

Mit diesen Vorstellungen läßt sich, wie in Arbeiten berichtet, erklären, daß

1. wäßrige Kontrastierungslösungen bei Zimmertemperatur, besonders bei Vestopal W, nur Oberflächenkontrastierungen ergeben;

2. alkoholische Kontrastierungslösungen, besonders bei erhöhter Temperatur, Tiefenkontrastierungen bewirken;

3. Kontrastierungen allgemein dazu neigen, die Oberflächenkomplexe stärker zu kontrastieren als das Innere.

Die Kontrastierungslösungen müssen das Einbettungsmittel mehr oder weniger erweichen. Dadurch besteht die Gefahr, daß sich Lagebeziehungen verändern. Schon die Anlagerung von Schwermetallatomen kann im Prinzip zu einer kleinen Lageveränderung führen.

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Vacuolar Membranes: Isolation from Yeast Cells

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Isolation, vacuolar membrane, yeast

α -Mannosidase was found associated with the vacuolar membranes of yeast. The vacuoles were isolated by flotation from osmotically disrupted spheroplasts of *Saccharomyces cerevisiae*. The enzyme was used as marker for isolating vacuolar membrane fragments directly from whole cells which were mechanically disintegrated. Over 90% of the total α -mannosidase was recovered in the particulate fraction. The enzyme was present in all of the fractions obtained upon differential centrifugation. Density gradient centrifugation in Urografin (5–20% w/v) of preparations obtained by differential centrifugation between 20 000 and 50 000 $\times g$ did not result in density equilibrium of the membrane. An isolation procedure involving a sedimentation velocity cut in Urografin gradients has, therefore, been worked out.

The vacuolar membrane (tonoplast) represents the boundary between vacuolar fluid (cell sap) and cytoplasmic matrix. Its possible functions are suggested by some unique properties of the cell sap. On the one hand it contains a number of hydrolases in a concentrated form; preparations of isolated vacuoles from baker's yeast contain high specific activities of proteases, RNase, esterase, β -glucosidase and other hydrolases^{1,2}, indicating a lysosomal function of this organelle. An important property of the tonoplast is, therefore, that it resists the attack of lytic enzymes present in the cell sap. On the other hand the cell sap contains a variety of com-

pounds that are temporarily removed from metabolism, *e.g.* purines³ and S-adenosylmethionin⁴. Recently Wiemken and Nurse⁵ have been able to demonstrate that storage pools of amino acids are localized in the vacuoles of the yeast *Candida utilis*. Although these compounds are very slowly metabolized they are in dynamic equilibrium with the sites of amino acid metabolism. Hence, an important function of the tonoplast is the mediation and control of exchange of metabolites between the cytoplasmic matrix and the cell sap. A difference between the tonoplast and the plasmalemma is indicated by the differential behaviour of these membranes in the presence of basic proteins that have been used for the stepwise extraction of yeast cells^{5,6}; the plasmalemma is disrupted whereas the tonoplast remains intact and impermeable for the micromolecules present in the cell sap⁷. These and

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other features of the vacuolar membrane point to interesting and perhaps unique properties which seemed to justify the reported attempt to its isolation.

Material and Methods

Organism and its culture

Strain LBG 1022 of *Saccharomyces cerevisiae* has been cultured as described previously⁸. Cells used for mechanical disruption were grown to early stationary growth phase. Growing cells used for preparing protoplasts and vacuoles were obtained by allowing one generation after addition of new culture medium to an early stationary phase culture.

Spheroplasts and vacuoles

These were prepared essentially according to Wiemken⁹.

