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Hydrolases in Vacuoles from Castor Bean Endosperm[1]

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ABSTRACT

Vacuoles were prepared from endosperm tissue of 4-day-old castor bean seedlings (*Ricinus communis* var. Hale) and purified on a stepped sucrose gradient. It was shown by assays of marker enzymes that there was only trace contamination of the final preparation by other organelles (mitochondria, glyoxysomes, nuclei, spherosomes, and plastids) and by cytoplasmic components. Hydrolytic enzymes (acid protease, carboxypeptidase, phosphodiesterase, RNAase, phytase and β-glucosidase) were present in the isolated vacuoles in amounts indicating a primarily vacuolar localization *in vivo*. The vacuoles also contained storage protein and high concentrations of sucrose. The over-all results indicate that the vacuoles from castor bean endosperm are the site of hydrolysis of the constituents of the protein bodies and are a temporary storage compartment for the sucrose produced from fat and protein reserves.

During the early growth of the castor bean seedling there is a massive production of enzymes and organelles concerned with the conversion of fat to sucrose in the endosperm (3). The amino acid precursors required for the synthesis of enzymes and other protein constituents arise from the hydrolysis of protein bodies present in the endosperm. Thus, in a single tissue active synthesis of specific proteins occurs concomitantly with massive protein breakdown. This raises the question of how the catalytically important protein components are protected from attack by the enzymes bringing about protein hydrolysis in the same cells. The most obvious possibility is that hydrolysis of storage protein occurs in a segregated compartment, the vacuole. In fact, early in germination small vacuoles are formed by the dissolution of the water-soluble protein matrix surrounding the protein crystalloid of the protein bodies and these coalesce to give a large central vacuole containing crystalloid remnants (2, 22). The concept of the vacuole as a discrete site of hydrolytic activity in higher plant cells is an old one and has received strong support from Matile (13) particularly from experiments with younger tissues.

Recently, methods have been developed for the isolation of vacuoles in quantities sufficient to allow a direct test of their storage and lytic function (4, 5, 7, 9, 26, 27). Somewhat surprisingly, in what the authors emphasize is the first detailed examination of this question in mature cells (from petals of *Hippeastrum*) assays for various acid hydrolytic enzymes revealed that they were not present in the vacuole but in the cytosol (5). However, in an earlier brief report Matile (12) had shown that the vacuoles that arise from protein bodies in germinating pea seeds contain an acid protease and other hydrolytic enzymes. The vacuoles in the endosperm tissue of young castor bean seedlings have a similar origin. The present paper describes a detailed investigation of vacuoles isolated in high purity from this tissue and it is shown clearly that they have both a lytic and storage function.

MATERIALS AND METHODS

Plant Material. Seeds of castor bean (*Ricinus communis* var. Hale) were soaked in running tap water for 1 day and germinated in moist Vermiculite at 30 C.

Preparation of Protoplasts. Fourteen endosperm halves, removed from 4-day-old seedlings, were used for preparation of protoplasts as described previously (15). The protoplasts (about 4 $\times 10^5$) were washed twice with 0.7 M mannitol. To obtain the protoplast extract, 0.5 ml of homogenizing medium (150 mM Tricine-KOH [pH 7.5], 15% sucrose, 0.1 mM EDTA) was added and the protoplasts were ruptured by sonication for 10 sec at 0 C. Appropriate amounts of this homogenate (5–200 μl) were used for the assays described below.

