Plant Physiol. (1979) 64, 74–78
0032-0889/79/64/0074/05/$00.50/0

Investigations of Vacuoles Isolated from Tobacco

I. QUANTITATION OF NICOTINE

Received for publication January 16, 1979 and in revised form March 19, 1979

JAMES A. SAUNDERS
Tobacco Laboratory, Plant Genetics and Germplasm Institute, United States Department of Agriculture, Beltsville, Maryland 20705

ABSTRACT

Nicotine was shown to be associated with mature vacuoles isolated from protoplasts of *Nicotiana rustica*. The vacuolar preparations also contained high levels of acid phosphatase, ATPase, and approximately 30% of the soluble protoplastic protein. The contamination of the vacuolar isolate by chlorophyll, succinate dehydrogenase, and NADPH cytochrome *c* reductase (markers for chloroplasts, mitochondria, and endoplasmic reticulum) was low. The enzymic activity associated with the vacuoles was not due to the exogenously supplied digestive enzymes used in the preparation of the protoplast. The relatively easy isolation of tobacco vacuoles makes this an excellent system for biochemical investigations of the vacuole.

The isolation of large numbers of vacuoles from leaf and petal tissue of higher plants has recently been reported by several investigators (3, 4, 9, 12, 17, 20, 21). Previous to those reports, studies on the biochemistry of vacuoles were restricted to unicellular systems, such as yeast (7, 13, 22), or to the small vesicular inclusions present in meristematic tissues, such as root tips (15). Due to the biochemical changes that occur during the ontogenic development of the vacuoles, it may not be appropriate to draw conclusions about the biochemistry of mature vacuoles in leaves on the basis of data obtained from provacuoles of meristematic cells in root tips. It is evident that direct biochemical analysis of the mature vacuole is necessary for a better understanding of the biochemical interactions of the various cellular components in a mature leaf cell.

In tobacco, nicotine is synthesized primarily in the roots, and the alkaloid is subsequently translocated to the foliar tissue through the xylem (19). The concentration of nicotine in the leaf depends on nitrogen availability and several environmental factors, including light, temperature, and moisture. Under appropriate conditions, nicotine can accumulate in concentrations as high as 5% of the air-dried leaf. At these concentrations, the vacuolar system is implicated in the accumulation of nicotine by virtue of its contribution to the volume of the cell.

Here, we report on the alkaloid content and the presence of several marker enzymes in the vacuoles of *Nicotiana rustica*.

MATERIALS AND METHODS

Plant Material. *N. rustica* variety brasilia was grown under greenhouse conditions and harvested 50 to 70 days after germination. During the summer months, the plants were transferred 1 week before harvest to an environmental growth chamber that provided 40% RH, 24 C, and a 14-h day length.

Preparation of Protoplasts. Four g of leaf tissue approximately 90 mm in length was painted with 150-mesh carborundum to facilitate infiltration of the digestion medium as previously described (17). The abraded leaf was digested in a mixture containing 1% (w/v) Macerase[1] plus 2% (m/v) Cellulysin in 25 mM Mes-NaOH (pH 5.7) with 0.6 M mannitol at 30 C for 3.5 to 4 h with gentle oscillations of 40/min. Protoplasts released by this treatment were filtered through one layer of cheesecloth and pelleted by centrifugation at 100*g* for 1 min in a swinging bucket rotor. The pellet was washed in a solution of 0.55 M mannitol, and 25 mM Tris-HCl buffer (pH 7.5) and recentrifuged at 100*g* for 1 min. The supernatant was discarded and the protoplasts collected by this procedure were resuspended in a known volume of the wash solution and an aliquot taken for protoplast quantitation and assays.