Extraction of cells

Cells were harvested by centrifugation, cooled to 0 °C and washed twice with 0.01 M Tris-HCl buffer pH 7.2. They were ruptured by vigorous shaking for 10 sec in the presence of Ballotini beads (diameter 0.3–0.45 mm) and a medium containing 0.5 M sorbitol and 0.01 M Tris-HCl buffer pH 7.2. After removal of the glass beads the extract was centrifuged for 5 min at 2000 × *g*. The supernatant cell free extract was thereafter subjected to

Differential centrifugation

A first sediment obtained after 10 or 30 min of centrifugation at 20 000 × *g* contained most of the mitochondria and plasmalemma fragments. A subsequent centrifugation at 50 000 × *g* (20 or 60 min) yielded a second sediment which was resuspended in sorbitol medium and used for density gradient centrifugation. This preparation was subjected to a low speed centrifugation (5 min 2000 × *g*) prior to loading onto gradients in order to remove some aggregated material. A third sediment was obtained by centrifuging the supernatant of the 50 000 × *g* step for 60 min at 100 000 × *g*. The final supernatant represented the soluble fraction.

Density gradient centrifugation

4.5 ml of linear density gradients of Urografin (methylglucamine salt of *N,N'*-diacetyl-3,5-diamino-2,4,6-triiodo benzoic acid) were loaded with 1.0 ml of resuspended particles and centrifuged in a Spinco SW-39 rotor at 100 000 × *g* for 1 to 5 hours. After centrifugation the gradients were fractionated into

ca. 16 fractions. Urografin was dissolved in sorbitol-medium; the gradients extended from 5 to 20% (w/v) Urografin (corresponding to a density range from 1.055 to 1.125 g cm⁻³) or from 5 to 15% (1.055 to 1.100 g cm⁻³).

Enzyme activities

α -Mannosidase activity (E.C. 3.2.1.24) was estimated using *p*-nitrophenyl- α -mannoside (1 mg/ml) as a substrate. 100 μ l of enzyme were mixed with 100 μ l of substrate and 0.3 ml of buffer. The incubation at 37 °C was stopped by adding 2.5 ml of 0.2 M Na₂CO₃. Optical density readings were made at 400 nm. In citrate-phosphate buffer the optimum of activity is at pH 6.1, the curve showing a broad maximum between pH 5.4 and 6.8. However, the activity was higher in the presence of Tris-HCl (0.01 M) and was optimal in this buffer at pH 7.2. These conditions were used routinely in the assays.

Other enzyme activities were estimated according to standard procedures.

Protein and RNA

Perchloric acid extracts of TCA-precipitates were analyzed for RNA using either the orcinol reaction¹⁰ or UV absorption at 260 nm. Urografin did not interfere with the orcinol reaction; RNA present in density gradients was, therefore, estimated directly in the fractions. Protein was estimated according to Lowry *et al.*¹¹.

Electron microscopy

Pellets of isolated tonoplast fragments were fixed according to Hess¹² and thin sections contrasted with lead citrate¹³.

Results and Discussion

The suitable marker enzyme

Incubation of whole cells of baker's yeast in the presence of *p*-nitrophenyl- α -mannoside yields virtually no reaction product. Evidently, α -mannosidase is localized intracellularly. Since many hydrolases are known to be concentrated in preparations of isolated vacuoles¹ this organelle prepared from osmotically lysed spheroplasts was analyzed for the presence of α -mannosidase activity. The result presented in the Table I indicates, indeed, a concentration of this activity in the fraction of isolated vacuoles, the specific activity being ca. 20 times higher than in the lysed spheroplasts. Similar ratios of specific activities in spheroplasts and vacuoles

Table I. Specific activities of α -mannosidase in lysed spheroplasts, isolated vacuoles and supernatant fluid obtained after flotation of vacuoles. Activities are given in $E_{400\text{ nm}}$ per min and per mg protein.

	Specific α -mannosidase activity	Ration of specific activities: Subfractions/spheroplasts
lysed spheroplasts	3.12	1.0
vacuoles	63.0	20.2
supernatant	3.72	1.19

Table II. Distribution of α -mannosidase and RNase activities between tonoplast and cell sap prepared from isolated vacuoles. Vacuoles suspended in the medium for flotation (7.5% Ficoll, 0.1 M sorbitol, 0.01 M citrate buffer pH 6.5) were sonicated for 5 sec. Centrifugation (60 min, 125 000 $\times g$) yielded a small gelatinous pellet. Total sedimentable and soluble activities are given in arbitrary units.

