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pH in Vacuoles Isolated from Castor Bean Endosperm[1]

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ABSTRACT

Vacuoles were prepared from germinating castor bean endosperm (*Ricinus communis* var Hale) and purified by filtration through a cotton layer under physiological osmolarity. The purity of vacuoles prepared by this method was comparable with that prepared by a sucrose step gradient centrifugation reported in a previous paper (Nishimura, Beevers 1978 Plant Physiol 62: 44–48). It was shown by assays of marker enzymes that the final preparation contained trace contamination of other organelles (glyoxysomes, mitochondria, and endoplasmic reticulum) and the cytosol. The isolated vacuoles were stained with neutral red, indicating that the intravacuolar pH is acidic. Intravacuolar pH of isolated vacuoles was determined by measuring the distribution of [^{14}C]methylamine in the vacuoles and by directly measuring the pH of vacuolar extracts. The pH of isolated vacuolar extracts was 5.7 to 5.9. Similar values were obtained by the methylamine method and it was shown that intravacuolar pH increased as the pH of the medium was increased.

The vacuoles of higher plant cells are known to be sites of deposition of salts and metabolites (11). Recently, methods for isolating vacuoles have been developed, and direct analysis of vacuolar extracts have revealed the dynamic aspects of vacuolar function in cellular metabolism (3, 5, 12, 13, 22).

We have previously purified vacuoles from germinating castor bean endosperm (13). The isolated vacuoles contain large amount of storage protein and sucrose which are produced from storage lipid during germination, showing that vacuoles have a storage function. The hydrolysis of the endogenous storage protein was also observed in isolated vacuoles, indicating that they have a lytic function (14). The optimum pH for the autolysis was 5.0 (14) and the vacuoles were stained with neutral red. These results suggest that the pH is acidic in vacuoles and the environment in vacuoles is quite different from that in cytosol. In this communication, I report on the determinations of pH values in vacuoles from castor bean endosperm by the [^{14}C]methylamine method and the direct pH analysis of vacuolar extracts. I also report a method for purification of vacuoles under physiological conditions, which might be useful for metabolite transport studies.

MATERIALS AND METHODS

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

Preparation of Vacuoles. The crude vacuole fraction was prepared from 4-d-old castor bean endosperm as described previously (13). The crude vacuole fraction (approximately 40 ml) prepared from 28 endosperm halves was centrifuged at 100*g* for 2 min. The precipitate contained vacuoles, protoplasts, and other cellular components. The precipitate, suspended in 3 ml of 0.7 M mannitol containing 5 mM EDTA (pH 7.0), was filtered through a small piece of absorbent cotton (ϕ = 0.5 cm) on a funnel (ϕ = 5 cm), followed by 5 ml of 0.7 M mannitol containing 5 mM EDTA. Most of the protoplasts formed aggregates and were removed by the filtration. The eluates were combined and centrifuged 100*g* for 2 min. The precipitate was washed twice with 5 ml of 0.7 M mannitol containing 5 mM EDTA. Protoplasts were unstable in high concentration of EDTA. The remaining protoplasts were disrupted during centrifugation and washing. Other contaminating organelles remained in the supernatant solution during centrifugation.

pH Measurements by Methylamine Method. The reaction mixture contained Hepes or Mes-Tris buffer at various pH levels; 10 μmol; $^{3}H_2O$; 7 μCi; [^{14}C]methylamine; 0.2 μmol (0.5 μCi); the vacuole preparation contained approximately 0.4 mg protein, and 175 μmol mannitol in a total volume of 250 μl. The samples were incubated at 25°C. Aliquots (60 μl) were taken after 1, 3, 5, and 10 min incubation and were subjected to silicone oil centrifugation.

