Purification of Vacuoles from *Neurospora crassa*

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The *Neurospora crassa* vacuole, defined by its content of basic amino acids, polyphosphate, protease, phosphatases, and α-mannosidase, was purified to near homogeneity. The procedure depends upon homogenization of snail gut enzyme-digested cells in a buffer osmotically stabilized with 1 M sorbitol, differential centrifugation of the extract, and sucrose density gradient centrifugation of the organellar pellet. Isopycnic centrifugation of vacuoles in 2.25 M sorbitol-Metrizamide density gradients yielded a peak (density, 1.31 g/cm³) of vacuolar markers coincident with ^{32}P-phospholipids, trichloroacetate-insoluble ^{14}C, and trichloroacetate-soluble ^{14}C. A trail of macromolecular markers in the lighter portions of the gradient reflected, at least in part, heterogeneity of the vacuoles. Almost no contamination by mitochondria or glyoxysomes was detected. Vacuoles were very heterogeneous in size as estimated by velocity sedimentation, but most were larger than mitochondria. Variations of the osmotic strength of the medium were found to alter the equilibrium density of vacuole preparations from 1.06 g/cm³ to over 1.3 g/cm³. This explains the great variation in density reported previously for the "vacuole," the "vesicle," and the "protease particle" of *N. crassa*, all of which appear to be the same entity.

In 1973, Weiss (35) and Subramanian et al. (28) reported the existence of an organelle in *Neurospora crassa* which sequestered basic amino acids. The organelle, called the "vesicle," was more dense than mitochondria (35) and invited comparison with the dense protease-containing particle, later called the vacuole (21), of Matile and co-workers (19). Because Weiss did not detect appreciable protease activity in his early work, and because the vacuole of *N. crassa* was characterized in certain conditions as a rather light organelle by other workers (17), the identity of the *N. crassa* vacuole remained open. In the meantime, Wiemken and his co-workers purified vacuoles from yeasts (11, 38). Although yeast vacuoles are of low density and quite large, they contain most of the cellular basic amino acids, polyphosphates, proteases, α-mannosidase, and other hydrolytic enzymes (39). Recently, we showed (9) that the basic amino acids are located in the same dense organelle as the polyphosphates of *N. crassa*. We report here the purification of the *N. crassa* vacuole, defined by its amino acid and polyphosphate content, and its association with protease, α-mannosidase, and certain other enzyme activities. Purity was assessed by enzyme-marker studies and by a rationale of macromolecule labeling used in the purification of synaptic vesicles (6) of mammals. Osmotically induced density changes were shown to be the cause of the source of variation in density (1.06 to 1.35 g/cm³) reported by previous workers (16, 17, 19, 21) for the *N. crassa* vacuole.

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MATERIALS AND METHODS

Strains, media, and growth conditions. The wild-type strain of *N. crassa*, 74A, was used throughout this work. The basic salt solution (medium N) of Vogel (32) was used as a medium, supplemented with 1.5% sucrose. For experiments involving labeling with $^{32}PO_4^{3-}$ (2 mCi/liter of culture), the unlabeled phosphate was reduced to 5 mM; for experiments involving $[U-^{14}C]$sucrose (0.2 mCi/liter of culture), the unlabeled sucrose was reduced to 0.2%. These conditions supported maximal growth rates for at least one doubling beyond the point at which cells were harvested. Cultures were inoculated with 7-day-old conidia (final concentration, 10^6 per ml) introduced into 1 liter of medium in a 1,000-ml boiling flask. They were aerated at 25°C for about 15 to 16 h, at which time the dry weight was 0.8 to 1.0 mg/ml (10).

Cell disruption. Vacuoles were usually isolated and purified from cells treated with snail gut enzyme to digest cell walls (9, 35, 37). This was followed by gentle homogenization three times in a fractionation buffer consisting of 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonate-NaOH (TES-NaOH), pH 7.5, with 1 mM ethylenediaminetetraacetate and 1.0 M sorbitol as an osmotic stabilizing agent

(9). For comparison in early experiments, cells were disrupted by grinding with sand in a mortar with the same homogenization buffer as above. In a few later experiments, to avoid possible contamination of vacuoles with snail-gut hydrolases, cells were disrupted with a 1.5-min grinding in a mill (Bead-Beater; Bio-Spec Products, Bartlesville, Okla.) with 0.4-mm glass beads. The increase of cell breakage here compensated for the decrease in vacuole survival, although the average size of the surviving vacuoles may have been smaller.

Vacuole purification: ultracentrifuge gradients. Vacuoles were purified as described previously (9). Briefly, homogenates were centrifuged for 5 min at $600 \times g$ to remove cell walls and debris. The supernatants, filtered through Miracloth (Chicopee Mills, Inc., Miltown, N.J.), were centrifuged at 15,000 $\times g$ for 20 min to obtain an organelle pellet (P15000). The P15000 was suspended in 0.2 to 1.0 ml of TES-NaOH buffer and subjected to density gradient centrifugation for 3 h at $100,000 \times g$ in a 30 to 60% (wt/wt; 1.0 to 2.25 M) sucrose gradient. The vacuoles formed a pellet in this gradient and were effectively pure, as shown below. Purity was tested by further centrifugation to equilibrium (4 to 6 h) on continuous sorbitol-Metrizamide gradients (2.25 M throughout, with 2.25 M sorbitol at the top to 1.59 M sorbitol plus 0.66 M Metrizamide at the bottom). All gradient centrifugation was done at 4°C.

