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Vacuoles from Sugarcane Suspension Cultures[1]

I. ISOLATION AND PARTIAL CHARACTERIZATION

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ABSTRACT

Vacuoles were isolated from suspension cultures of sugarcane (*Saccharum* sp.) cells by centrifugation of protoplasts at high *g* force against a 12% (w/v) Ficoll solution. Distribution of marker enzymes and Concanavalin A binding showed an 11% contamination of the vacuole preparation by cytoplasmic components, mitochondria, and endoplasmic reticulum, and 18% contamination by plasma membrane. Acid phosphatase, carboxypeptidase, protease, peroxidase, and ribonuclease activities were enriched in isolated vacuoles. Carboxypeptidase was tonoplast-bound, whereas the other enzymes were soluble. Sucrose, reducing sugars, and free amino acids were measured in protoplasts and vacuoles during growth of cells in suspension culture. Sucrose and reducing sugar content of vacuoles increased as the culture aged, while free amino acids decreased sharply.

Until recently, vacuoles were among the least well understood organelles of higher plants because they could not be investigated by direct isolation. This situation has changed in recent years as vacuoles have been isolated from a number of plant tissues. Among the tissues used for isolation are leaves (1, 3, 12, 23, 28, 29) petals (1, 28), beet roots (4, 10), endosperm (20), yeast cells (5), and cell cultures (1, 12, 16, 22). Various methods have been employed for vacuole release depending on source of the vacuoles and the purpose of their isolation. Methods have included the slicing of tissue (4, 10), enzymic release of protoplasts followed by osmotic lysis (1, 16, 22, 23, 28), polybase-induced lysis (3, 5), or high shear force in an ultracentrifuge (6, 9, 12).

In stalk parenchyma tissues of sugarcane plants, vacuoles play an essential role in sugar uptake and storage. The mechanism of sucrose accumulation in vacuoles has been investigated in storage tissue of red beet (4). Also, 3-*O* methylglucose transport into mesophyll vacuoles of peas (6) has been reported. In the present report we describe the isolation and partial characterization of vacuoles from sugarcane cells grown in suspension culture. Cell cultures were selected for this work because previous investigations (14, 15) provided considerable background information on sugar uptake by these cells. In addition, these cultures resemble rapidly growing apical tissue of intact sugarcane plants in that they can accumulate up to 25% of their dry weight as sucrose.

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MATERIALS AND METHODS

Plant Material. Sugarcane (*Saccharum* sp.) cell suspension cultures were isolated from meristematic stalk tissue of cultivar H50-7209. Stock cultures were maintained by transfer at 14-day intervals into White's basal salt medium supplemented with 0.1% (w/v) yeast extract, 50 mM sucrose, 0.4 mM arginine, and 9 μM 2,4-D (19).

Isolation of Protoplasts. Protoplasts were isolated from 4- to 17-day-old cultures by the following method: Cells (4 g fresh weight) were incubated in 25 ml 10 mM Hepes buffer (pH 5.6) containing 0.4 M mannitol, 2% (w/v) Cellulysin (Calbiochem), 2% (w/v) Driselase (Kyowa Hakko Kogyo Co., Ltd., Costa Mesa, CA), 0.5% (w/v) Rhozyme HP150 (Rohm and Haas) on a rotary shaker at 100 rpm at 30°C for 2 h. The suspension was filtered through 84 μm nylon mesh cloth to remove intact cells. Protoplasts were washed 3 times in buffered mannitol by centrifugation at 50*g*.

Vacuole Isolation. Vacuoles were isolated from protoplasts using a modification of the method described by Lörz (12). Briefly, the protoplast suspension was layered over 1 ml 12% Ficoll (Type 400DL, Sigma) made up in buffered 0.4 M mannitol in a cellulose acetate tube and centrifuged for 30 to 120 min in a Beckman SW50.1 rotor at 49,000 rpm. Vacuoles were recovered at the 0/12% Ficoll interface and washed three times in buffered 0.4 M mannitol at 50*g*.

