATP Hydrolysis (pH 6.5)	ATP Hydrolysis (pH 9.0)	Malate Dehydrogenase	Cyt *c* Oxidase	NADH-Cyt *c* Reductase
	mg	*μmol Pi released/mg protein·h*				*μmol product/mg protein·min*		
1. Protoplast	89	21	11	19	16	8	0.4	0.3
2. Filtered lysate	51	56	30	33	18	7	0.6	0.8
3. 500*g* supernatant	49 (97.1)[a]	42 (79)	24 (81)	27 (84)	16 (90.9)	7 (98.4)	0.4 (97.8)	0.7 (99.2)
4. Ficoll gradient layers	0.6 (1.3)	280 (7)	146 (6)	103 (4)	32 (2.4)	5 (0.9)	0.4 (1.2)	0.3 (0.5)
5. Ficoll gradient pellet	0.2 (0.3)	217 (1)	155 (2)	93 (1)	14 (0.2)	2 (0.1)	0.1 (0.1)	0.4 (0.2)
6. Vacuole preparation	0.6 (1.3)	559 (13)	263 (11)	243 (10)	87 (6.5)	4 (0.6)	0.3 (0.9)	0.02 (0.1)

[a] Numbers in parentheses indicate per cent of total activity or protein recovered from filtered lysate.

Observation of isolated vacuoles with the electron microscope revealed membranes which were weakly stained by uranyl acetate and lead citrate but were not stained by the PACP procedure (Fig. 3). These characteristics were also observed for the tonoplast of intact protoplasts (Fig. 4). Of particular interest was the reaction of the PACP procedure with the electron-dense particles in the vacuole. Electron-dense bodies are commonly observed within the vacuoles of plant cells stained with lead citrate and uranyl acetate. The identity of these deposits is not established and they have been variously ascribed to tannins or polyphenolics (1), proteins (26), or proteins precipitated by polyphenolics (29). The ability of the PACP staining procedure to reduce the electron density of these inclusions apparently has not been reported.

ATP Hydrolytic Activity. The ATP hydrolytic activity of the isolated vacuoles was examined for possible characteristics similar to those of the plasma membrane ATPase (12, 13). The hydrolytic activity showed no preference for ATP as a substrate (Table II). This was also true when just KCl-stimulated activity was considered. Divalent cations inhibited the rate of ATP hydrolysis and were neither required nor specific for KCl stimulation of enzyme activity (Table III). There were no specific effects of monovalent salts (except for inhibition by KF) on ATP hydrolytic activity (Table IV). Similar results were observed for the effects of mono- and divalent salts on *p*-nitrophenol phosphatase activity (Tables III and IV). The results suggest that the bulk of the salt-stimulated ATP hydrolytic activity of the purified vacuole fraction was associated with nonspecific acid phosphatase and not transport ATPase. It is conceivable that a low level of ion-stimulated substrate-specific ATPase activity associated with the tonoplast may have been masked by an active, nonspecific phosphatase in the vacuole. Several attempts to distinguish between a putative "tonoplast ATPase" and the soluble vacuole phosphatase were not successful (19).

Sucrose Gradient Centrifugation of Isolated Vacuoles. The vacuole fraction was layered on top of a linear sucrose gradient (10–45%, w/w) and centrifuged for 15 h at 80,000*g*. The majority of the ATP hydrolytic activity and acid phosphatase remained near the top of the gradient associated with the peak in *A* at 280 nm (Fig. 5). ATP hydrolytic activity and acid phosphatase at the top of the gradient was slightly separated from malic dehydrogenase activity. A relatively small amount of phosphatase and malic dehydrogenase activities was found at about 36 to 39% sucrose, indicating that mitochondrial contamination in the vacuole fraction was small. Treatment of the vacuole preparation with sonication or diafiltration (Amicon XM-300 membrane filter) before centrifugation did not affect the distribution of these enzyme activities in the sucrose gradient (not shown). Centrifugation of the vacuole preparation in a linear sorbitol gradient (10–45%, w/w) produced results similar to those for the sucrose gradient (not shown).