Other gradients used were (i) sorbitol-Ficoll (1 M sorbitol with a 0 to 20% Ficoll gradient and a 65% [wt/wt] sucrose cushion); (ii) 5 to 45% sucrose; (iii) sorbitol-sucrose (1 M each; density range, 1.08 to 1.13 g/cm³); and (iv) sorbitol-Metrizamide (uniform molarities; different gradients having from 0.5 to 2.25 M solute throughout). All gradient materials contained 10 mM TES-NaOH (pH 7.5) and 1 mM ethylenediaminetetraacetate.

Labeling. In many experiments, the vacuolar amino acid pool was specifically labeled with [³H]- or [¹⁴C]arginine. Cycloheximide (10 µg/ml of culture) was added 3 to 5 min before the addition of 1 µCi of carrier-free L-[2,3-³H]arginine or L-[*guanidino*-¹⁴C]arginine. At 10 min after the latter addition, cells were harvested and processed as usual. Under these conditions, virtually all label enters the vacuole; only 0.5% is degraded or used for protein synthesis (28). In other experiments, cells were grown in KH₂³²PO₄ or [¹⁴C]-sucrose for whole cell labeling as noted above.

Extraction methods and assays. Polyphosphates, amino acids, and protein were extracted from cells or cell fractions and were processed and determined as described previously (9). In almost all cases, preparations began with about 3 g (dry weight equivalent) of cells grown to that weight in 3 liters of medium. ³²P- or ¹⁴C-labeled cultures, however, were grown to the same density in 500 ml of medium. ³²P-phospholipids were extracted from gradient fractions by the method of Ames (1) by using 0.6 ml of a fivefold-concentrated preparation of a stationary *Escherichia coli* culture per ml of sample as a carrier. Polyphosphate size distribution was determined by gel filtration on a Sephadex G-75 column (1.5 by 30 cm) calibrated with polyphosphates of known chain length (12).

Amino acid analysis of purified vacuoles was done in the laboratory of R. L. Weiss, Department of Chemistry, University of California, Los Angeles. Routine analyses of arginine were done by the Sakaguchi method (31) after purification of samples on Dowex-50 ion-exchange columns (9). Polyamines were determined by Thomas Paulus of this laboratory by an isotope dilution method (25).

The following enzyme activities were assayed as follows: general protease, by the azocoll method (15); succinate dehydrogenase, by the method of Pennington (26); α-mannosidase, by the method of Van der Wilden et al. (30); ornithine aminotransferase, by the method of Weiss and Anterasian (36), except that 0.5% Triton X-100 was included; isocitrate lyase, by the method of G. H. Dixon and H. L. Kornberg (Biochem. J. **72**:3P, 1959), except that the reaction was terminated by addition of an equal volume of 10% trichloroacetic acid; acid phosphatase, by the method of Scott et al. (27); alkaline phosphatase, by the method of Burton and Metzenberg (5), except that 5 mM Mg²⁺ was added; and β-glucosidase, by the method of Wiemken et al. (39).

Estimation of radioactivity. All radioactive samples (0.1 to 2.0 ml) were counted in 15 ml of ACS fluor (Amersham Corp., Arlington Heights, Ill.) in a Beckman LS-230 liquid scintillation counter with quench corrected or standardized. In double-label experiments, the radioactivity of each isotope was determined by the method of Cooper (8).

Chemicals. All chemicals were of reagent grade. Most biochemicals, including β-glucuronidase, Ficoll-400, and straight-chain polyphosphates of chain lengths 5, 15, 25, 35, 45, 65, and 200, were purchased from Sigma Chemical Co. (St. Louis, Mo.). Metrizamide (analytical grade) was obtained from Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y. Isotopes were obtained from New England Nuclear Corp. (³H and ¹⁴C) and from International Chemical and Nuclear Co. (³²P).

RESULTS

Stability, size heterogeneity, and density. The stability of vacuoles in the P15000 (crude organellar pellet) was tested by measuring the sedimentability of arginine and, in some cases, of polyphosphate. Over 80% of the arginine was initially re-sedimentable upon gentle resuspension in fractionation buffer; only 60% was sedimentable after 2 h at 4°C. If the molarity of sorbitol was reduced from 1.0 to 0.7 M, only 22% of the arginine remained sedimentable after 30 min. Most of the loss appeared to be due to lysis of vacuoles, because polyphosphate, a macromolecule, showed the same pattern. In fact, once vacuoles are stabilized at a given osmotic strength, they tend to lyse upon resuspension in lower osmotic strength. Sucrose, sorbitol, Metrizamide, and glycine all stabilized vacuoles, indicating that they were relatively impermeant solutes. Both TES-NaOH (10 mM) and

tris(hydroxymethyl)aminomethane-citrate (10 mM) could be used as buffers; vacuoles were stable over a broad pH range (5.0 to 8.0). However, potassium phosphate and tris-2(*N*-morpholino)ethanesulfonate buffers were somewhat deleterious above pH 6.5.