	α -Mannosidase	RNase
sedimentable	0.410 (83.7%)	0.095
soluble	0.080	6.80 (98.6%)

have been observed in the case of hydrolases such as protease and RNase that are possibly localized exclusively in the vacuoles. If isolated vacuoles are disrupted by ultrasonication the fragments of the tonoplast can be sedimented and separated from the soluble vacuolar constituents. As shown in the Table II a large proportion of the α -mannosidase activity is associated with the sedimentable membranes whereas RNase activity is soluble almost completely. Hence, α -mannosidase seems to represent a suitable marker enzyme for tonoplasts which can be used for isolating this membrane directly from whole cells.

Isolation of tonoplast fragments

The association of α -mannosidase with membranes is also demonstrated by the observation that over 90% of the total activity present in cell free extracts is sedimentable. A preliminary trial using a comparatively steep density gradient of Urografin² has shown that the enzyme was contained in a mixed fraction of light membranes equilibrating at low densities of Urografin. It was, therefore, decided to use flat gradients in order to separate these membranes; in addition it seemed advisable to start density gradient centrifugation with a rather small fraction of sedimentable material containing a high proportion of α -mannosidase. Hence, differential centri-

Table III A. Distribution of α -mannosidase activity among subcellular fractions obtained by differential centrifugation. Activities are given in arbitrary units.

Fraction	Total activity in fraction	Percentage of total activity in extract
sediment 1 (30 min 20 000 $\times g$)	3.240	35.7
sediment 2 (60 min 50 000 $\times g$)	3.805	41.8
sediment 3 (60 min 100 000 $\times g$)	1.210	13.3
soluble fraction	0.816	9.0

Table III B. Distribution of α -mannosidase activity and RNA among subcellular fractions obtained by differential centrifugation. Total and specific activities are given in mMoles of substrate hydrolyzed in 60 min, and per mg of protein, respectively.

Fraction	Protein [mg]	α -Mannosidase activity			RNA
		Total	Per-centage of total	Specific	
cell free extract	45.6	1.73	100	0.038	1.0
Sediment 1 (10 min 20 000 $\times g$)	9.19	0.445	25.7	0.0485	0.20
sediment 2 (20 min 50 000 $\times g$)	2.88	0.326	18.8	0.113	0.107
sediment 3 (60 min 100 000 $\times g$)	9.06	0.675	39.0	0.0745	0.512
soluble fraction	25.7	0.147	8.5	0.0057	0.182
recovery	103%	92%	—	—	—

fugation yielding the results listed in the Table III A was initially employed. Sediment 2 obtained between 30 min 20 000 $\times g$ and 60 min 50 000 $\times g$ contains ca. 42% of the total activity. It was used in the subsequently described density gradient centrifugation.

Of the various gradient materials tested (sucrose, Ficoll, sorbitol and Urografin) by far the best separation of membranes could be achieved in gradients of Urografin. After 2.5 hours of centrifugation in a gradient ranging from 5 to 20% (w/v) Urografin, α -mannosidase was concentrated in a band corresponding to a density of ca. 1.08 g cm⁻³. The distribution curve (Fig. 1) corresponds with peaks of ATPase and NADH-DIP oxidoreductase. It also overlaps partially the distribution curve of RNA suggesting an incomplete separation of free ribosomes and tonoplast fragments. Indeed, electron micrographs of pelleted material from the α -mano-