Electron micrographs of the material which remained near the top of the sucrose gradient showed the presence of large membrane vesicles which were weakly stained by lead citrate-uranyl acetate (Fig. 6A) and did not stain with PACP (Fig. 6B). The results suggest that the vacuoles did not significantly enter the sucrose gradient employed in these experiments.

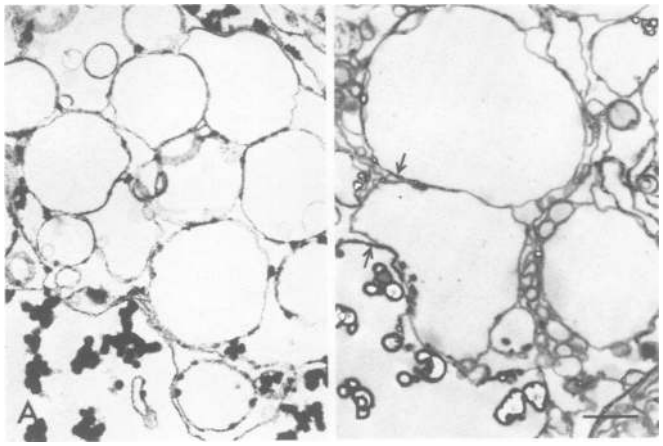

FIG. 3. Electron micrographs of the purified vacuole preparation from tobacco protoplasts. A: poststained with lead citrate and uranyl acetate. Note the presence of electron-dense bodies and the weak staining of the membrane. B: stained with the PACP procedure. Note the clearing in center of electron-dense bodies. In some regions (arrows) thin layers of electron-dense material are associated with the membrane. The tonoplast itself is not stained (compare with density of PACP-stained plasma membrane in Fig. 4). Bar represents 1.0 μm.

Sucrose Gradient Centrifugation of Protoplast Lysate. Gentle lysis of the protoplasts may be a desirable method for obtaining a cell homogenate to be used in membrane or organelle purification. The protoplast lysate fraction remaining after sedimentation of vacuoles at 500*g* was centrifuged at 13,000*g* (15 min) and 40,000*g* (90 min) to obtain crude mitochondrial and microsomal fractions, respectively. These fraction were subjected to sucrose density gradient centrifugation.

As expected, the crude mitochrondrial fraction (500–13,000*g* pellet from the protoplast lysate) showed a sharp peak of *A* (280 nm) which coincided with the mitochondrial marker, malate dehydrogenase at about 42% sucrose (Fig. 7). ATP hydrolytic activity showed two peaks, one near the top of the gradient which corresponded to a peak in *A* at 280 nm, and a second at about 34% sucrose which was not associated with a major *A* peak. Electron micrographs of the material near the top of the gradient showed the presence of relatively large (0.5–3 μm in diameter) membrane vesicles which were weakly stained by lead citrate-uranyl acetate, and not stained by the PACP procedure (Fig. 6, C and D). We conclude that these vesicles represented small vacuoles not sedimented at 500*g*. The membrane identity of the peak of ATP hydrolytic activity at about 34% sucrose is unknown.

Centrifugation of the 13,000 to 40,000*g* fraction from lysed protoplasts in a sucrose gradient gave the distribution of enzyme activities shown in Figure 8. The presence of malate dehydrogenase activity suggested that this fraction still contained some mitochondrial contamination. NADH-Cyt *c* reductase, a marker for smooth ER, showed a sharp peak of activity at about 23% sucrose (about 1.09 g/cc) which coincided with the major peak in *A* at