The sand grinding and snail gut enzyme methods of cell disruption were compared. Sand grinding broke more cells, but only 22% of the arginine liberated was sedimentable; 65% of the arginine liberated by the gentler enzyme method was sedimentable. In neither case was ornithine aminotransferase, a cytosolic enzyme, found in the P15000 (crude organellar pellet). This indicated that the methods removed spheroplasts from this fraction. The populations of vacuoles liberated by the sand grinding and enzyme methods were compared in a double-label experiment. The vacuolar arginine of parallel cultures was labeled with [^{14}C]arginine or [^{3}H]arginine. The cultures were ground with sand (^{3}H) or were homogenized after enzyme treatment (^{14}C). Their low-speed supernatants were combined, and a single P15000 was prepared. The resuspended P15000 was layered on sorbitol-Ficoll gradients and centrifuged for 15 min or 8 h at

100,000 × *g*. The data (Fig. 1) indicate that the enzyme method liberated a population of faster-sedimenting, and thus larger, vacuoles which were almost absent in the sand grinding method. This is consistent with the lower yield noted previously for the latter method. Figure 1 also shows that whereas the size of vacuoles was quite heterogeneous (15-min gradient), the density of vacuoles was high and more homogeneous (8-h gradient). We attribute the fact that increasing amounts of vacuoles penetrated the cushion and pelleted to hydrostatically induced uptake of sucrose (see below).

The size heterogeneity of vacuoles of the P15000 was tested in a gradient in which mitochondria and glyoxysomes were also monitored (Fig. 2). During 1 h of centrifugation, there was a progressive increase in arginine in the pellet at the expense of a trail of vacuolar arginine throughout. By contrast, mitochondria and glyoxysomes sedimented more slowly and more
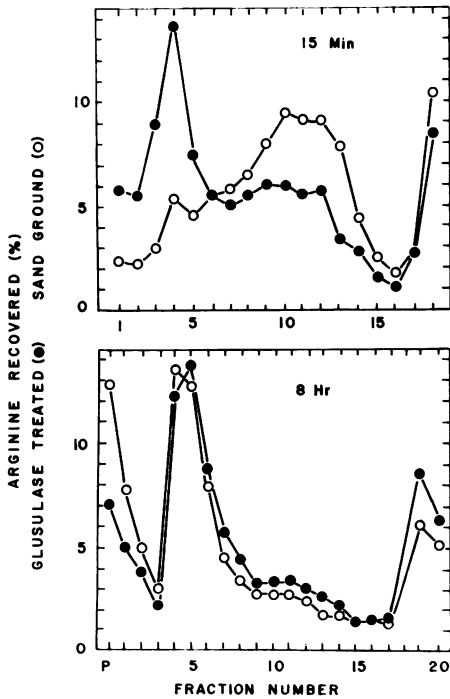

FIG. 1. *Distribution of vacuolar arginine on 0 to 20% Ficoll gradients (with 65% sucrose cushions) containing 1 M sorbitol, after centrifuging for 15 min or 8 h at 100,000 × g. Cells were disrupted by homogenization after snail gut enzyme treatment or by sand grinding. Sedimentation is to the left; P, pellet.*
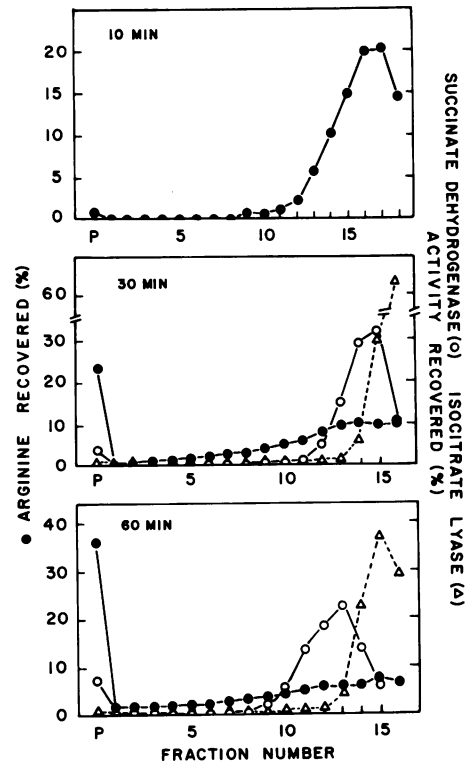

FIG. 2. *Velocity sedimentation of organelles of the P15000 in 1 M sucrose-sorbitol gradients (1.08 to 1.13 g/cm³). Gradients were centrifuged for 10, 30, and 60 min at 4,250 × g. Arginine, succinate dehydrogenase, and isocitrate lyase were measured as markers for vacuoles, mitochondria, and glyoxysomes, respectively. Recoveries are expressed as percentages of the total measured in gradient fractions. Sedimentation is to the left; P, pellet.*

homogeneously, although a slight amount of succinate dehydrogenase was found in the pellet. Most isolated vacuoles, therefore, appeared to be larger than mitochondria in gradients in which their densities were similar.