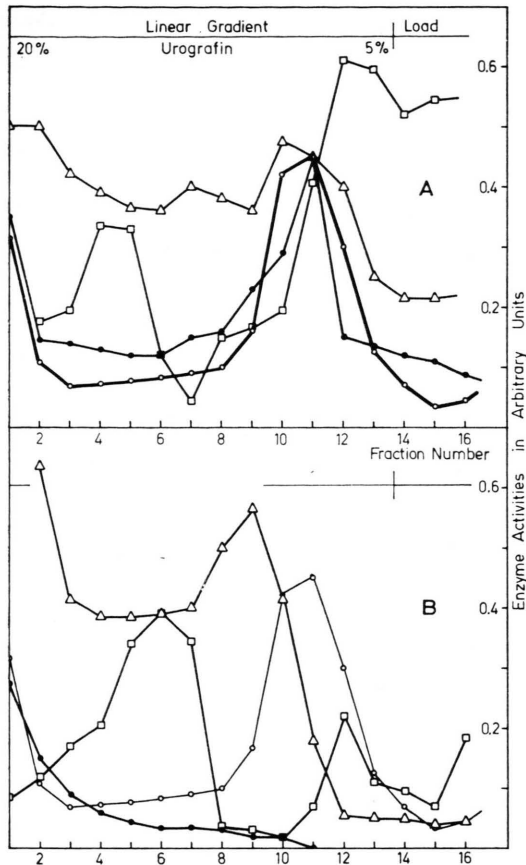


Fig. 1. Distribution of various enzyme activities present in sediment 2 in density gradients of Urografin. A. Distribution of α -mannosidase (\bigcirc — \bigcirc), NADH-DIP oxidoreductase (\bullet — \bullet), β -glucosidase (\square — \square) and ATPase (\triangle — \triangle). B. Distribution of α -mannosidase (\bigcirc — \bigcirc), NADPH-cytochrome c-oxidoreductase (\square — \square), succinate dehydrogenase (\bullet — \bullet) and RNA (\triangle — \triangle). The gradients were loaded with particles present in sediment 2 (obtained between 30 min $20\,000\times g$ and 60 min $50\,000\times g$) and centrifuged for 2 hours.

sidase peak demonstrate the presence of both, membrane vesicles and ribosomes forming two distinct layers in the pellet (Fig. 4 A *). In order to induce a further sedimentation of free ribosomes the same gradient system was now centrifuged for prolonged periods of time. The result was unexpected as not only the RNA but also the α -mannosidase activity moved towards higher densities of Urografin; after 12.5 hours of centrifugation both, RNA and α -mannosidase were contained in the sediment. This result suggested that a separation could possibly be achieved by taking advantage of the different sedi-

mentation velocities of free ribosomes and tonoplast fragments.

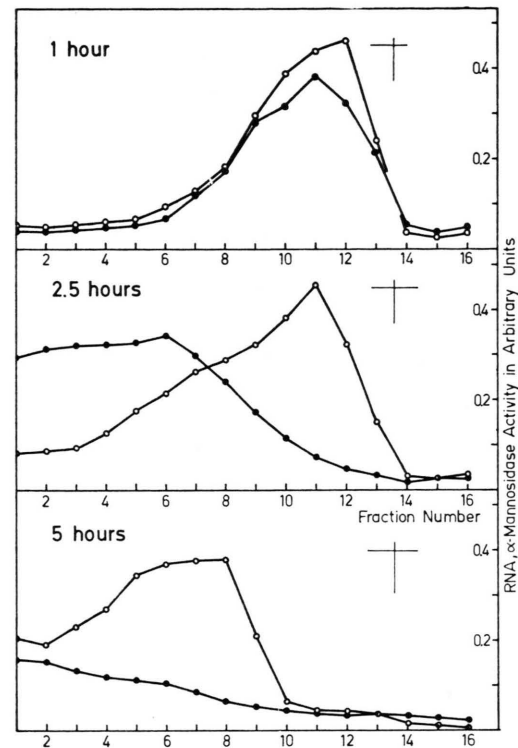


Fig. 2. Distribution of α -mannosidase containing membranes and RNA in density gradients of Urografin after 1, 2.5, and 5 hours of centrifugation. α -Mannosidase: \bigcirc — \bigcirc , RNA: \bullet — \bullet . The gradients (5–15% Urografin) were loaded with particles present in sediment 2 obtained between $20\,000\times g$ (30 min) and $50\,000\times g$ (60 min).