Enzyme Assays. Glucose-6-P dehydrogenase (9), phosphoglucoisomerase (24), and NADH malate dehydrogenase (21) were assayed spectrophotometrically by monitoring changes in optical density at 340 nm. Cyt *c* oxidase and NADH Cyt *c* reductase were assayed by recording the change in optical density due to oxidation and reduction of Cyt *c*, respectively (7). Acid phosphatase was assayed with *p*-nitrophenyl phosphate as substrate at pH 5.0 (1). ATPase activity was determined at pH 6.0 by a modified Hodges and Leonard method (7). Ammonium molybdate (100 μM) was added to the incubation mixture to inhibit acid phosphatase activity (11). Carboxypeptidase was assayed by measuring hydrolysis of N-carbobenzoxy phenylalanylalanine (20). Ribonuclease was determined by a modification of the method of Tang and Maretzki (25). Acid protease was assayed using casein hydrolysate as substrate. Peroxidase was determined by oxidation of guaiacol in the presence of H_2O_2. Acid and neutral invertases were assayed by the hydrolysis of sucrose at pH 5 and 7, respectively.

Chemical Assays. Sucrose was determined by the anthrone method after destruction of reducing sugars with KOH (27). Reducing sugars were determined by reduction of alkaline copper sulfate (18). Amino acids were determined by the ninhydrin method (17). Protein was determined by the Lowry method (13) after precipitation with TCA. Vacuole and protoplast numbers were determined by counting samples of an appropriate dilution in a Sedgewick-Rafter counting chamber. The experimental error was ±15% and was largely due to the difficulty of counting the fragile protoplasts and vacuoles. In all cases, experiments were conducted in parallel. Cells from at least two cultures were used

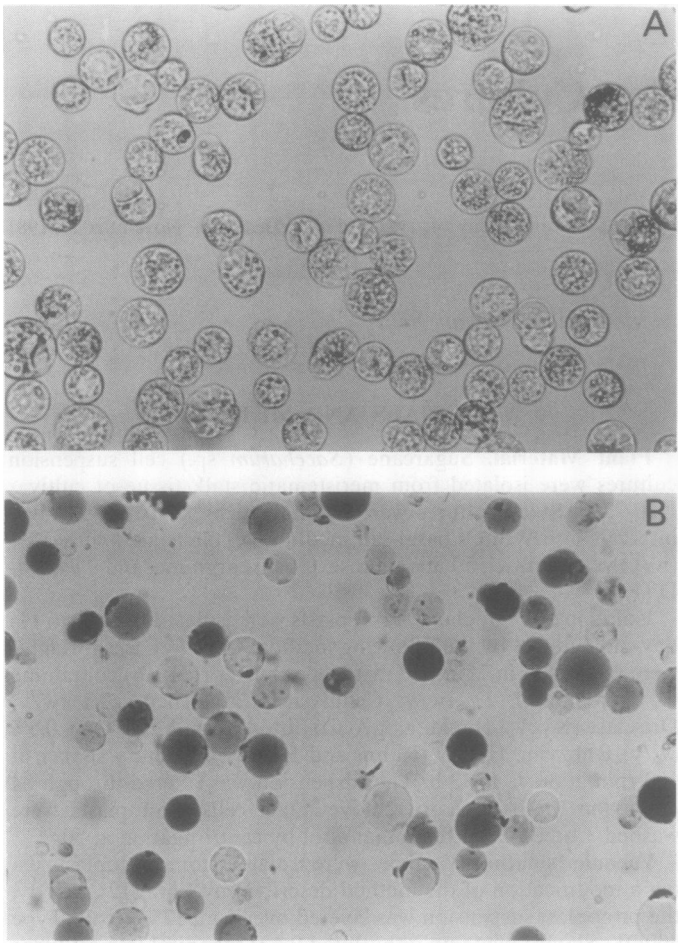

FIG. 1. Photomicrograph of protoplasts and vacuoles isolated from suspension cultures of sugarcane cells. *A*, protoplasts, *B*, vacuoles. Vacuoles were stained with neutral red.

to prepare protoplasts, and for all comparisons vacuoles and protoplasts from the same batch of cells were used.

Labeling of Protoplasts with Con A.[3] Protoplasts were incubated with [^{3}H]Con A for 45 min at 4°C and washed 4 times in buffered mannitol. Vacuoles were isolated from labeled protoplasts and radioactivity was determined by scintillation spectrometry. Protoplasts were labeled with FITC-Con A (16 μg/ml) in a manner similar to that described for [^{3}H]Con A. Protoplasts and vacuoles in this case were examined by fluorescence microscopy, using a Zeiss FITC excitation filter in combination with a Zeiss barrier filter 47.