The effect of osmoticum upon density was tested to determine whether variation among previous reports of vacuolar density (see above) could be attributed to isolation and fractionation methods. Log-phase cells were fractionated as usual, except that the fractionation buffer contained only 0.5 M sorbitol. This sorbitol concentration is not optimal for isolation, but it allowed a test of buoyant density in gradients of all solute concentrations greater than 0.5 M. The P15000 was resuspended in 0.5 M sorbitol buffer, and samples were centrifuged on isomolar sorbitol-Metrizamide gradients of various solute concentrations ranging from 0.5 to 2.25 M. The equilibrium density of vacuoles was 1.065 g/cm^3, considerably less than that of mitochondria (1.122 g/cm^3), in a gradient containing 0.5 M osmoticum (Fig. 3). At the other extreme, vacuoles

were denser (1.212 g/cm^3) than mitochondria (1.152 g/cm^3) in 2.25 M gradients. Thus, the response of vacuoles to osmoticum concentration was more pronounced than that of mitochondria.

When the same experiment was done with vacuoles isolated from cells suspended after enzyme digestion in 1.0 M sorbitol, the density of vacuoles at all osmotic strengths tested was about 0.02 g/cm^3 higher. This may reflect osmotic adjustments in vivo during the enzyme digestion step. Thus, various preparations of vacuoles may have densities ranging from 1.06 to 1.2 g/cm^3; as shown below, the vacuolar density can be as high as 1.3 g/cm^3 if sucrose gradient centrifugation is used to isolate the organelle.

Vacuole purification. Vacuoles were resolved from mitochondria and glyoxysomes by centrifuging the P15000 fraction on 30 to 60% sucrose gradients. The distribution of diagnostic markers is shown in Fig. 4. Except for the sample zone and a slight trail throughout the gradient, all of the arginine was found in the pellet. In contrast, succinate dehydrogenase (mitochondrial) and isocitrate lyase (glyoxysomal) were found in a peak about one-third of the way down the gradient. Less than 0.04% of the recovered activity of these enzymes was detected in the pellet. In a separate gradient, activities of α-mannosidase and protease, enzymes known to be vacuolar in yeasts (39) and in plants (2, 24) were also found in the pellet (Fig. 4). All sedimentable polyphosphate, also vacuolar (13, 29), was present in the pellet (9) (data not shown). Finally, protein was distributed largely in the upper portion of the gradient, coinciding with the turbid mitochondrial band (Fig. 4); less than 5% of the protein was associated with the pellet. Thus, a substantial purification of the vacuoles was effected, with removal of virtually all mitochondrial and glyoxysomal material.

The purity of vacuoles was assessed by a criterion which included isopycnic banding and whole cell labeling to detect unknown organelles for which no marker enzymes could be tested. The sucrose gradient pellets from cells fully labeled during growth with $^{32}PO_4^{3-}$ or [^{14}C]sucrose were suspended in 2.25 M sorbitol and layered on 2.25 M sorbitol-Metrizamide gradients. The distribution of ^{32}P-phospholipids, trichloroacetate-soluble ^{14}C, and trichloroacetate-insoluble ^{14}C, which might reveal contaminating organelles, was compared with the distribution of vacuolar markers. (In all such experiments, considerable lysis of vacuoles takes place as the sucrose pellet is applied to the sorbitol-Metrizamide gradients, because the osmotic strength of 2.25 M sucrose is substantially
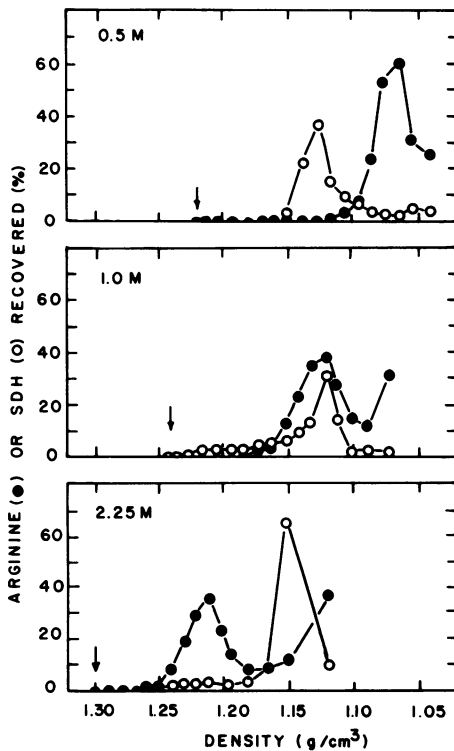