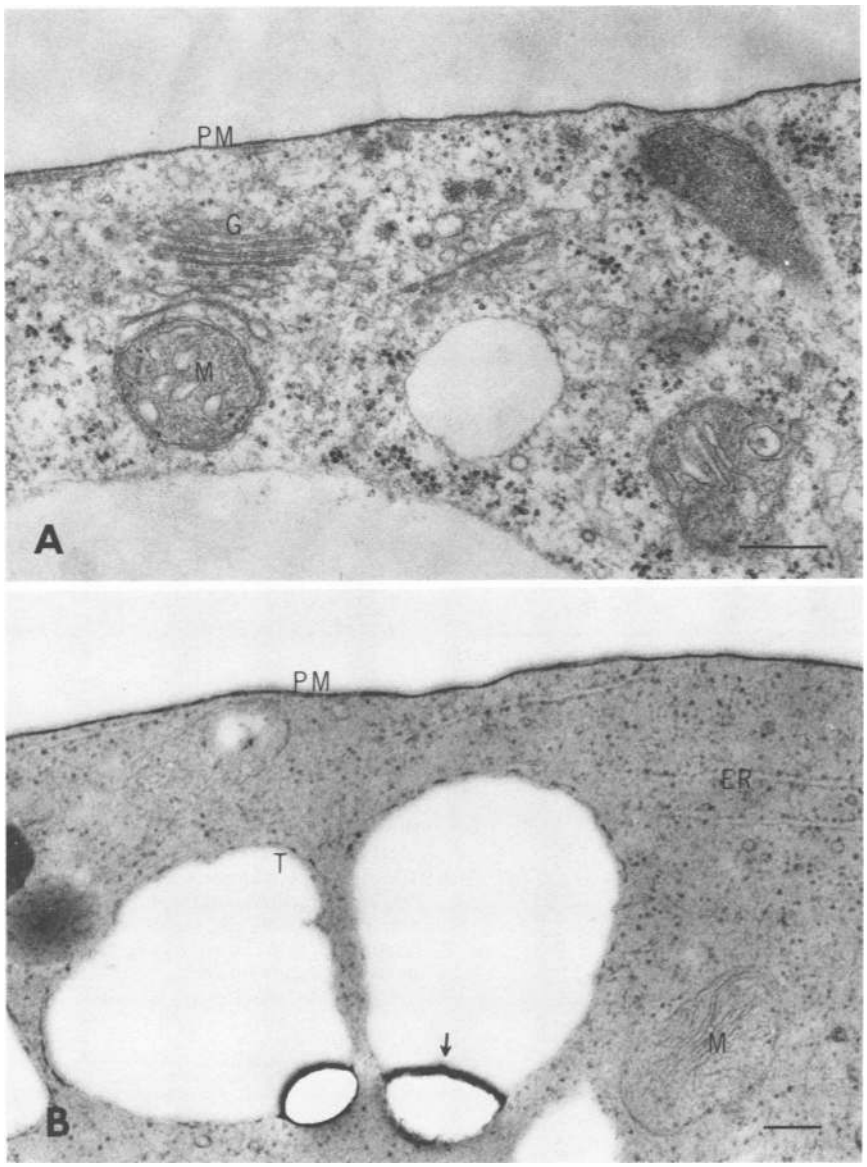

FIG. 4. Electron micrographs of tobacco protoplasts. A: typical appearance of protoplasts poststained with lead citrate and uranyl acetate showing well defined plasma membrane (PM), Golgi (G), mitochondria (M), and other cytoplasmic constituents. The tonoplast is weakly stained. B: protoplast stained with the PACP procedure. The plasma membrane is heavily stained while the membranes of the ER, mitochondria (M), and the tonoplast are not stained. Note the internal clearing and peripheral staining of the normally electron-dense bodies in the vacuoles (arrow). Bars represent 0.25 μm in A and B.

Table II. *Activity of Vacuole Fraction Isolated from Tobacco Protoplasts with Various Substrates*

Assay contained 3 mM substrate (Na salt), 30 mM Tris-Mes (pH 6.0), 3 mM $MgSO_4$ and 50 mM KCl (when added).

Substrate	Activity		
	−KCl	+KCl	KCl stimulation
	μmol Pi/mg protein·h		
ATP	271	290	19.6
CTP	199	217	17.2
GTP	206	234	28.0
ITP	194	204	10.4
UTP	195	193	0
ADP	290	318	28.5
GDP	232	247	15.1
IDP	202	225	23.0
UDP	272	283	11.0
p-Nitrophenyl-P	265	278	13.0

Table III. *Effect of Divalent Cations on ATP Hydrolytic Activity and Acid Phosphatase of the Vacuole Fraction Isolated from Tobacco Protoplasts*

Assay contained 3 mM ATP or *p*-nitrophenyl-P, 30 mM Tris-Mes (pH 6.0 or 5.0), 3 mM divalent ions, and 50 mM KCl (when added).