Isolation of Vacuoles. For release of the vacuoles, the protoplast suspension was diluted to a final concentration of 0.2 M mannitol with 25 mM Tris-HCl (pH 7.5) and gently pipetted three or four times over a 2-min interval. The suspension was then loaded onto the top of a four-step discontinuous gradient consisting of 1.5, 7, 12.5, and 20% (w/w) Ficoll 400 in 25 mM Tris-HCl (pH 7.5) and 0.5 M mannitol. The gradient was centrifuged in a Sorvall OTD-50 ultracentrifuge at 26,000 rpm (90,000*g*) for 2 h at 4 C using an AH-627 swinging bucket rotor. Mature intact vacuoles that banded at the 1.5 and 7% (w/w) Ficoll interface were recovered by the use of a Buchler Auto-Densi Flow sample fractionator (Fig. 1A).

Protoplast and vacuolar preparations were examined and quantitated by the use of an A/O Spencer Bright Line Hemacytometer with a Nikon inverted phase contrast microscope.

Enzyme Assays. NADPH Cyt *c* reductase activity was used as a marker of the ER and was assayed as previously described (18).

The mitochondrial enzyme succinic acid dehydrogenase was assayed by the method of Pennington (16).

Acid phosphatase activity was similar to the procedure of Walker-Simmons and Ryan (21) in which 1.2 ml of 0.2 M Na-acetate buffer (pH 5.0) was preincubated with 100 μl of the enzyme preparation for 10 min at 35 C. The assay was started with the addition of 250 μl of 32 mM *p*-nitrophenyl phosphate and an aliquot taken every 5 min for 30 min. The reaction was terminated by the addition of 3 ml cold 1 M Tris-Na-phosphate buffer (pH 8.5) and the change in *A* was recorded at 420 nm. Standards contained *p*-nitrophenol without enzyme.

ATPase activity was determined by modifications of the technique of Leonard and Van der Woude (10) in which the 1-ml reaction mixture contained: 3 mM $MgSO_4$, 50 mM KCl, 30 mM Tris-Mes buffer (pH 6.5), 3 mM ATP-Tris salt, and 50 to 150 μg enzyme preparation. The enzymic reaction was terminated at 10-