Silicone Oil Centrifugation. The principle of silicone oil centrifugation was described by Klingenberg and Pfaff (4). Samples (60 μl) were quickly layered on a gradient composed of 20 μl of 40 % w/w sorbitol and 50 μl of silicone oil (Dow Corning 704 diffusion pump fluid) in 250 μl polyethylene tubes (Beckman 682823). The centrifugation was performed at maximum speed for 10 s using Beckman Microfuge B at room temperature. The polyethylene tubes were directly put into Dry Ice-acetone and frozen. The bottom layer was cut off with a razor blade and placed in a counting vial containing 1 ml of 0.1 % Triton X-100. After the addition of 10 ml of scintillator, the radioactivity was measured by dual-channel counting in a Beckman scintillation counter.

Direct Measurements of pH of Vacuolar Extracts. In this experiment vacuoles were prepared by a sucrose step gradient centrifugation as described in a previous paper (13). The vacuoles (approximately 4 mg progein) were diluted with 0.7 M mannitol to 1 ml and sonicated for 10 s. The pH of the diluted and the sonicated samples was measured with a pH meter (Orion 601A).

Enzyme and Chemical Assays. The methods used were those described in the literature as follows: catalase (9), fumarase (15), NADH-malate dehydrogenase (1), NADH-Cyt *c* reductase (7), acid phosphatase (13), phosphodiesterase (13), and protein (8).

RESULTS AND DISCUSSION

To check the purity of the vacuoles, some marker enzyme activities were measured in the vacuolar extracts (Table I). The vacuole fractions purified by the filtration method contained less than 1% of the cellular catalase, fumarase, NADH-malate dehydrogenase, and NADH-Cyt *c* reductase. On the other hand, acid phosphatase and phosphodiesterase activities which had been demonstrated to be vacuolar enzyme were found to the same

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Table I. *Enzyme Content in Vacuoles and Protoplasts Isolated from Castor Bean Endosperm*

The preparation of the crude vacuoles and the purified vacuoles are described in text. The following marker enzymes were used: catalase (glyoxysomes), fumarase (mitochondria), NADH-malate dehydrogenase (glyoxysomes, mitochondria, cytosol), NADH-Cyt *c*-reductase (ER), acid phosphatase, and phosphodiesterase (vacuoles). Enzymes were assayed on the vacuole and protoplast extracts and gave the specific activities (amount of substrate utilized or product produced/min·mg protein). The values for enzyme activity/10^6 vacuoles and protoplasts were calculated from the measured relationship between numbers of vacuoles and protoplasts and their protein contents.

Enzymes	Crude Vacuoles	Purified Vacuoles		Protoplasts[a]		II:IV	III:V
	I	II	III	IV	V		
	/mg protein	*/mg protein*	*/10^6 vacuoles*	*/mg protein*	*/10^6 protoplasts*	*ratio*	
Catalase (mmol/min)	0.368	0.00757	0.0145	0.539	4.04	0.014	0.0036
Fumarase (μmol/min)	0.0240	ND[b]	ND	0.143	1.07	0	0
NADH-malate dehydrogenase (μmol/min)	1.10	0.196	0.376	10.5	78.8	0.019	0.0048
NADH-Cyt *c*-reductase (μmol/min)	0.00546	0.000835	0.00160	0.210	1.50	0.0040	0.0011
Acid phosphatase (μmol/min)	5.56	4.35	8.35	5.50	41.3	0.790	0.202
Phosphodiesterase (μmol/min)		0.322	0.618	0.128	0.960	2.52	0.643

[a] Data from Ref. 13.
[b] ND, Not detectable.