RESULTS AND DISCUSSION

Vacuole Isolation. The modified method of Lörz (12) resulted in the release of a large number of vacuoles from protoplasts which, in turn, were prepared from a sugarcane cell suspension. A procedure employing osmotic lysis proved to be unsatisfactory for sugarcane cells, not only because large numbers of protoplasts (30% or greater) contaminated the preparation, but also because resuspension of protoplasts in 0.17 or 0.20 M K_2HPO_4 (pH 8) increased the size of the protoplast by about 100%. Stretching of the plasma membrane and the isolated tonoplast would create an undesirable condition for nutrient uptake, the process we wished to study. Lysis of protoplasts induced by a polycation, such as DEAE dextran, is also likely to damage the tonoplast. Figure 1A is a photomicrograph of a protoplast suspension and Figure 1B shows a preparation of vacuoles. The average size of isolated vacuoles, measured as the water-accessible but dextran-excluding space, was approximately 60% of the protoplast volume. This agreed well with an *in vivo* vacuolar volume measurement of 62% (26). The yield of vacuoles was usually 20 to 30% of the number of protoplasts from which they were released, but could be increased to 60% of the initial protoplast number by careful handling during the washing procedures. The results obtained with the isolated vacuoles can be assumed to be representative of properties of vacuoles in the majority of the original cells (more than 80% of cells give rise to protoplasts) and presumably do not merely represent a specific cell population. Further purification might decrease the level of cytoplasmic contamination but it would also decrease the yield of vacuoles and might even select for vacuoles of a certain size or density, in other words, for a nontypical population of vacuoles.

Purity of Vacuoles. The distribution of marker enzymes and Con A binding in protoplasts and the various fractions obtained by the vacuole isolation procedure are shown in Table I. At least 80% of all marker enzymes were recovered in the fractions. Glucose-6-P dehydrogenase and phosphoglucoisomerase, which are believed to be exclusively located in the cytosol, contaminated the vacuole preparation by 26 and 11%, respectively. Cyt *c* oxidase was used as a marker of mitochondria, and NADH Cyt *c* reductase as a marker of the ER. Compared with protoplasts, each vacuole contained approximately 11% of these enzymes. Con A should bind to the plasma membrane only and should not enter the protoplasts (2); it was used, therefore, as a potential marker for plasma membrane contamination. When vacuoles were isolated from protoplasts which had been incubated with [^{3}H]Con A, 22.8% of the Con A bound to each protoplast was found in the vacuole. However, when vacuoles were isolated from protoplasts which had been incubated simultaneously with Con A and 0.1 M α-methyl mannoside, 74.5% of the Con A bound to each protoplast was in the vacuole (Table II). Specific binding (difference in radioactivity bound in the absence and presence of α-methyl mannoside) showed that vacuole preparations were contaminated 18.5% by plasma membrane. Experiments using [^{3}H]Con A cannot distinguish among the possible types of plasma membrane contamination in the preparations. Three possibilities exist: (a) 18% of the vacuoles are surrounded by plasma membrane (called "vacuoplast" by Lörz [12]); (b) membrane fragments are attached to the vacuoles; or (c) membrane fragments are present as aggregates of protoplasmic material floating free in the vacuole preparation. To resolve this question, vacuoles were isolated from protoplasts which had been incubated with FITC-Con A. Examination of these vacuoles under fluorescence microscopy showed that about 20% of the vacuoles contained fluorescent material attached to a small portion of the tonoplast. The vacuole itself showed very faint or no fluorescence. The preparation also contained amorphous fluorescent material floating free in the preparation. Therefore, the plasma membrane contamination does not envelop the vacuole and should have little effect on uptake properties. As pointed out previously, there is contamination by all cell compartments, amounting to 11%, in addition to the larger contamination by plasma membrane. It is possible, but not probable, that the vacuole preparation contained 11% of intact or enucleated protoplasts. Photomicrographs of vacuole suspensions show a virtual absence of intact protoplasts and the FITC-Con A data further confirm this conclusion. It is assumed, therefore, that most of the contamination in the vacuole preparation is present as aggregates of protoplasmic material. This assumption is consistent with the fact that a large amount of contaminating enzymes appear in the wash solution used to purify the vacuoles (Table I). Inasmuch as more than half of the vacuoles are broken during

[3] Abbreviations: Con A, Concanavalin A; FITC-Con A; Concanavalin A—fluorescein isothiocyanate

Table I. *Distribution of Marker Enzymes*

Protoplasts were layered onto Ficoll and centrifuged for 30 min. The fractions were separated into the supernatant, the Ficoll phase, the pellet, and the interface containing the vacuoles. The vacuoles were washed 3 times and the enzyme activities in purified vacuole preparation, vacuole wash solution, and fractions from the Ficoll centrifugation were determined.