Preparation of Vacuoles. Sliced tissue from 14 endosperm halves was incubated for a total of 5 hr in the enzyme solution (0.5% Macerozyme R-10 and 2% cellulose "Onozuka" R-10 in 0.7 M mannitol) and the enzyme solution was removed (15). Seven ml 0.7 M mannitol was added to the slices and the flask was briefly swirled by hand. This treatment released vacuoles and other components from the slices. The mannitol solution was collected by filtration through nylon bolting cloth (35 mesh) and the process was repeated using fresh mannitol solution until 40 ml of filtrate had been collected. Microscopic examination showed the presence of protoplasts and other components in this crude vacuole fraction (see Fig. 1B). Twenty ml of the filtrate was layered on 10 ml of 40% (w/w) sucrose-containing 0.1 mM EDTA in a 50-ml tube. The tubes were centrifuged at 300*g* for 5 min. Only vacuoles were sedimented; protoplasts and other cell components remained in the 0.7 M mannitol layer. To obtain the vacuolar extract the purified vacuole fraction containing about 7×10^5 vacuoles was mixed with 0.5 ml of homogenizing medium (as above) and ruptured by sonication for 10 sec at 0 C. Samples of this homogenate were used directly for the enzyme and other assays described below. For the hydrolytic enzymes 10 to 100 μl (equivalent to about 10^4–10^5 vacuoles) were used. Larger amounts, up to 500 μl, were used for the other assays.

When vacuoles were prepared for sucrose analysis, 40% (w/w) sorbitol containing 0.1 mM EDTA was used in place of 40% (w/w) sucrose containing 0.1 mM EDTA, and sucrose was omitted from the homogenizing medium.

Enzyme and Chemical Assays. All hydrolytic enzyme assays were essentially those of Tully (22). The extracts were not dialyzed and rates of reaction were corrected for zero time blank values. Acid protease was assayed with bovine hemoglobin as substrate. The reaction mixture contained the following components in 1 ml: K-malate (pH 3.5), 100 μmol; bovine hemoglobin, 10 mg. After incubating for 30 min at 37 C, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid. The solutions were centrifuged and the supernatant solutions were assayed for amino-N by the ninhydrin method (21) using glycine as a standard. Carboxypeptidase was assayed by hydrolysis of Z-Phe-Ala.[3] The reaction mixture contained the following components in 1 ml: K-acetate

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[3] Abbreviations: bis-PNPP: bis-(*p*-nitrophenyl phosphate); PNP: *p*-nitrophenol; PNPP: *p*-nitrophenyl phosphate; PNP-β-D-Glu: *p*-nitrophenyl-β-D-glucoside; Z-Phe-Ala: carbobenzoxy phenylalanylalanine.

(pH 4.5), 100 μmol; Z-Phe-Ala, 1 μmol. The reactions were stopped by adding 1 ml of 10% trichloroacetic acid after incubation at 37 C for 30 min and the alanine assayed with ninhydrin. Acid phosphatase was assayed with PNPP as substrate. The reaction mixture contained the following components in 0.5 ml: K-acetate (pH 5), 50 μmol; PNPP, 2.5 μmol. After incubating for 5 min at 37 C, the reaction was stopped and the free PNP color developed by adding 2.5 ml of 0.2 N NaOH. The *A* at 400 nm was measured, and PNP concentration determined from a standard curve. Phosphodiesterase was assayed by hydrolysis of bis-PNPP. The reaction mixture contained the following components in 0.5 ml: K-malate (pH 5), 50 μmol; bis-PNPP, 2.9 μmol. After incubation for 10 min at 37 C the reaction was stopped with 2.5 ml 0.2 N NaOH. Phosphodiesterase activity was calculated by measuring the *A* at 400 nm and correcting for PNPPase activity. RNA was assayed by hydrolysis of *Torula* RNA following the method of Pitt and Combes (18). The reaction mixture contained the following components in 0.2 ml: K-acetate (pH 5), 20 μmol; RNA, 0.1 μg. After incubation for 30 min at 37 C the reaction was stopped by the addition of 200 μl of 4% $HClO_4$ plus 0.25% uranyl-Mg-acetate, and the mixture was chilled on ice for 30 min. After adding 2.6 ml of H_2O, the insoluble protein and RNA were removed by centrifugation, and the *A* at 260 nm was measured. Phytase was assayed by the hydrolysis of Na-phytate. The reaction mixture contained the following components in 0.5 ml: K-malate (pH 5), 50 μmol; Na-phytate, 5 μmol. After incubation for 30 min at 37 C, the reaction was stopped with 0.5 ml of 10% trichloroacetic acid. The Pi released was measured by the method of Fiske and Subbarow (6). β-Glucosidase was assayed by hydrolysis of PNP-β-D-Glu. The reaction mixture contained the following components in 0.5 ml: K-acetate (pH 5), 50 μmol; PNP-β-D-Glu, 1.25 μmol. After 1 hr of incubation at 37 C, the reaction was stopped and PNP determined as for acid phosphatase.