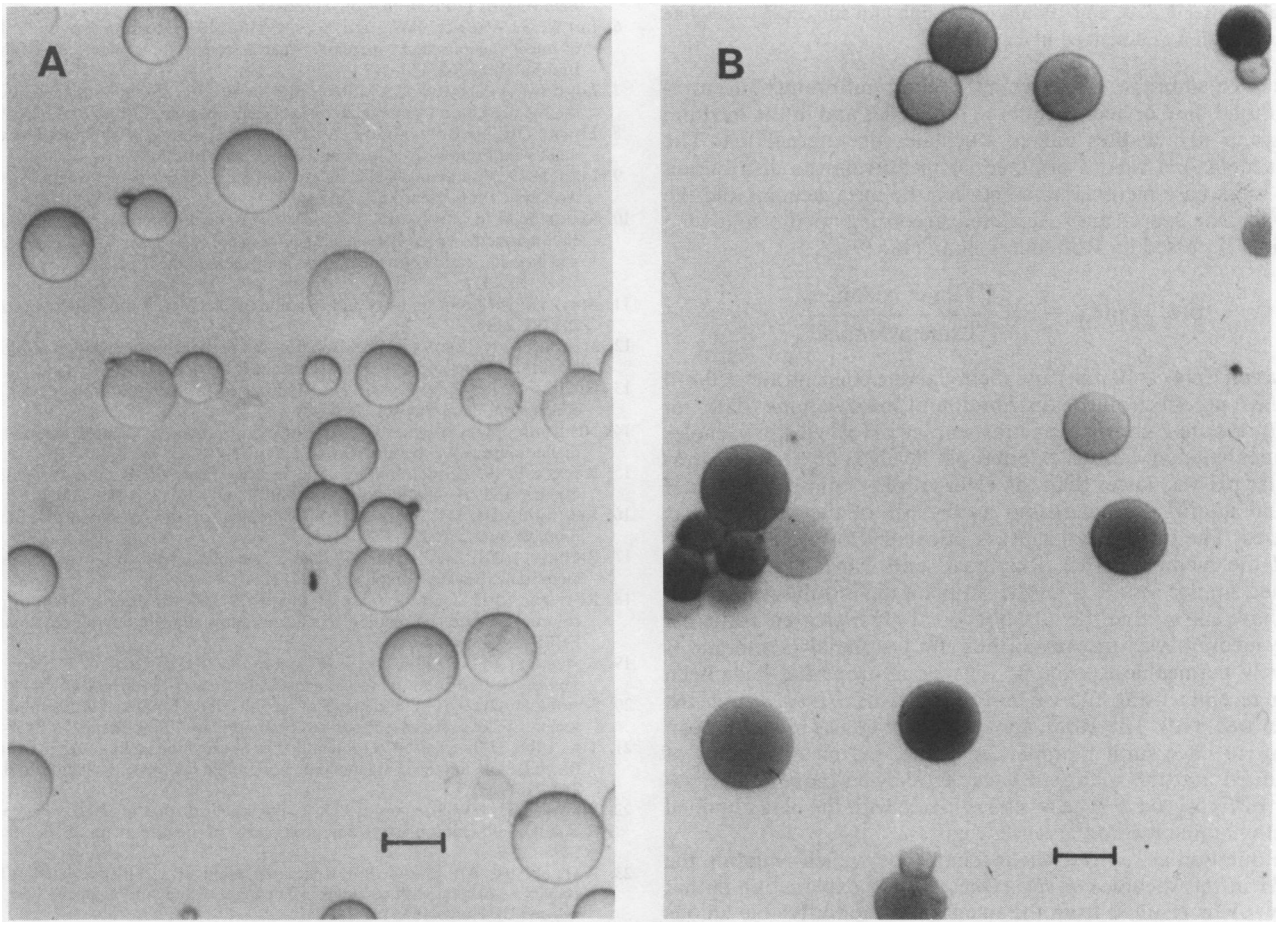

FIG. 1. Photomicrographs of vacuoles incubated with (B) and without (A) neutral red. Bars, 50 μm.

extent as reported in a previous paper (13). These data indicate that vacuoles were prepared in a highly purified form at physiological osmolarity by this method.