FIG. 3. *Isopycnic density gradient centrifugation of organelles on sorbitol-Metrizamide gradients of different total solute concentration. The arrows designate the bottom of the gradients on the common density scale (abscissa). Arginine and succinate dehydrogenase (SDH) were measured as markers for vacuoles and mitochondria, respectively.*
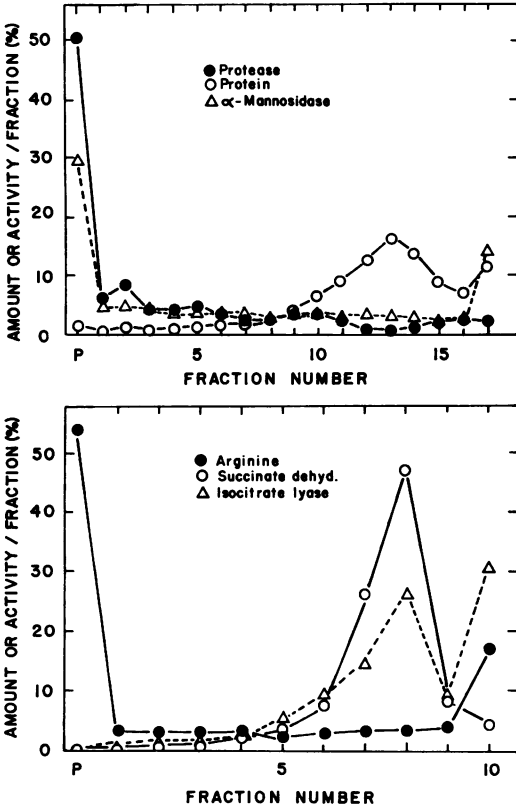
FIG. 4. *Sucrose density gradient (30 to 60%, wt/wt) centrifugation (100,000 × g, 3 h) of the resuspended organellar pellet (P15000). Two gradients, in which different sets of markers were measured, are shown. In the top panel, succinate dehydrogenase was measured, and its peak coincided with that of protein. Sedimentation is to the left; P, pellet.*

greater than that of the 2.25 M sorbitol at the top of the latter gradient. The sample zones of the sorbitol-Metrizamide gradients accordingly contained vacuolar markers. It is formally possible that differential lysis defines two components of a heterogeneous population.)

(i) ^{32}P-phospholipids. The sucrose gradient pellet derived from a ^{32}PO$_4$-labeled culture was applied to a sorbitol-Metrizamide gradient and centrifuged to equilibrium. The distributions of arginine and total ^{32}P in the gradient were superimposable, as reported previously (9). However, because over 96% of the phosphate in the sucrose gradient pellet is polyphosphate, the distribution of ^{32}P-phospholipids was masked. Therefore, the phospholipids of each gradient fraction were extracted and specifically determined. The ^{32}P-phospholipid trailed the arginine peak somewhat, suggesting a contaminant in or a heterogeneity of the vacuole preparation (Fig. 5).

(ii) Trichloroacetate-soluble and trichloroacetate-insoluble ^{14}C. The sucrose gradient pellet derived from a [^{14}C]sucrose-labeled culture was centrifuged in a 2.25 M sorbitol-Metrizamide gradient. The distributions of ^{14}C and arginine were almost superimposable, but the ^{14}C trailed the arginine slightly (data not shown). Each gradient fraction was then treated with cold trichloroacetate (5%, final concentration) and centrifuged for 10 min at 1,450 × g, and the distributions of trichloroacetate-soluble and trichloroacetate-insoluble ^{14}C were determined (Fig. 6). The soluble ^{14}C was superimposable with arginine, consistent with the known high concentration of basic amino acids in vacuoles
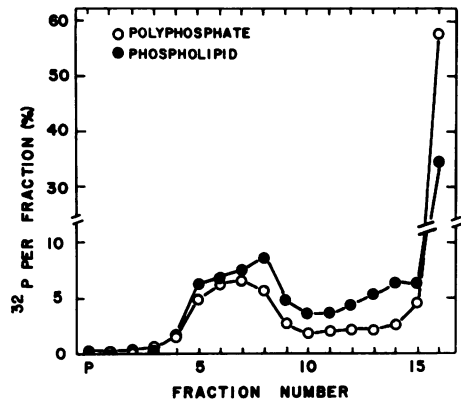

FIG. 5. *Distribution of ^{32}P-polyphosphate and ^{32}P-phospholipid of the sucrose-density gradient pellet in a 2.25 M sorbitol-Metrizamide gradient (100,000 × g, 3 h). As noted in the text, the material in the sample zone is the contents of vacuoles lysed by osmotic shock in the transition from 60% sucrose to 2.25 M sorbitol-Metrizamide. Sedimentation is to the left; P, pellet.*
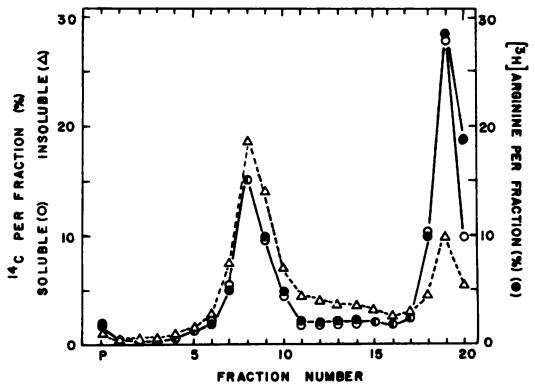

FIG. 6. *Distribution of [^{3}H]arginine, trichloroacetate-soluble ^{14}C, and trichloroacetate-insoluble ^{14}C of the sucrose density gradient pellet in a 2.25 M sorbitol-Metrizamide gradient. Sedimentation is to the left; P, pellet.*

(14, 28, 35). The insoluble ^{14}C was not superimposable upon the arginine peak and was qualitatively similar to the ^{32}P-phospholipid profile in this respect. The existence of a macromolecular carbon component which trailed arginine suggested an impurity which did not include small molecules.