Fraction	Glucose-6-P dehydrogenase	Phosphoglucoisomerase	Cyt *c* Oxidase	NADH Cyt *c* Reductase	Acid Phosphatase
		μmol·min⁻¹		*mmol·min⁻¹*	*μmol·min⁻¹*
Protoplast	1.377	12.97	544.3	13.89	2.12
Supernatant	0.080	4.36	0	2.67	0.46
Ficoll	0.140	1.42	23	2.92	0.13
Pellet	0.513	3.76	441	3.70	0.11
Vacuole wash	0.579	2.89	0	1.19	0.68
Vacuole	0.068	0.21	9	0.22	0.47
Total recovered	1.380	12.64	473	10.30	1.85
	nmol·min⁻¹ 10⁶ protoplasts or vacuoles⁻¹		*μmol·min⁻¹ 10⁶ protoplasts or vacuoles⁻¹*		*nmol·min⁻¹ 10⁶ protoplasts or vacuoles⁻¹*
Protoplast	30.73	241	10.13	258.6	47.3
Vacuole	7.86 (25.6%)	27 (11.3%)	1.14 (11.3%)	29.4 (11.4%)	54.5 (115.2%)

Table II. *Concanavalin A Binding to Protoplasts and Vacuoles Isolated from Sugarcane Suspension Cultures*

	Protoplasts	Vacuoles	% Vacuoles
	cpm		
+ Con A	3041	648	22.8
+ Con A, + α methyl mannoside	159	115	74.5
Specific binding	2882	533	18.5

Table III. *Enzyme Activities and Protein Content in Protoplasts and Vacuoles Isolated from Sugarcane Cell Suspension Cultures*

	Protoplasts	Vacuoles	Activity in Vacuoles
			%
Acid phosphatase[a]	0.34	0.32	96
Mg, K-ATPase (pH 6)[a]	5.69	3.59	63
Peroxidase[b]	2.31	1.63	71
Carboxypeptidase[a]	0.51	0.25	49
Protease[c]	0.23	0.11	48
Ribonuclease[c]	2.99	1.02	34
Acid invertase[a]	0.08	0.007	9
Neutral invertase[a]	0.18	0.01	6
NADH malate dehydrogenase[a]	0.19	0.01	5
Protein[d]	195.2	30.5	16

[a] $\mu mol \cdot hr^{-1} \cdot (10^6$ protoplasts or vacuoles$)^{-1}$.
[b] $\Delta OD \cdot min^{-1} \cdot (10^6$ protoplasts or vacuoles$)^{-1}$.
[c] $\Delta OD \cdot hr^{-1} \cdot (10^6$ protoplasts or vacuoles$)^{-1}$.
[d] $\mu g \cdot (10^6$ protoplasts or vacuoles$)^{-1}$.

washing (as can be estimated from the release of vacuole-specific enzymes such as acid phosphatase), we had to arrive at a reasonable compromise between vacuole yield and purity.

Enzymes in Protoplasts and Vacuoles. The activities of some hydrolytic enzymes were determined in the protoplast and vacuole preparations (Table III). Nearly all the acid phosphatase of the protoplast was located in the vacuole. The vacuoles also contained high levels of ATPase, carboxypeptidase, protease, peroxidase, and ribonuclease activities. These results confirm that vacuoles of higher plant cells contain enzymes capable of degrading cytoplasmic components. Activities of acid and neutral invertases and

Table IV. *Distribution of Enzymes and Protein Between Tonoplast and Soluble Components of Vacuoles*

	% Membrane Bound
Carboxypeptidase	87
Protease	52
Peroxidase	37
Ribonuclease	12
Acid phosphatase	11
Protein	67