Figure 1 A and B are photomicrographs of vacuoles, showing that the isolated vacuoles were stained with neutral red. Apparently the nonionized form of neutral red can pass through the tonoplast, and since the intravacuolar pH is acidic, the ionized form of neutral red accumulates in the vacuoles. The same principle is applicable for estimating intravacuolar pH. It is assumed that the biological membrane is freely permeable to the nonionized form of the weak base or acid, but not to the ionized species. Since the distribution of the ionized species will depend on the pH

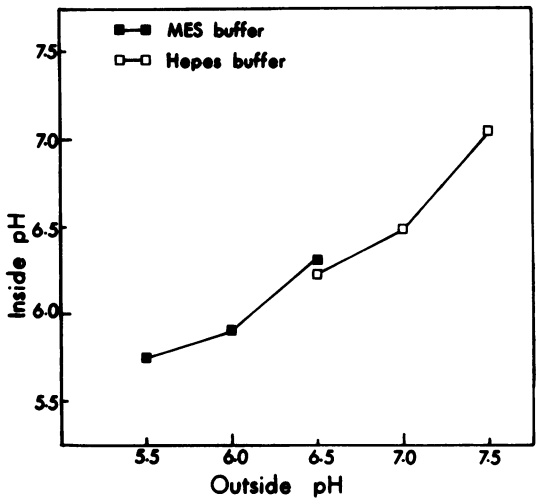

FIG. 2. Intravacuolar pH as a function of pH of medium. pH measurements were carried out by the methylamine method. Reaction mixture contained: Hepes or Mes-Tris buffer at various pH levels, 10 μmol; ^3H-H_2O, 7 μCi; [^{14}C]methylamine, 0.2 μmol (0.5 μCi); the purified vacuoles as described in text, approximately 0.4 mg protein; and mannitol, 175 μmol in a total volume of 250 μl. Sample as incubated at 25°C. Aliquots (60 μl) were taken after 1, 3, 5, and 10 min incubation and subjected to silicone oil centrifugation as described in text.

across the membrane, measurement of the equilibrium concentration of total base or acid within the organelles and in the medium at a given pH enables one to calculate the internal pH. The intravacuolar pH was determined by measuring the distribution of the weak base methylamine between the intravacuolar and the extravacuolar space, and calculated according to the following equation discussed by Rottenberg et al. (18, 19):

$$pH_{in} = pH_{out} - \log \frac{[^{14}C]\text{methylamine}_{in}}{[^{14}C]\text{methylamine}_{out}} \quad 1$$

Incubation time (1–10 min) and methylamine concentration (0.8–4 mM) have no effect on the distribution of methylamine (data not shown). Figure 2 shows the intravacuolar pH when the vacuoles were incubated at various external pH levels at 25°C. The intravacuolar pH was lower than the external pH (from pH 6.0 to pH 7.5) and internal pH increased as the pH of the medium was increased. The intravacuolar pH is calculated to be 5.8 when the pH of the medium is 6.0. Reijngoud and Tager (16, 17) have obtained similar results from the study on the intralysosomal pH. They have shown that the intralysosomal pH increased as the pH of the medium was increased, thus the lysosomal membrane is relatively permeable to protons. The same properties have been shown recently using the vacuo-lysosomes of *Hevea brasiliensis* (rubber tree) (10). Therefore, apparently tonoplasts of castor bean endosperm have similar properties. Direct pH measurements of the diluted and the sonicated vacuoles showed that the intravacuolar pH is 5.7 to 5.9. The results coincide with the data obtained by methylamine method given in Figure 2.

The question arises as to the mechanism(s) which maintain the low pH in the vacuoles *in vivo*. One possible explanation is that the low pH is resulted from the retention of nondiffusible anions within the vacuoles and the setting up of a Donnan equilibrium. Inasmuch as since the vacuoles come from protein bodies in germinating castor bean endosperm (14, 21), they contain phytin or polyphosphate as a polyanion. The other possibility is an ATP-dependent proton pump to maintain a low intravacuolar pH. In fact the existence of ATPase in tonoplasts has been reported in *Amaryllis* (6), red beet (23), and rubber tree (2). ATP-dependent acidification was demonstrated in lysosomes of rat liver (20) and in vacuo-lysosomes of *Hevea brasiliensis* (10). Further studies such as the effect of ATP and various ionophores on the vacuolar pH may lead us to clarify the mechanism(s).

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