Divalent Salt	ATP Hydrolysis pH 6.0		Acid Phosphatase pH 5.0	
	−KCl	KCl stimulation	−KCl	KCl stimulation[a]
	μmol Pi/mg protein·h			
None	312	32	221	139
$MgSO_4$	230	46	354	25
$NiSO_4$	119	53	345	20
$MnSO_4$	162	74	306	71
$ZnSO_4$	81	4	91	1
$CaSO_4$	290	25	307	73
$CoSO_4$	128	20	–[b]	–[b]

[a] Calculated by subtracting the activity in absence of KCl from that in the presence of KCl.

[b] Not determined.

Table IV. *Effect of Monovalent Ions on ATP Hydrolytic Activity and Acid Phosphatase of the Vacuole Fraction Isolated from Tobacco Protoplasts*

Assay contained 3 mM ATP or *p*-nitrophenyl-P, 30 mM Tris-Mes (pH 6.0 or 5.0), 3 mM $MgSO_4$, and 50 mM monovalent salts.

Monovalent Salt	Ion-stimulated[a] ATP Hydrolysis (pH 6.0)	Ion-stimulated[a] Acid Phosphatase (pH 5.0)
	μmol Pi/mg protein·h	
KCl	46	25
KBr	48	24
KI	50	39
KF	−153	−324
KNO_3	38	59
NaCl	44	33
LiCl	37	62
CsCl	53	61
RbCl	55	51
NH_4Cl	55	45
Tris-Cl	41	–[b]
Choline Cl	56	–
K-Mes	32	–
Na-acetate	37	–
25 mM KCl + 25 mM NaCl	50	–

[a] Activities in the presence of $MgSO_4$ alone for ATP hydrolysis and acid phosphatase were 230 and 354 μmol Pi/mg protein·h, respectively. Ion-stimulated activity was calculated by subtracting the activity in the absence from that in the presence of monovalent ions.

[b] Not determined.

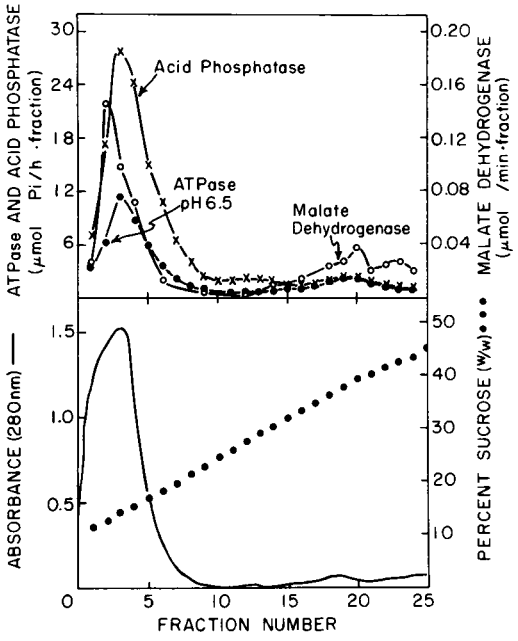

FIG. 5. Distribution of ATPase (pH 6.5), acid phosphatase, and malate dehydrogenase obtained when a vacuole preparation isolated from tobacco protoplasts was centrifuged for 15 h in a linear sucrose gradient.