An attempt to follow the sedimentation by analyzing gradients after various periods of centrifugation has yielded the results illustrated in the Fig. 2. After 1 hour the peaks of RNA and mannosidase had roughly the same position in the gradient; however, after 5 hours the enzyme was present in a relatively broad band in the region of ca. 13% Urografin whilst the bulk of RNA had sedimented. It was now clear that the isolation and purification of tonoplast fragments had to be based on a sedimentation velocity cut. In addition, it seemed necessary to eliminate the bulk of free ribosomes by modifying the initial differential centrifugation. As shown in Table III B this could be achieved by reducing the duration of the second centrifugation at $50\,000\times g$ from 60 min to 20 min. As a consequence the yield of α -mannosidase is re-

* Figs 4 A and 4 B see Table p. 420 a.

duced from 42% to 18% of the total activity. The high specific activity in sediment 2 as well as the low RNA content justify, however, the loss of quan-

titative yield. Using this material the distribution curves in density gradients centrifuged for 5 hours depicted in the Fig. 3 were obtained.

Thin sections from pelleted particles present in fractions 5–7 (Fig. 3) demonstrate the absence of a layer of free ribosomes. The comparatively homogeneous preparation consists largely of membrane vesicles with diameters ranging from 0.1 to 0.4 μm (Fig. 4 B). Whether ribosomes that can still be detected in the preparation are free or bound to the membranes is unknown. In any case Wiemken⁹ has been able to observe polysome-like structures in electron micrographs of isolated vacuoles which had been stained with uranyl acetate. Interpretations of data produced upon the biochemical analysis will have to take into account that the preparation may be contaminated as suggested by the presence of structured material in some of the membrane vesicles.

The specific activities of enzymes estimated in the pelleted membrane fragments are listed in the Table IV. α -Mannosidase activity in the isolate is about 12 times higher than in the cell free extract. However, only this marker enzyme is concentrated to such an extent in the final preparation. RNase is completely absent; as mentioned before it is localized in the cell sap. In contrast, β -glucosidase which is known to be localized in the extracellular space as well as in vacuoles and secretory vesicles² is present in the isolate at a comparatively high specific activity, whereas invertase whose vacuolar localization has also been reported¹⁴ has been largely eliminated in the course of tonoplast isolation. Unspecific aggregation of these hydrolases, which are both predominantly localized extracellularly, with isolated membranes is rather unlikely since α -glu-

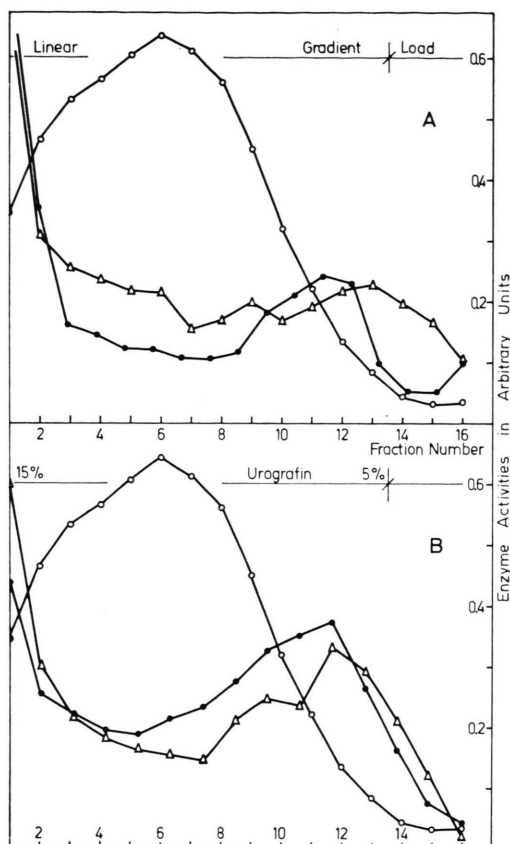


Fig. 3. Distribution of various enzyme activities present in sediment 2 in density gradients of Urografin. A. α -Mannosidase (\circ — \circ), NADH-DIP oxidoreductase (\bullet — \bullet) and ATPase (\triangle — \triangle). B. α -Mannosidase (\circ — \circ), β -glucosidase (\bullet — \bullet) and invertase (\triangle — \triangle). The gradients were loaded with particles present in sediment 2 (obtained between 10 min $20\,000\times g$ and 20 min $50\,000\times g$) and centrifuged for 5 hours.