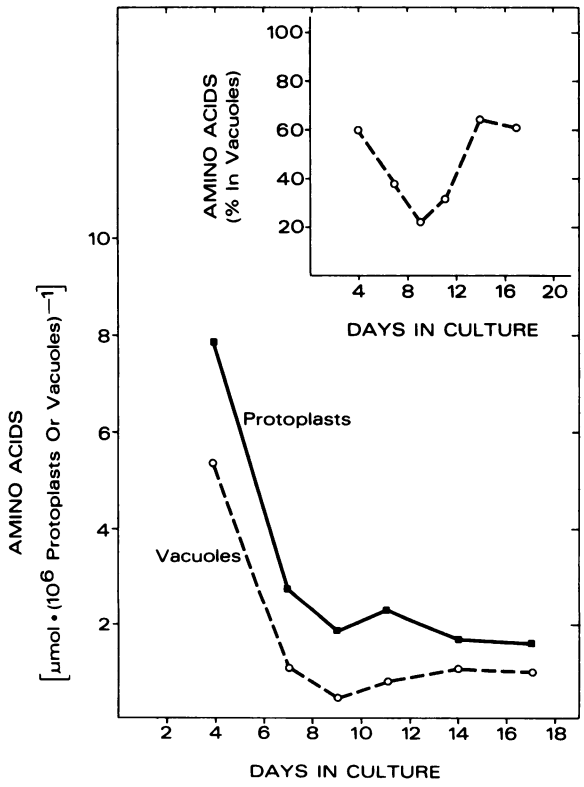

FIG. 2. Amino acid content in protoplasts and vacuoles during the growth cycle.

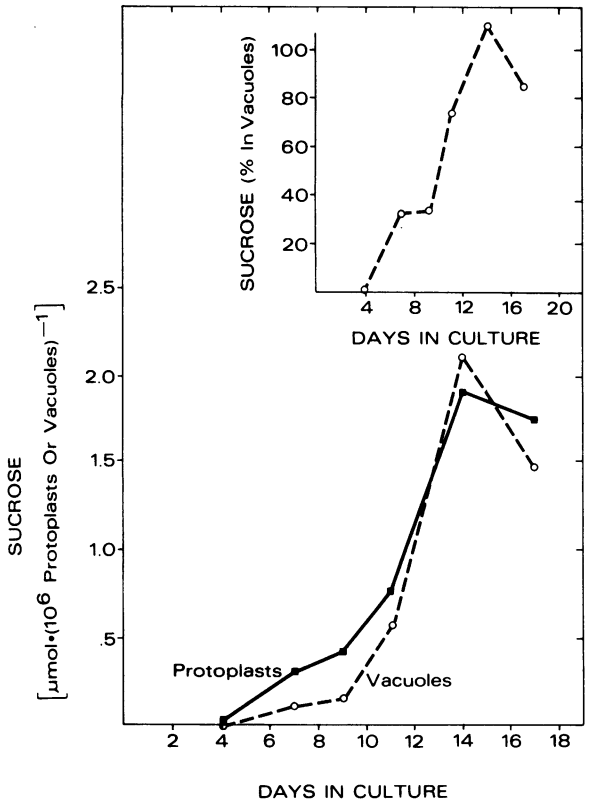

FIG. 3. Sucrose content in protoplasts and vacuoles during the growth cycle.

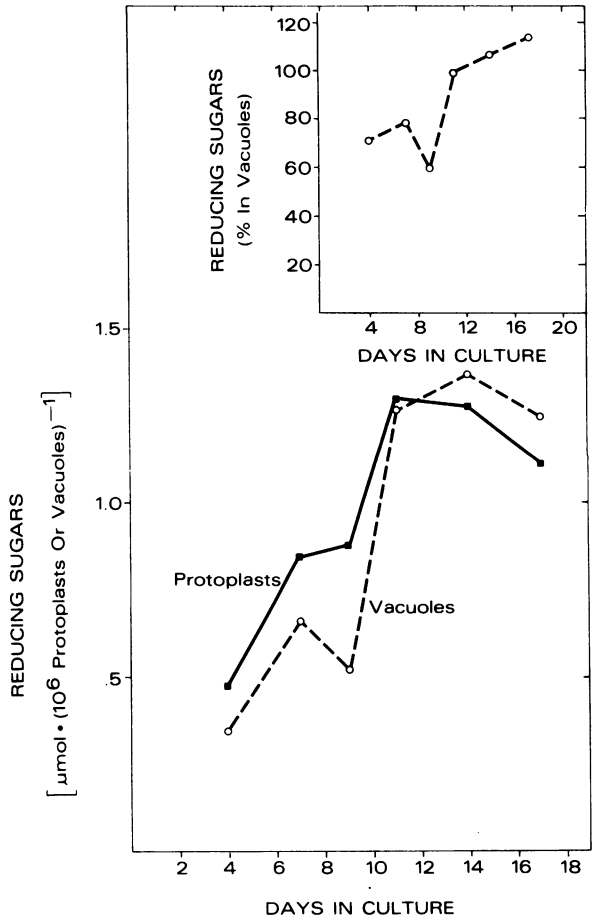

FIG. 4. Reducing sugar content in protoplasts and vacuoles during the growth cycle.