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

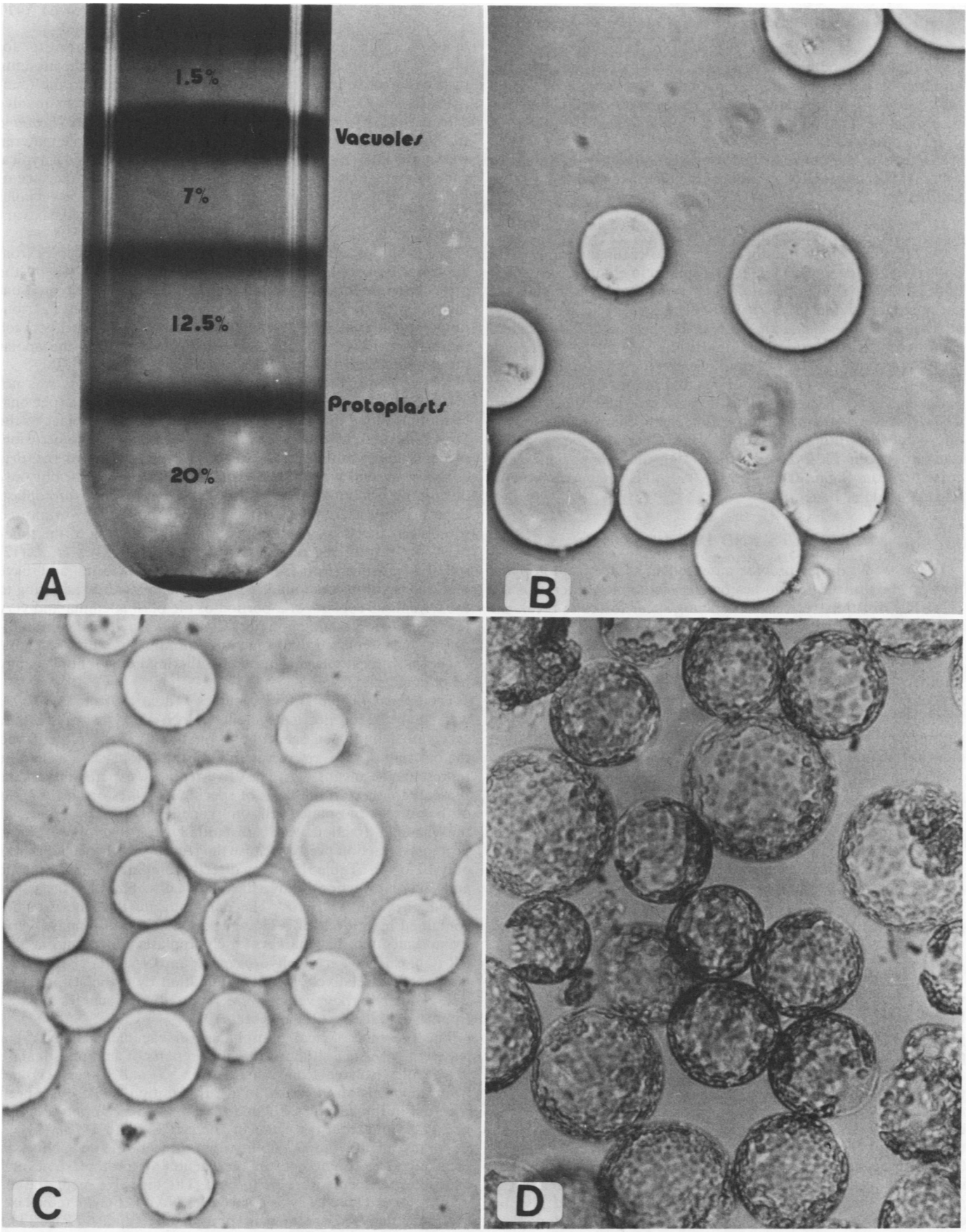

FIG. 1. A: distribution of cellular components of tobacco leaf on a discontinuous Ficoll gradient centrifuged at 90,000*g* for 2 h. The main vacuolar band is found at the interface between 1.5/7% (w/w) Ficoll with a mixture of vacuoles and protoplasts at the 7/12.5% (w/w) Ficoll interface. Unbroken protoplasts band at the 12.5/20% (w/w) Ficoll interface and the pellet consisted of chloroplasts and other cellular debris. The vacuoles were stained with neutral red to facilitate photography. B and C: vacuoles taken from the 1.5/7% (w/w) Ficoll interface of the discontinuous gradient (× 500). D: protoplasts characteristic of *Nicotiana* leaf before lysis to release the vacuoles (× 575).

min intervals for 30 min by the addition of 2 ml of cold 1% ammonium molybdate in 2 N H_2SO_4. Pi was determined by the addition of 400 μl of 1-amino-2-naphthol 4-sulfonic acid (Fisher Scientific Co.), incubation at room temperature for 35 min, and recording the *A* at 660 nm. Standards contain 1 μmol/ml KH_2PO_4 in place of enzyme.

Controls in the above assays consisted of boiled protoplast or vacuolar preparations or aliquots of the digestive medium without leaf tissue.

Chl was determined by the method of Arnon (1), and protein was assayed by the technique of Bradford (2).

Nicotine was quantified by thin layer co-chromatography with known standards on Silica Gel IB-F plates in chloroform-methanol (100:20, v/v). The alkaloid was visualized by spraying the chromatogram with 0.5% (w/v) *p*-aminobenzoic acid in 95% (v/v) ethanol prior to placing the thin layer sheet in an atmosphere saturated with cyanogen bromide. Estimations of the nicotine in protoplastic and vacuolar samples were made by visual comparison of the thin layer chromatograms to dilutions of standards.

Chemicals and Reagents. The digestive enzymes Macerase and Cellulysin were obtained from Calbiochem, the reagents for the phosphate determinations from Fisher Chemical Co., nicotine standards from Eastman Chemical Co., and all enzymes and cofactors from Sigma Chemical Co. All other chemicals were of reagent grade or better.