Other enzyme and chemical assay methods were those described in the literature as follows: acid lipase (14), alkaline lipase (14), catalase (11), fumarase (19), NADH-Cyt *c* reductase (8), NADH-malate dehydrogenase (1), RuP_2 carboxylase (16), triose-P isomerase (17), and DNA (7). Sucrose was measured by the resorcinol method (20) after removal of glucose and fructose (24). Protein was assayed by the Lowry method (10) after precipitation and washing in trichloroacetic acid (10%, w/v).

RESULTS

Preparation of Vacuoles. Figure 1A is a photomicrograph of the protoplast preparation showing the size range and Figure 1B shows the crude vacuole fraction obtained from the predigested endosperm slices in 0.7 M mannitol. The vacuoles range in diameter from 10 to 100 μm corresponding roughly with the protoplast dimensions. The vacuoles in 0.7 M mannitol are labile and rupture within 20 min. Separation from other components is achieved by centrifuging the vacuoles through 40% sucrose or 40% sorbitol as described under "Material and Methods." Figures 1C and 1D are photomicrographs of the purified vacuole fractions. The vacuoles in 40% (w/w) sucrose containing 0.1 mM EDTA are stable for at least 3 to 4 hr by microscopic inspection. The vacuoles shrink during purification and range in size from 8 to 90 μm in the final preparation. The vacuoles stain with neutral red. From analysis of total protein and from hemacytometer counts of appropriate samples it was determined that the protein content of 10^4 protoplasts was 75 μg and that of 10^4 vacuoles was 19.2 μg. Thus, if only a single vacuole arises from one cell, roughly 25% of the total protein is vacuolar; if more than one vacuole is released from a single cell, the value would be greater than this.

Enzymes and Vacuoles. The activities of marker enzymes of various organelles and cytoplasm were measured in order to assess the purity of the isolated vacuoles (Table I). Specific activities (enzyme activity/unit protein) of vacuole extracts (column I) and protoplast extracts (column III) were measured and from the known relationship between protein content and numbers of protoplasts and vacuoles, the other values in Table I were obtained. The marker enzymes were as follows: fumarase (mitochondria), catalase (glycoxysomes), alkaline lipase (glyoxysomes), RuP_2 carboxylase (plastids), acid lipase (spherosomes), NADH-malate dehydrogenase (glyoxysomes, mitochondria, cytoplasm), triose-P isomerase (plastids, cytoplasm, NADH-Cyt *c* reductase (ER, mitochondria) and DNA (nuclei).

As shown in Table I, fumarase, RuP_2 carboxylase, acid lipase, and alkaline lipase were present in the protoplast extracts but below the limits of detection in the vacuoles. As shown in column V, the four marker enzyme activities that were measurable in the vacuole extracts, catalase, NADH-malate dehydrogenase, NADH-Cyt *c* reductase, and triose-P isomerase, were present at less than 1% of their activity in the protoplasts. The protoplasts contained 5.9 μg DNA/mg of protein (44.4 μg/10^6 protoplasts) and none was detectable in the vacuoles. Thus, it is clear that the procedures employed yield vacuoles that are virtually uncontaminated by other cellular constituents.