NADH malate dehydrogenase found associated with the vacuoles could be accounted for by cytoplasmic contamination. It must be noted that organelles such as mitochondria and ER contaminated the vacuole preparation by 11%. Therefore, values reported in Table III for the vacuole preparation include the contribution made by these contaminants. Only 16.0% of the protein found in protoplasts was located in the vacuoles.

Vacuoles were lysed by resuspension in 10 mM Hepes buffer without mannitol (pH 5.6) in order to determine the localization of the above five vacuole enzymes on and within the vacuoles. The tonoplast membrane was separated from soluble components by centrifugation at 100,000*g* for 90 min. Acid phosphatase, ribonuclease, and peroxidase were associated mainly with the soluble components of the vacuole, while carboxypeptidase was largely membrane-bound (Table IV). Of the total vacuolar protein, only 33% was soluble.

Solute Concentration Changes in Vacuoles During the Growth Cycle. Sucrose, reducing sugars, and amino acids were examined in vacuoles and protoplasts isolated from 4- to 17-day-old cultures. The concentrations of these solutes during the growth cycle have been measured previously on intact cells (26). The free amino acid content was 7.8 μmol/10^6 protoplasts and 5.4 μmol/10^6 vacuoles (Fig. 2) in 4-day-old cultures and decreased rapidly in older cultures, suggesting that amino acids were mobilized out of vacuoles during the period of most rapid growth. The rate of amino acid decrease between days 4 and 7 could not be explained by dilution of vacuole content because this decrease was greater than the rate of cell mass increase. The free amino acid content in the vacuoles was never more than 60% of that in the protoplasts and decreased sharply in 7- to 11-day-old cultures (Fig. 2, inset). Amino acid concentration in the vacuoles was about 220 mM in 4-day-old cultures; but it must be kept in mind that the protoplast volume in 0.4 M mannitol is less than it is under natural conditions.

Sucrose content of sugarcane protoplasts increased from about 0.1 to 2 μmol/10^6 protoplasts during the growth cycle (Fig. 3). The percentage of sucrose stored in the vacuoles (Fig. 3, inset) also increased as the culture aged until 100% of the sucrose was found in the vacuoles in stationary phase cells. Reducing sugars were also accumulated in protoplasts and vacuoles as the culture aged (Fig. 4). However, a higher percentage of reducing sugars was found in vacuoles than in protoplasts even in 4-day-old cultures. Neutral sugars (sucrose, glucose, and fructose) increased in the vacuole from 35 mM at day 7 to 135 mM at day 14, an increase of 100 mM, again under the conditions of reduced volume caused by a 0.4 M mannitol concentration in the surrounding medium. Vacuolar volume in intact cells increased by 9% between day 7 and 14 (26).

The percentage of solute in the vacuole is an indication of solute accumulation by the vacuole. Total amino acid concentration seems to be in equilibrium between cytoplasm and vacuoles. A similar situation seems to exist for hexoses in younger cells, but in older cells almost all the hexose is found in the vacuole. Sucrose concentration in vacuoles is lower than in cytoplasm in young cultures and is clearly accumulated in old cultures. The fact that certain substances are found entirely in the the vacuole fraction has two implications: (a) Because 100% of a compound could be found in the vacuole, the vacuole preparation procedure does not cause opening and reclosing of the tonoplast; therefore the method is suitable for analysis of vacuolar content. (b) There is likely to be active transport of sugars into the vacuole, either of hexoses, or of sucrose, or both.

CONCLUSIONS

Vacuoles isolated from protoplasts released from sugarcane suspension culture can be obtained in high yield by centrifugation

on a Ficoll cushion. There is apparently no loss of low mol wt compounds, such as sugars, during the isolation procedure. The data suggest active sugar transport at the tonoplast. Isolation and characterization of sugarcane vacuoles are necessary prerequisites for further studies of sugar transport across the tonoplast and for studies of energetics on the tonoplast.

Note added in proof. R. L. Berkowitz and R. L. Travis (1981 Plant Physiol 68: 1014–1019) have reported Con A-binding methods that helped to furnish additional confirmation of the purity of our vacuole preparation.

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