The ^{32}P-phospholipid and trichloroacetate-insoluble ^{14}C data suggested three possibilities. First, a contaminating organelle, somewhat less dense than vacuoles, might be present. Second, vacuolar or other organellar membranes might sediment to a position slightly higher in the gradients than intact vacuoles. Third, some intact vacuoles may be deficient in acid-soluble contents (e.g., polyphosphate and trichloroacetate-soluble ^{14}C). In testing these possibilities, it was reasoned that if the vacuolar markers protease and α-mannosidase trailed the major arginine-containing peak, it would identify the trailing material as vacuolar, at least in part. Further, a test of the sedimentability of these enzymes in hypoosmotically-lysed vacuoles would show whether vacuolar membranes alone would account for the trailing material. This test is valid if, as we show below, a portion of the activities of the enzymes is membrane bound.

The association of α-mannosidase and protease activities with vacuolar membranes was tested. A vacuole preparation from a 30 to 60% sucrose gradient was lysed by suspension in sorbitol-free buffer. The preparation was sedimented on a 5 to 45% sucrose gradient for 7 and 14 h. A peak constituting 17% of the total protease activity and 22% of the α-mannosidase activity was seen at a density of 1.17 g/cm^3. All arginine and all polyphosphate were found at the top of the gradient, together with the remainder of the α-mannosidase and protease activities. The peak at 1.17 g/cm^3 did not change position between 7 and 14 h. The two enzymes at the top of the gradient, however, moved independently and slowly into the gradient, as would be expected of free proteins. The results show that some of the markers were membrane associated.

The standard sorbitol-Metrizamide gradients were used for sedimentation of intact and lysed vacuolar preparations. The lysed material was prepared by suspending a sucrose gradient pellet in fractionation buffer without sorbitol. The arginine, protease, and α-mannosidase of the lysed preparation failed to enter the gradient (data not shown), consistent with the finding above that vacuolar membranes were less dense than the 1.21 g/cm^3 at the top of the gradient. The distribution of the same markers in the intact preparation showed that protease and α-mannosidase trailed the arginine peak (Fig. 7). The
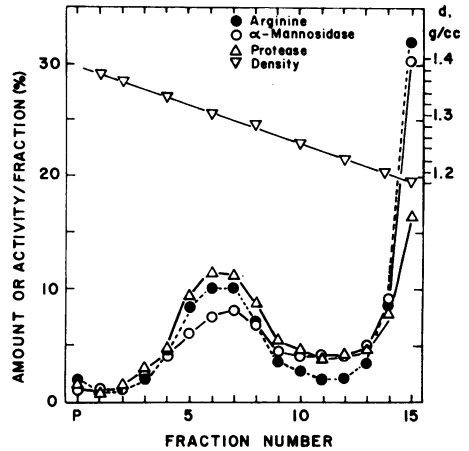

FIG. 7. *Distribution of arginine and two vacuolar markers of the sucrose density gradient pellet in a 2.25 M sorbitol-Metrizamide gradient. Sedimentation is to the left; P, pellet.*

behavior of the enzymes was qualitatively the same as that of the ^{32}P-phospholipids and trichloroacetate-insoluble ^{14}C. Thus, the trailing material cannot be made up wholly of vacuolar membranes or wholly of contaminating organelles. The results demonstrate heterogeneity of vacuoles without, however, proving the absence of contaminants.

Contents. (i) Phosphorus compounds. As noted previously (9), most of the phosphorus in vacuoles of normal mycelia is inorganic polyphosphate. Assays of phosphorus compounds in a purified vacuole preparation (sucrose gradient pellet) identified 96% as inorganic polyphosphate, 2% as inorganic orthophosphate, and 2% as phospholipid. No acid-stable phosphorus, except phospholipid, was seen; no evidence of nucleic acid or nucleotide base was obtained by tests of absorbance at 260 nm or adsorption of phosphorus on Norit A.