Table II shows that the vacuole extracts contain high activities of phosphodiesterase, RNAase, carboxypeptidase, acid phosphatase, phytase, acid protease, and β-glucosidase. In spite of the high protein content of the vacuoles the specific activities of most of these enzymes are 2.5- to 3-fold higher than the corresponding values for the protoplast extracts (column V). Thus, it is clear that these enzymes are in fact predominantly present in vacuoles and the values for enzyme activity based on numbers of vacuoles or protoplasts (column VI) show that except for acid phosphatase, at least 60 to 70% of these enzyme activities are vacuolar.

Sucrose Content of Vacuoles. Analysis for sucrose yielded the data shown in Table III. The vacuolar extracts contain 3.7 times as much sucrose as protein and at least 62% of the sucrose is restricted to the vacuoles.

DISCUSSION

Matile (13) has eloquently summarized the evidence supporting the view that the vacuoles of higher plant cells are a site of deposition of some metabolites and of hydrolytic enzyme activities. Much of the evidence is indirect and he has emphasized that quantitative conclusions about the role of the large central vacuoles in mature cells have been limited because methods for their separation have not been available. Recently, methods have been developed for the isolation of such vacuoles (4, 5, 7, 9, 26, 27). Butcher *et al.* (5) first investigated the question of vacuolar localization of a series of hydrolases using vacuoles derived from protoplasts of *Hippeastrum* petals and other mature tissues. They showed that the vacuoles were not a primary site of localization for most of the enzymes, and, in a valuable discussion of the general question, they argued that in such cells the enzymes must be sequestered within the cytoplasm. In earlier experiments with the cotyledons of germinating pea seedlings Matile (12) observed that the specific activities of some hydrolytic enzymes were higher than those in the cell extracts, but no estimate could be made of the proportion of the total activities present in the vacuoles.

It is clear from the present results that in the cells of the endosperm of the germinating castor bean, the vacuoles do contain hydrolytic enzymes, including some examined by Butcher *et al.* (5). The vacuoles were shown not to be significantly contaminated with enzymes from other organelles or cytosol (Table I) and yet they contained from 64 to 77% of six of the seven hydrolases examined (Table II, column VI). This estimate is based on the amounts of enzyme present in equal numbers of vacuoles and protoplasts, and to the degree that each protoplast contains more than one vacuole the true values will be higher than the 64 to 77% observed. Indeed, microscopic examination of the endosperm tissue has shown that in the early stages of germination many small vacuoles containing protein bodies are present in each cell and that these later coalesce into a large central vacuole (22). The