RESULTS AND DISCUSSION

Leaves of *N. rustica* provide an excellent source of protoplasts that can be utilized for studies on the metabolic contents of the cells. The disruptive effects of applying shear forces to break the cell wall are avoided by employing wall-digesting enzymes. Using a technique developed to isolate vacuoles from protoplasts of *Sorghum* (17), vacuoles have been isolated from *N. rustica* in large numbers (10^6–10^7), with recoveries as high as 35%.

Protoplasts, prepared by enzymic digestion as described under "Materials and Methods," were lysed by osmotic shock, which ruptured over 90% of the protoplasts. The mannitol concentration causing maximum lysis of the protoplasts from different plants with minimum rupture of the vacuoles appears to be species-specific. For the release of vacuoles from tobacco protoplasts, the optimum concentration of mannitol was 0.2 M. With *Sorghum* and *Hordeum* protoplasts a somewhat lower concentration of mannitol (0.175 M) is more effective for releasing the vacuoles (17).

The tonoplast of *Nicotiana* vacuoles could withstand the low osmotic potential of the lysing medium for only a relatively short period (less than 45 min). Therefore, for osmotic stability at each gradient step the lysed protoplast preparation was rapidly layered onto a discontinuous Ficoll gradient which contained 0.5 M mannitol.

Figure 1A depicts a typical discontinuous Ficoll gradient in which the main vacuolar band is centered at the 1.5/7% (w/w) Ficoll interface, with a mixture of the vacuoles and unlysed protoplasts accumulating at the 7/12.5% (w/w) interface. Protoplasts and chloroplasts accumulate at the 12.5/20% (w/w) interface and the pellet contains the main fraction of chloroplasts and membranous debris. The amount of protoplastic contamination in the vacuolar band, and the ratio of protoplasts to vacuoles in the band at the 7/12.5% (w/w) Ficoll interface varied inversely with the extent of the protoplastic lysis. After centrifugation a well lysed protoplast preparation was pale amber at the vacuolar interface, pale green at the intermediate, and darker green at the protoplastic interface. The vacuolar preparations could also be contaminated by cytoplasmic material adhering to the tonoplast at specific points on the membrane. Such a contamination is evident in vacuoles isolated from *Sorghum* (17), tomato (6, 21), *Petunia* (12), and beets (9). The adherence of cytoplasmic material to the tonoplast has been reported to be reduced by the addition of BSA, which is thought to act as a nonspecific competitor for the sites of the membrane adherence (17). Although the albumin may be effective in reducing cytoplasmic adherence to the tonoplast, its addition is undesirable when the investigations involve calculations of a specific activity based on protein. In *Nicotiana* the degree of contamination by the adhering cytoplasmic material was quite low (Fig. 1, Band C). These figures depict a typical vacuolar preparation from the 1.5/7% (w/w) Ficoll interface of the discontinuous gradient. The diameter of the isolated vacuoles ranged from 40 to 60 μm, whereas that of the protoplasts vary from 30 to 50 μm (Fig. 1D).

Buser and Matile (3) described the isolation of vacuoles from *Bryophyllum* in which protoplasts were induced to lyse by the presence of 0.5 mg/ml DEAE-dextran. The authors pointed out that the protoplasts of *Bryophyllum* have a very uneven distribution of cytoplasm. These "polar" protoplasts are apparently much more susceptible to the polybase-induced lysis than the apolar protoplasts characteristic of the *Nicotiana* system (Fig. 1D).

In our hands the lysis of the *N. rustica* protoplasts was not affected by 0.5 mg/ml DEAE-dextran, and Matile stated (personal communication) that *Nicotiana tabacum* protoplasts react to the DEAE-dextran treatment in a variable manner. On occasion, protoplasts resembling the polar ones characteristic of the *Bryophyllum* system were observed in the tobacco system, but only during the osmotic lysis of the more common apolar protoplast. Polar protoplasts in *Nicotiana* are therefore interpreted as ruptured protoplasts that have cytoplasmic material adhering to the tonoplast and do not represent unlysed protoplasts. Lorz *et al.* (12) isolated vacuoles from several higher plant species and have reported that the contaminating cytoplasmic material adhering to the tonoplasts consists of a portion of the plasmalemma and very few other cytoplasmic remnants. Conn *et al.* (personal communication) have reported that some polar protoplasts which resemble vacuoles with cytoplasmic material adhering to the tonoplast may be of epidermal origin.