Table IV. Specific activities of enzymes present and absent in preparations of isolated tonoplasts. Specific activities are calculated on the basis of 1 mg protein. Sediment 2 was obtained between $20\,000\times g$ (10 min) and $50\,000\times g$ (20 min). Isolated tonoplasts correspond to fractions 5–7 (Fig. 3) pooled from several gradients.

Enzyme	Unit of activity	Cell free extract	Sediment 2	Isolated tonoplasts
α -mannosidase	mmoles mannose/60 min	0.0503	0.202	0.616
RNase	$\Delta E_{260\text{ nm}}/60\text{ min}$	1.432	0.508	0
invertase	mmoles sucrose/60 min	0.849	0.0187	0.0448
β -glucosidase	$\mu\text{moles glucose}/60\text{ min}$	85.8	32.5	64.9
NADH-DIP-oxidoreductase	$\Delta E_{660\text{ nm}}/\text{min}$	7.25	18.75	10.68
NADH-cytochrome c-oxidoreductase	$\Delta E_{550\text{ nm}}/\text{min}$	3.62	15.85	8.18
ATPase, Mg^{++} dependent	mmoles $\text{PO}_4^{--}/60\text{ min}$	0.327	0.106	0.214
α -glucosidase	mmoles glucose/60 min	1.92	0.111	0
cytochrome c-oxidase	$\Delta E_{550\text{ nm}}/\text{min}$	0.114	0.117	0