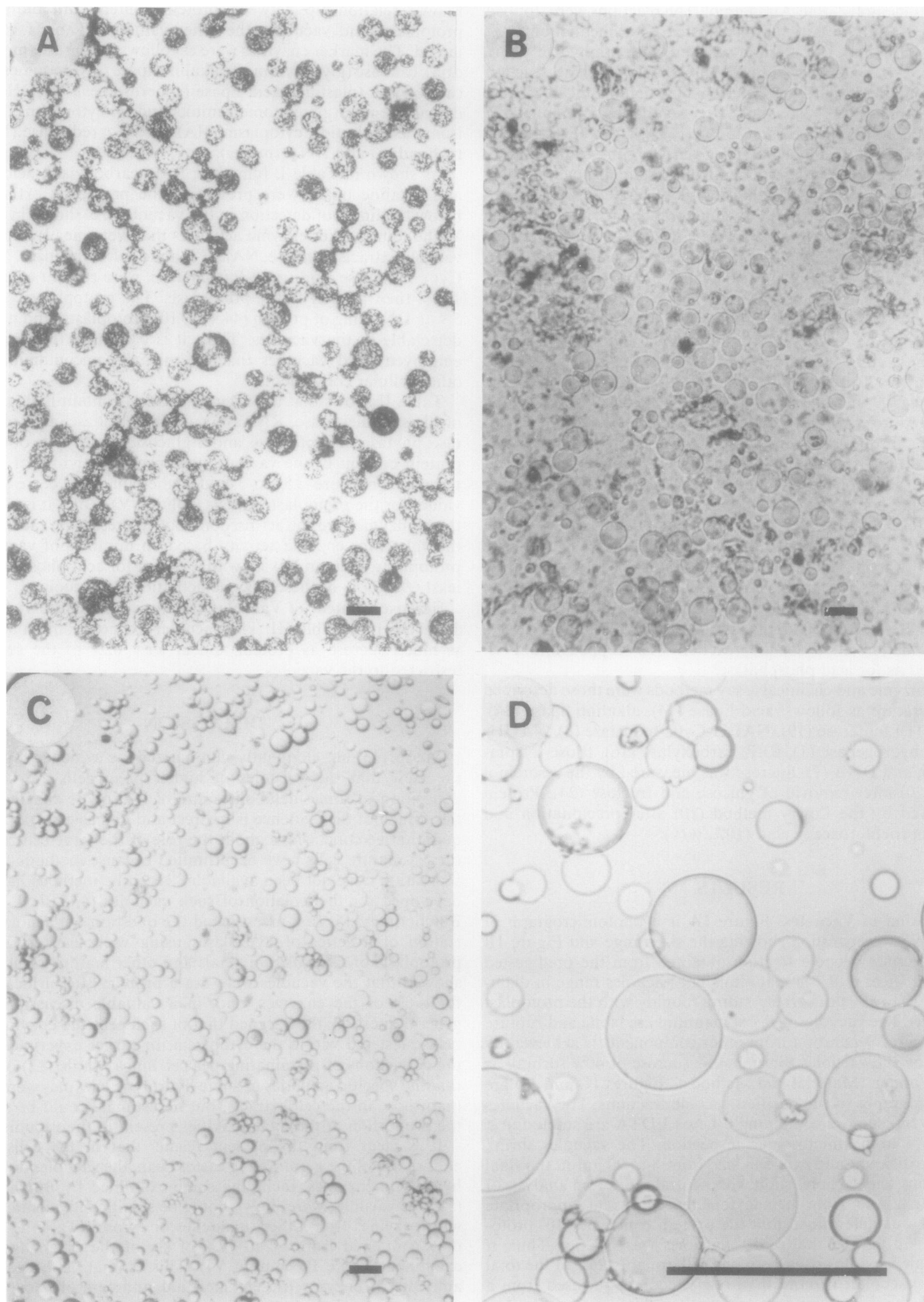

FIG. 1. Photomicrographs of: A, protoplast preparation; B, crude vacuole fraction; C and D, purified vacuole fraction from 4-day-old castor bean endosperm. Bars are 100 μm.

Table I. Enzyme content of vacuoles and protoplasts from castor bean endosperm. Enzymes were assayed on vacuole and protoplast extracts and gave the specific activities (amount of substrate utilized or product produced/min mg protein) shown. The values for enzyme activity/10^6 vacuoles and protoplasts were calculated from the measured relationships between numbers of vacuoles and protoplasts and their protein contents.

	I	II	III	IV	V
Enzyme	Activity in vacuoles		Activity in protoplasts		Ratio
	/mg protein	/10^6 vacuoles	/mg protein	/10^6 protoplasts	II/IV
Fumarase (μmoles/min)	N.D.	-	0.143	1.07	-
Catalase (mmoles/min)	0.00347	0.00666	0.539	4.04	0.0016
RuP_2 carboxylase (nmoles/min)	N.D.	-	7.63	57.2	-
NADH-malate dehydrogenase (μmoles/min)	0.202	0.388	10.5	78.8	0.0049
NADH Cyt c-reductase (μmoles/min)	0.00190	0.00365	0.201	1.50	0.0024
Triose phosphate isomerase (μmoles/min)	0.0482	0.0925	7.09	53.2	0.0017
Acid lipase (nmoles/min)	N.D.	-	5.36	40.2	-
Alkaline lipase (nmoles/min)	N.D.	-	9.65	73.4	-

N.D. not detectable

Table II. Hydrolases in vacuoles and protoplasts from castor bean endosperm. Expression of results as in Table I.