The identity and chain length of the polyphosphates were studied. The polyphosphates were heterogeneous on polyacrylamide gels (data not shown) and stained pink, as expected, with toluidine blue. The size distribution of polyphosphates on a Sephadex column indicated that most were in the 3- to 45-U size range (Fig. 8). Some long-chain material was excluded from the column and there was a peak in the tri- to pentaphosphate area. Because the polyphosphate chain length distribution may have been affected by phosphatases of the vacuoles during preparation, the size distribution of these molecules in the living cell cannot be inferred with confidence from these data. The gel filtration results, however, were consistent with the separate determination of chain length by gel elec-

trophoresis noted above. Finally, it should be noted that, at all stages of purification, the arginine/polyphosphate-phosphorus ratio remained constant; neither is selectively lost during isolation of vacuoles (9).
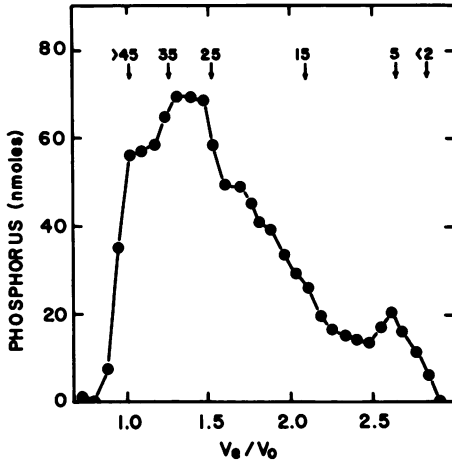

FIG. 8. *Gel filtration (Sephadex G-75, 1.5 by 30 cm) of polyphosphates from vacuoles purified by sucrose density gradient centrifugation. Inorganic phosphate and synthetic polyphosphates of various chain lengths (n = 3 to 200) were used to calibrate the column, and their peak positions are indicated at the top of the figure.*

(ii) Amino acids and amines. Samples of a cell-free lysate, a crude organellar pellet, and vacuoles purified by means of sucrose density gradient centrifugation were analyzed for amino acids. The results (Table 1) showed that only the basic amino acids remained strongly associated with vacuoles throughout purification. Many neutral amino acids may have been associated with the vacuole in vivo, and some proportion of these may retain their association with the organellar pellet (P15000, Table 1). However, the last stage of purification yielded a preparation having significant absolute amounts only of the basic amino acids and perhaps glutamine. The retention of basic amino acids probably reflects a charge interaction with polyphosphate, as noted in previous work (9, 12). Moreover, the disproportionately low recovery of glutamine and alanine in the cell-free lysate probably reflects metabolic changes which occurred during the 30-min glusulase incubation step.

The polyamines putrescine and spermidine were not specifically associated with vacuoles. About 15 to 20% of the polyamines found in the cell-free lysate were found in the organellar pellet; one-third of the organellar polyamines were associated with vacuoles during sucrose density gradient centrifugation. This may have been adventitious, because unlike arginine, more polyamines were found in the mitochondrial

TABLE 1. *Amino acid pools of intact cells, cell fractions, and purified vacuoles (sucrose gradient pellet)*

Amino acid[a]	Whole cell (μmol)	Cell-free lysate[b] (μmol)	Crude organelle pellet (P15000)		Purified vacuoles	
			μmol	% of lysate	μmol	% of lysate
Ornithine	69.7	11.9	6.7	56	2.32	20
Arginine	52.1	10.9	5.8	53	1.55	14
Lysine	12.4	2.0	1.1	53	0.33	17
Histidine	10.5	2.0	1.2	61	0.09	4
Isoleucine	3.9	0.6	0.2	33	0.03	5
Leucine	6.4	1.2	0.4	33	0.06	5
Glutamine	510.0	17.6	1.8	11	0.22	1
Glycine	6.4	2.2	0.4	16	0.03	1
Serine	24.4	1.8	0.4	19	0.03	1
Alanine	393.0	40.5	2.3	6	0.13	<1
Asparagine	13.9	1.1	0.1	11	<0.01	<1
Glutamate	174.0	16.5	0.7	5	0.01	<1
Methionine	8.1	0.6	0.1	20	<0.01	<1
Threonine	12.0	0.9	0.2	25	<0.01	<1
NH₃	93.3	11.1	1.4	13	<0.01	<1
Aspartate	<0.2	—[c]	—	—	—	—
Phenylalanine	<0.2	—	—	—	—	—
Tryptophan	<0.2	—	—	—	—	—
Tyrosine	<0.2	—	—	—	—	—
Valine	<0.2	—	—	—	—	—

[a] Citrulline, proline, and cysteine were not measured, but citrulline and proline are each present in whole cells at one-tenth the level of the arginine pool (14).

[b] Sum of S15000 and P15000.

[c] —, Not detected in cell lysate or later fractions.

than the vacuolar region of the gradient. Polyamines are known to bind nonspecifically to structures with polyanionic components (7).

(iii) Enzymes. Purified vacuoles have α-mannosidase and protease; as noted above, a portion of these enzyme activities was associated with the vacuolar membrane. Three other enzymes were detected in purified vacuoles. The preparations originated with glass bead homogenization; sucrose gradient pellets were placed on 2.25 M sorbitol-Metrizamide gradients, and the gradients, after centrifugation to equilibrium, were analyzed for β-glucosidase, acid phosphatase, and alkaline phosphatase activities. All activities showed a peak coincident with arginine. In contrast to protease and α-mannosidase, a much lesser proportion of the whole-cell activity of these enzymes was found in the vacuole than in other cell fractions. Nevertheless, these activities were definitely associated with the vacuole, and the result was not compromised by use of snail gut enzyme (which contains all three activities) in preparing cell homogenates. Protease and α-mannosidase activities were found, as usual, in such preparations.