Protoplasts (Table I) and vacuoles (Table II) from *N. rustica* were analyzed for nicotine content, Chl, NADPH Cyt *c* reductase, succinate dehydrogenase, acid phosphatase, ATPase, protein, and the per cent of recovery of vacuoles from protoplasts. Each value represents the means of at least five replicate experiments and is presented either on the basis of specific activities or on the basis of the number of isolated cells or organelles. The total activities of the vacuolar fraction were substantially less than those of the protoplast fraction partly because the recovery of the vacuoles was only 10 to 40%. Table III, which directly compares the protoplastic and vacuolar fractions, shows the relative distribution of the assays based on vacuolar yields. The data indicate that the vacuoles contained high levels of ATPase activity, acid phosphatase activity, and nicotine. The markers for chloroplasts, mitochondria, and ER (Chl, succinic acid dehydrogenase, and NADPH Cyt *c* reductase) showed relatively low contamination by cytoplasmic material. Similar marker values were reported for vacuoles isolated from tomato leaves (21) and beets (9).

The presence of acid phosphatase in vacuoles isolated from leaves of *N. rustica* supports previous reports of acid phosphatase in vacuoles isolated from tomato fruits (5) and leaves (21) and from the petals of *Hippeastrum* (4). In addition ATPase has been reported in the vacuoles isolated from yeast *Saccharomyces cerevisiae* (22) and from the petals of *Hippeastrum* and *Tulipa* (11).

To investigate the possibility that Macerase and Cellulysin contributed to the activity in the vacuole or protoplast, several control experiments were performed. The digestive media minus plant source (Table IV) was assayed for NADPH Cyt *c* reductase, succinate dehydrogenase, acid phosphatase, and ATPase. The total activities of the acid phosphatase and the ATPase present in the digestive medium were insufficient to account for the activity found in either the protoplast or the vacuole. Succinate dehydrogenase, while somewhat higher in total activity in the medium,

TABLE I. ANALYSIS OF PROTOPLASTS ISOLATED FROM TOBACCO LEAVES

Leaves were incubated 4 hours in Cellulysin and Macerase to release protoplasts. Total activity (total assay/mg protein for non-enzymatic assays), and total acivity/10^6 protoplasts are means of a least 5 separate experiments. Values for the number of cells and protein are means based on the total number (29) of experiments.

	Total Activity	(S.E.)*	Total/mg Protein	(S.E.)*	Total/10^6 Protoplast	(S.E.)*
NADPH Cyto C Reductase ΔOD_{550}/min	1.70	(0.47)	0.362	(0.27)	0.350	(0.16)
Succinate Dehydrogenase ΔOD_{490}/min	0.312	(0.22)	0.067	(0.10)	0.055	(0.09)
Nicotine µg	243.9	(4.61)	32.9	(1.86)	43.5	(2.25)
Chlorophyll µg	1566.4	(9.81)	108.4	(2.78)	200.6	(3.30)
Acid Phosphatase µ moles/min	3.25	(0.42)	0.16	(0.11)	0.31	(0.12)
ATPase n moles/min	830.1	(8.19)	68.1	(2.33)	83.9	(2.24)
Number of Cells x 10^6	7.45	(0.41)				
Protein mg	11.60	(0.60)			1.67	(0.21)

* Standard Error

TABLE II. ANALYSIS OF VACUOLES OBTAINED FROM PROTOPLASTS OF TOBACCO LEAVES

Analysis of vacuoles obtained by the osmotic lysis of the tobacco protoplasts and centrifugation on a discontinuous Ficoll gradient. Total activity, specific activity (total assay/mg protein for non-enzymatic assays), and total activity/10^6 vacuoles are means of at least 5 separate experiments. Values for the number of vacuoles and protein are means based on the total number of experiments (29).