280 nm. Latent IDPase (assayed after 7 days of storage at 4 C), a presumed marker for Golgi apparatus membranes (21, 24), was distributed in two peaks of activity. One was associated with the marker for ER at about 23% sucrose, and a second at about 31% sucrose (about 1.13 g/cc). ATPase and acid phosphatase activities were broadly distributed throughout the gradient with an enrichment in ATPase relative to acid phosphatase at about 36% sucrose (about 1.16 g/cc), a density typical of plasma membrane vesicles (14, 22). Some ATPase, IDPase, and acid phosphatase activity remained at the top of the gradient. The distribution of marker

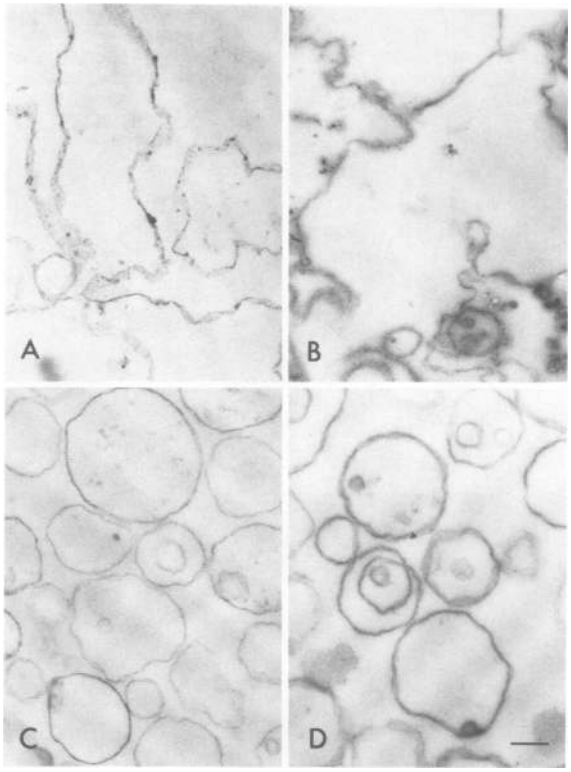

FIG. 6. Electron micrographs of membrane fractions separated by centrifugation in linear sucrose gradients. A: fractions 2 through 7 (1.04–1.06 g/ml) from gradient loaded with purified vacuoles as shown in Figure 5, stained with lead citrate and uranyl acetate. B: same as A, stained with PACP. C: fractions 3 through 8 (1.04–1.06 g/ml) from gradient loaded with 500 to 13,000*g* preparation from lysed protoplasts as shown in Figure 7, stained as in A. D: same as C, stained with PACP. In all micrographs, the bar represents 1.0 μm.

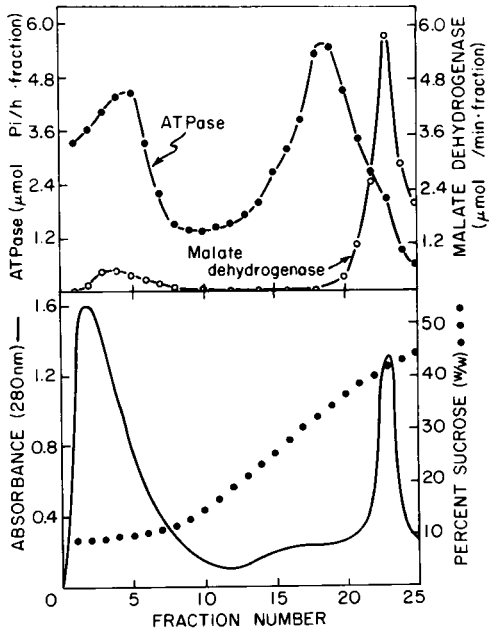

FIG. 7. Distribution of ATPase (pH 6.5) and malate dehydrogenase obtained after centrifugation of 500 to 13,000*g* fraction from tobacco protoplasts (lysed during vacuole isolation) for 15 h in a linear sucrose gradient.