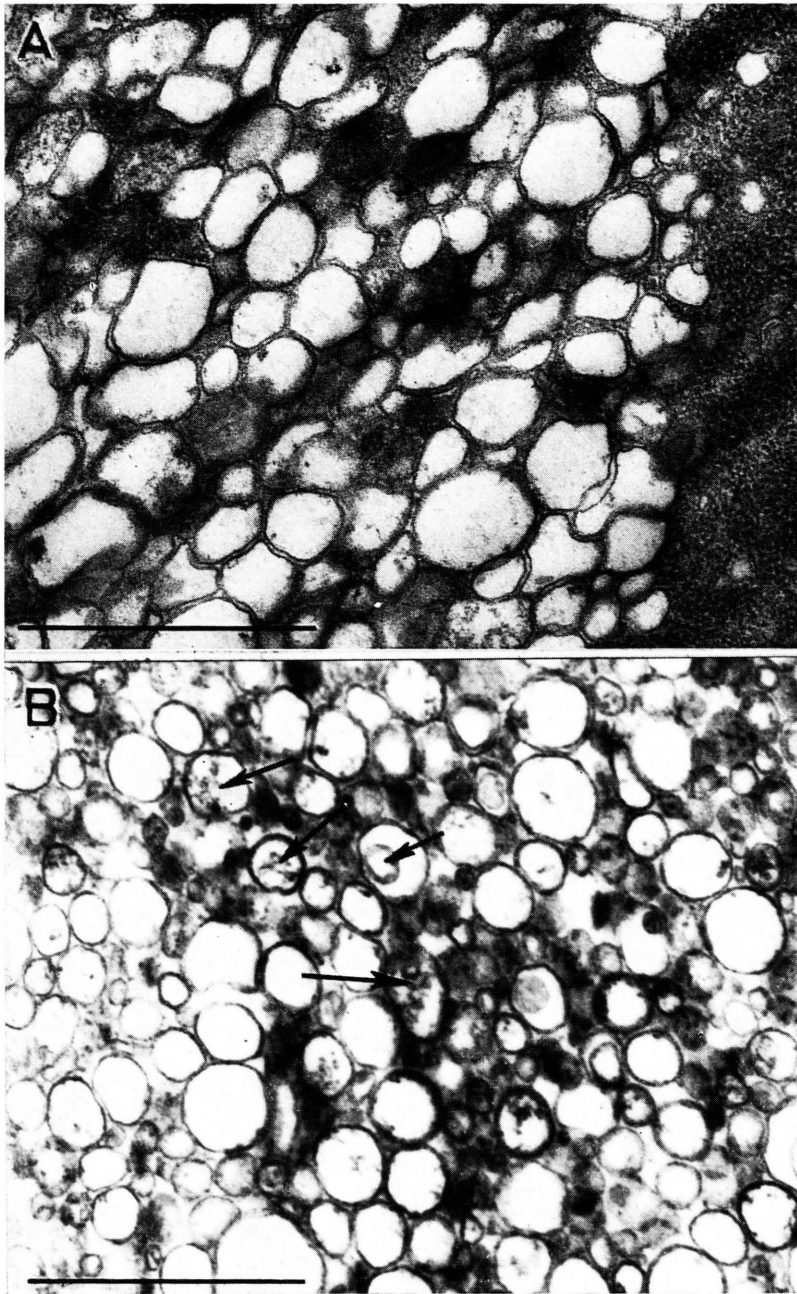


Fig. 4. Electron micrographs of isolated tonoplasts. A. Preparation corresponding to fractions 10 and 11 from gradients such as depicted in Fig. 1. Note the layer of free ribosomes at the centripetal edge of the pellet. B. Preparation corresponding to fractions 5–7 from gradients such as depicted in Fig. 3. Arrows point to contaminating material present within vesicles. The bars indicate 1 μ m.

cosidase, a soluble cytoplasmic enzyme is completely absent from the isolate. On the other hand the distribution curves of invertase and β -glucosidase (Fig. 3) do not suggest a specific association with α -mannosidase marking the position of tonoplast fragments. Still another problem concerns ATPase and oxidoreductases which have previously been detected in preparations of isolated vacuoles^{1,9}. These enzymes are known to be also localized in mitochondria, endoplasmic reticulum and (ATPase only) in plasmalemma. They are present in the isolate at rather high specific activities; nevertheless the distribution curves (Fig. 3) are not decisive. There are certainly several distinct types of membranes containing these enzymes which are present in the preparation used for gradient centrifugation. Contaminating mitochondrial membranes in the isolate can be neglected as documented by the absence of cytochrome oxidase. A membrane containing oxidoreductases bands in the fractions 10–12 (Fig. 3); it is possibly endoplasmic reticulum in nature. It should be mentioned that NADPH-cytochrome c oxidoreductase which has been described as a marker enzyme of the yeast endoplasmic reticulum¹⁵ is practically absent from the isolated membranes (see Fig. 1). Hence, it is possible that the activities of the NADH-dependent oxidoreductases present in this preparation are constituents of the isolated membranes, and this would

correspond with results obtained with isolated vacuoles^{1,9}.

Spheroplasts and vacuoles prepared therefrom seemed to be an unsatisfactory source of vacuolar membranes. Not only would the corresponding isolation procedure be laborious and inefficient, it would also be restricted to the culture conditions necessary for the production of spheroplasts. Studies of the properties of the tonoplast under different conditions of lysosomal activity and metabolic compartmentation would require an isolation procedure of tonoplasts directly from cells avoiding the interfering step of spheroplast formation. The discovery of the marker enzyme α -mannosidase has established the basis for an isolation procedure such as the one described in the present report.

The functional significance of α -mannosidase associated with the yeast tonoplast is unknown. One possibility is indicated by the presence in isolated vacuoles of mannan-proteins such as invertase^{14,16} which are possibly degraded in the lysosomal cell compartment.

This report is dedicated to the memory of the late Miss Ruth Rickenbacher who has performed the electron microscopical work.

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