	Total Activities	(S.E.)*	Total/mg Protein	(S.E.)*	Total/10^6 Vacuoles	(S.E.)*
NADPH Cyto C Reductase ΔOD_{550}/min	0.043	(0.076)	0.117	(0.096)	0.040	(0.053)
Succinate Dehydrogenase ΔOD_{490}/min	0.009	(0.057)	0.040	(0.128)	0.005	(0.047)
Nicotine µg	55.4	(3.1)	100.4	(3.3)	40.6	(1.9)
Chlorophyll µg	3.3	(0.7)	5.5	(1.0)	3.2	(0.7)
Acid Phosphatase µ moles/min	0.48	(0.19)	0.56	(0.19)	0.29	(0.18)
ATPase n moles/min	194.4	(4.6)	274.4	(4.6)	109.1	(3.0)
Number of Vacuoles x 10^6	1.27	(0.21)				
Protein mg	0.48	(0.12)			0.48	(0.13)

* Standard Error

TABLE III. RATIOS OF THE VACUOLAR FRACTION (VF) VS THE PROTOPLASTIC FRACTION (PF)

	$\frac{\text{Specific Activity of VF}}{\text{Specific Activity of PF}}$	$\frac{\text{Activity/}10^6\text{ Vacuoles}}{\text{Activity/}10^6\text{ Protoplasts}}$
		% *
NADPH Cyto C Reductase	0.32	11.4
Succinic Acid Dehydrogenase	0.59	9.1
Chlorophyll	0.05	1.6
Acid Phosphatase	3.40	95.6
ATPase	4.03	129.9
Nicotine	3.05	93.3
Protein		28.7
Yield of Vacuoles		17

* The values represent the percent of protoplastic activities or concentrations recovered in the vacuolar fraction.

TABLE IV. ANALYSIS OF THE PROTOPLAST DIGESTIVE ENZYME MEDIUM

This table shows the total and specific activities for the protoplast digestive medium which contains 1% (w/v) macerase and 2% (w/v) Cellulysin in 25 mM MES-NaOH buffer pH 5.7 with 0.6 M Mannitol without any leaf tissue for 24 ml of digesting medium.

Assay	Total Activity	Total Activity per mg Protein
NADPH Cyto C Reductase ΔOD_{550}/min	0.035	0.001
Succinate Dehydrogenase ΔOD_{490}/min	1.68	0.042
Acid Phosphatase µ moles/min	0.48	0.013
ATPase n moles/min	210.0	5.23

was present in the vacuole in only minor concentrations. The activity of NADPH Cyt *c* reductase in the digestive medium was very low in relation to that in the protoplast or vacuole.

To assure further that the enzyme activity found in the vacuole was endogenous to that organelle, the protoplasts were washed twice during the isolation procedure. The resulting centrifugation during the washing procedure, however, did rupture some of the protoplasts prior to lysis, thus reducing the yield of vacuoles recovered.

The vacuoles of *Nicotiana* contained high concentrations of nicotine (Table II). Although nicotine is known to accumulate in the leaf in high concentrations (19) very little has been reported on the direct accumulation of nicotine in specific subcellular compartments. Matile *et al.* (14) reported that vacuoles taken from the specialized latex cells of *Chelidonium majus* contained about 70% of the major alkaloid of that species. As the vacuole is known to accumulate several kinds of secondary metabolic compounds (8, 17, 21) its accumulation of nicotine may not be surprising. It must be cautioned that nicotine bound to the cell wall or lost through a semipermeable plasmalemma may not be recovered in the protoplast fraction. Nicotine is thought to be synthesized in the roots of tobacco and transported to the leaf through a poorly understood mechanism (19). Experiments are currently under way to investigate further the localization, transport, and biosynthesis of nicotine by specific, antibody precipitation tests.