DISCUSSION

In this and previous work (9), we have developed simple methods for the extraction, stabilization, and purification of *N. crassa* vacuoles. The steps include digestion of cell walls, high concentration of osmoticum, collection of organelles, and purification of vacuoles in a 30 to 60% sucrose gradient. We have used a relatively labile marker, the soluble arginine pool, in the purification. Our procedure leads to copurification of three other vacuolar markers, inorganic polyphosphate, protease, and α-mannosidase. There was virtually no cross-contamination of the vacuolar pellet of 30 to 60% sucrose gradients with mitochondrial or glyoxysomal markers, and there was no indication of a minor peak of the four vacuolar markers in the mitochondrial band. The vacuolar pellet, upon centrifugation to isopycnic equilibrium in sorbitol-Metrizamide gradients, appeared to be highly purified; little positive evidence of contamination by another organelle was obtained. The possibility of such contamination was not wholly excluded, however.

Our major goals were to purify the vacuole in some form and to give it an operationally valid identity. The first goal was met by showing that all vacuolar markers had coincident peaks, which in turn were coincident with ^{32}P-phospholipids and ^{14}C when vacuoles originated from cells systemically labeled with ^{32}PO$_4^{3-}$ or [^{14}C]-sucrose, respectively, during growth. Trailing of ^{32}P-phospholipid and macromolecular ^{14}C was correlated with trailing of vacuolar enzymes. The purity of the organelle preparation was comparable to or better than that of vacuoles from yeasts (39) or higher plants (2, 20, 24, 33) and approached the purity of the best chromaffin granule (23), synaptic vesicle (3, 6), or lysosome preparations. The identity of the vacuole as such was established by the fact that virtually all of the organellar protease activity detectable with azocoll moved to the sucrose gradient pellet, together with all basic amino acids, polyphosphate, and α-mannosidase activity. All of these components define the vacuole of yeasts, and many are found in vacuoles of higher plants as well (18). The detection of phosphatases and β-glucosidase in the purified vacuole reinforces the point.

The demonstrable heterogeneity of vacuolar size is to be expected from cytological observations. Most vacuoles have polyphosphate granules which become obvious with proper staining (22, 40; C. L. Cramer, unpublished observations). We have not measured the size of isolated vacuoles because the size would be heavily influenced by the osmotic strength, and because vacuoles in the population isolated are just barely visible by light microscopy. It should be emphasized that larger vacuoles, being more vulnerable to breakage during isolation, are doubtless underrepresented even in our best preparations, in which 80% of the vacuolar contents remain sedimentable (9). A previous report by Martinoia et al. (16) reported the isolation of very large vacuoles from a *N. crassa* variant (*slime*) lacking cell walls. By the use of polybase-induced cell lysis techniques, these vacuoles could be liberated in 0.5 M sucrose and purified by flotation. They were 5 to 6 μm in diameter and had a density in these conditions of less than 1.06 g/cm^3. By contrast, our vacuoles, isolated from young mycelia, were less than 1 μm in diameter and had densities which varied greatly with the osmoticum. The contents of the preparation of Martinoia et al. were similar to those reported here.

The identity of isolated *N. crassa* vacuoles has been unclear, owing to the variety of markers studied by various workers and the different particle densities reported (16, 17, 19, 21, 35). We have shown here that vacuolar density varied from 1.06 to over 1.23 g/cm^3, depending upon the gradient medium. Further, when vacuoles were centrifuged in 30 to 60% sucrose gradients, their subsequent equilibrium density in 2.25 M sorbitol-Metrizamide gradients was 1.3 g/cm^3. This point deserves comment because most pre-

vious investigators used sucrose gradients to isolate *N. crassa* vacuoles. The high vacuolar density reported here and previously is due in part to the polyphosphate content, since polyphosphate-depleted vacuoles are quite light (Cramer and Davis, unpublished data). Given the high density of the contents, isopycnic equilibrium is never reached during centrifugation in a sucrose gradient. Vacuolar density will increase continuously because of osmotic water loss as vacuoles move through the gradient and because of the probable entry of the sucrose solution under the hydrostatic pressure characteristic of high-speed centrifugation. Pressure-induced permeability to sucrose is well known in the case of mammalian mitochondria (4, 34). Other reasons for density changes during preparation may also apply, such as changes in the hydration state of polyphosphate and other components of the vacuoles as vacuoles lose water. Therefore, the vacuoles we have purified cannot be expected to have the contents, the permeability, and the physical characteristics of vacuoles newly liberated from living cells. Nevertheless, our data reconcile the findings of others and render the "vesicle" (35), the "protease particle" (19), and the "vacuole" (17, 21) of earlier work synonymous. It is probable that the vacuole of *N. crassa*, like that of yeasts, is the major storage and hydrolytic compartment of the cell (39), and reserves and hydrolases other than those described here are undoubtedly present within it in vivo. Work is in progress to further define the contents and to study the behavior and the roles of the organelle.

ACKNOWLEDGMENTS

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