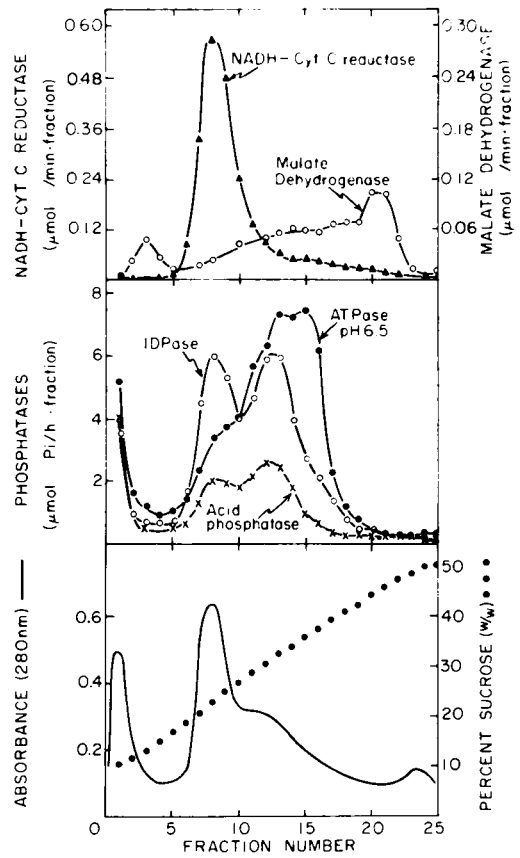

FIG. 8. Distribution of malate dehydrogenase, NADH-Cyt *c* reductase, ATPase (pH 6.5), acid phosphatase, and latent IDPase obtained when a 13,000 to 40,000*g* fraction from tobacco protoplasts (lysed during vacuole isolation) was centrifuged for 15 h in a linear sucrose gradient.

enzymes was similar to that reported for other plant tissues (11, 14, 22). The 13,000 to 40,000*g* protoplast lysate fraction would not be a particularly good source for the purification of the various microsomal membranes. The similarity of these results for gently lysed cells to those obtained from tissue homogenates suggests that overlapping distributions of the various microsomal membranes in a sucrose gradient are not a function of shearing forces associated with various tissue disruption procedures.

DISCUSSION

Vacuoles isolated from tobacco protoplasts exhibited high specific activities of acid phosphatase. From calculation involving the recovery of vacuoles and their associated phosphatase activity it was concluded that the majority of the phosphatase activity of tobacco protoplasts was of vacuolar origin. This may not be the case for other plant tissues (4, 23).

Recent results which suggest that a Mg^{2+}-requiring, monovalent cation-stimulated ATPase on the plasma membrane is involved in ion transport have led to the proposal that such a transport ATPase may be associated with the tonoplast as well. Some evidence has been presented purporting to demonstrate ATPase activity on isolated tonoplast membranes (5, 15). An evaluation of the substrate specificity (Table II) and response to divalent and monovalent ions (Tables III and IV) showed conclusively that the bulk of the ATP hydrolytic activity of isolated vacuoles from tobacco protoplasts was not characteristic of a transport ATPase, but was due to the highly active acid phosphatase of this cell component. It is possible that the activity of a transport ATPase on the tonoplast was hidden by the presence of acid phosphatase in the vacuole.

An attempt to determine the effective density of tonoplast membranes in a sucrose gradient gave equivocal results. After 14 h of centrifugation the vacuoles, as marked by acid phosphatase (Fig. 5) and electron microscopy (Fig. 6) were found near the top of the sucrose gradient. This suggests that the effective density of tonoplast vesicles is low, perhaps about 1.06 g/cc. However, phosphatase (as well as the ATP-hydrolyzing activity) appears to be a soluble enzyme and not an integral tonoplast protein (above and ref. 2). It is conceivable that the majority of the tonoplast vesicles equilibrated further into the sucrose gradient than would be indicated by the phosphatase distribution. The presence of large membrane vesicles in the electron micrographs of material from the peak acid phosphatase region of the sucrose gradient suggests that at least some vesicles of tonoplast have a low (less than 1.1 g/cc) effective density in sucrose. This is in agreement with the results of Boller and Kende (2) which indicate that choline-labeled tonoplast vesicles band in sucrose at a density of 1.10 g/cc. If this observation can be verified with an unequivocal marker for the tonoplast, then the tonoplast of plant cells would be the "lightest" cellular membrane known and would be expected to have a lipid to protein ratio that is dramatically different from that of the plasma membrane (effective density in sucrose, 1.14–1.17 g/cc).

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