	I	II	III	IV	V	VI
Enzyme	Activity in vacuoles		Activity in protoplasts		Ratio	
	/mg protein	/10^6 vacuoles	/mg protein	/10^6 protoplasts	I/III	II/IV
Acid phosphatase (μmoles/min)	6.23	12.0	5.50	41.3	1.13	0.290
Phosphodiesterase (μmoles/min)	0.320	0.614	0.128	0.960	2.51	0.640
RNAase (ΔOD_{240}/min)	0.370	0.710	0.146	1.10	2.53	0.645
Carboxypeptidase (nmoles/min)	35.0	67.2	13.1	98.3	2.67	0.683
Phytase (nmoles/min)	4.73	9.08	1.75	13.1	2.70	0.693
Acid protease (nmoles/min)	112	215	37.0	278	3.03	0.773
β-glucosidase (nmoles/min)	27.5	52.8	11.0	82.5	2.50	0.640

Table III. Sucrose content of vacuoles and protoplasts from castor bean endosperm. Expression of results as in Table I.

I	II	III	IV	V	VI
Sucrose in vacuoles		Sucrose in protoplasts		Ratio	
mg/mg protein	mg/10^6 vacuoles	mg/mg protein	mg/10^6 protoplasts	I/III	II/IV
3.70	7.10	1.51	11.3	2.45	0.628

fact that there is a wide range of sizes in the isolated vacuoles (Fig. 1) suggests that some may have originated from cells with more than one vacuole. Thus, it is quite possible that the six hydrolases are entirely localized in vacuoles in the endosperm tissue. By contrast, the clearly lower value for acid phosphatase (Table II) confirms that this enzyme is not confined to vacuoles (25).

There are several other features of the vacuoles from the endosperm tissue that distinguish them from most others that have been examined previously (4, 5, 7, 9, 26, 27). The first is that they sediment rapidly through very dense solutions as noted by Matile in his preparation from young pea cotyledons (12). One major reason for this is the very high protein content of the vacuoles derived from protein bodies. Whereas the protein content of *Hippeastrum* petals was less than 4% of that of the protoplasts, the endosperm vacuoles contained at least 25% of the protein in the protoplasts. This is undoubtedly due to the presence of the crystalloid remnants of the protein bodies in the process of digestion which is not completed until day 6; such crystalloids have a density of 1.29 g/ml (23). The high protein content reflects the origin of the vacuoles in these tissues (2, 22) and the presence of hydrolases shows their major function as a localized domain of protein breakdown.

As a result of the high protein content of the vacuoles the

specific activities of the hydrolases in the vacuoles from the endosperm, while clearly higher than those in the protoplasts, are only 2- to 3-fold higher (Table II, column V). Corresponding ratios for some of the vacuolar hydrolases from yeast are in the range of 20 to 40 (13).

Another feature of the endosperm vacuoles contributing to their density is their high sucrose content. Sucrose is the major end product of fat utilization in the endosperm and as shown in Table III, the vacuoles contain at least 62% of the cellular sucrose. It is reasonable, as for the hydrolytic enzymes, to adjust this figure upward because on the average more than one vacuole arises from one protoplast. Buser and Matile (4) have recently shown directly that the malate in the cells of *Bryophyllum* leaves is essentially confined to the vacuoles and it is quite conceivable that a similar distribution of sucrose occurs in the endosperm cells.

Thus, it is clear that the vacuoles in the endosperm cells of young castor bean seedlings are a primary intracellular site of various enzymes concerned with the hydrolysis of protein, carbohydrates and phytin, and also a temporary storage site of sucrose. These vacuoles appear to play a dynamic role in the breakdown of protein that is a feature of the endosperm metabolism; distinctions between them and other vacuoles that have been examined are probably related to differences in their origin and function.

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