Acknowledgments—The author gratefully acknowledges the aid of H. A. Skoog for the preparation and care of the greenhouse material, and Mary E. Engelhaupt for her valuable laboratory assistance.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
3. BUSER C, P MATILE 1977 Malic acid in vacuoles isolated from *Bryophyllum* leaf cells. Z Pflanzenphysiol 82: 462–466
4. BUTCHER HC, GJ WAGNER, HW SIEGELMAN 1977 Localization of acid hydrolyases in protoplasts. Plant Physiol 59: 1098–1103
5. DOI E, T MATOBA, C OHTSURU 1975 Lysosomal nature of plant vacuoles. I. Apparent absence of lysosomal particles in tomato fruit and leaf homogenates. Plant Cell Physiol 16: 571–580
6. GREGORY DW, EC COCKING 1966 Studies on isolated protoplasts and vacuoles. I. General properties. J Exp Bot 17(50): 57–67
7. HASILIK A, H MÜLLER, H HOLZER 1974 Compartmentation of the tryptophan-synthase-proteolyzing system in *Saccharomyces cerevisiae*. Eur J Biochem 48: 111–117
8. HRAZDINA G, GJ WAGNER, HW SIEGELMAN 1978 Subcellular localization of enzymes of anthocyanin biosynthesis in protoplasts. Phytochemistry 17: 53–56
9. LEIGH RA, D BRANTON 1976 Isolation of vacuoles from root storage tissue of *Beta vulgaris L.* Plant Physiol 58: 656–662
10. LEONARD RT, WJ VAN DER WOUDE 1976 Isolation of plasma membranes from corn roots by sucrose density gradient centrifugation. Plant Physiol 57: 105–114
11. LIN W, GJ WAGNER, HW SIEGELMAN, G. HIND 1977 Membrane-bound ATPase of intact vacuoles and tonoplasts isolated from mature plant tissue. Biochim Biophys Acta 465: 110–117
12. LORZ H, CT HARMS, I POTRYKUS 1976 Isolation of "vacuoplasts" from protoplasts of higher plants. Biochem Physiol Pflanzen 169: 617–620
13. MATERN H, H BETZ, H HOLZER 1974 Compartmentation of inhibitors of proteinases A and B and carboxypeptidase Y in yeast. Biochem Biophys Res Commun 60(3): 1051–1057
14. MATILE P, B JANS, R RICKENBACHER 1970 Vacuoles of *Chelidonium* latex: lysosomal property and accumulation of alkaloids. Biochem Physiol Pflanzen 161: 447–458
15. MATILE P, H MOOR 1968 Vacuolation: origin and development of the lysosomal apparatus in root-tip cells. Planta 80: 159–175
16. PENNINGTON RJ 1961 Mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. Biochem J 80: 649–654
17. SAUNDERS, JA, EE CONN 1978 Presence of the cyanogenic glucoside dhurrin in isolated vacuoles from *Sorghum*. Plant Physiol 61: 154–157
18. SAUNDERS JA, EE CONN, CH LIN, M SHIMADA 1977 Localization of cinnamic acid 4-monoxygenase and the membrane-bound enzyme system for dhurrin biosynthesis in *Sorghum* seedlings. Plant Physiol 60: 629–634
19. TSO TC 1972 Physiology and Biochemistry of Tobacco Plants. Dowden, Hutchinson and Ross Inc, Stroudsburg, Pa. pp 231–258
20. WAGNER GJ, HW SIEGELMAN 1975 Large-scale isolation of intact vacuoles and isolation of chloroplasts from protoplasts of mature plant tissues. Science 190: 1298–1299
21. WALKER-SIMMONS M, CA RYAN 1977 Immunological identification of proteinase inhibitors I and II in isolated tomato leaf vacuoles. Plant Physiol 60: 61–63
22. WIEMKEN A 1975 Isolation of vacuoles from yeast. Cell Biol 12: 99–109