Analytical Characterization of Beetroot Vacuole Membrane

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ABSTRACT Vacuoles from beetroot (*Beta vulgaris* L. var. *esculenta* Gürke) isolated by a mechanical procedure were osmotically lysed to separate the membrane and sap components for analysis. Approximately 62% of the vacuole proteins, 70% of the nondialyzable carbohydrates and almost all of the phospholipids and sterols were recovered in the membrane fraction. The vacuole membrane had a phospholipid:protein ratio of 0.68 and a sterol:phospholipid ratio of 0.21. 17 complex polar lipids including phosphatides and glycolipids have been tentatively identified. Phosphatidylcholine (54%) and phosphatidylethanolamine (24%) were the most prominent phosphoglycerides besides phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid (1, 4, 5, and 12%, respectively). A putative sulfoglycoside and two major ceramide glycoside-like lipids, resembling those of animal lysosomes, were identified by thin-layer chromatography. High-resolution SDS-acrylamide gel electrophoresis of the polypeptides from the vacuole revealed 15 major bands with apparent molecular weights ranging from 91,000 to 12,000. Selective elution experiments delineated those polypeptides that were peripheral membrane proteins or sap proteins adsorbed to the membrane, and those that exhibited hydrophobic interactions with the lipid core. Lectin labeling results indicated that most of the polypeptides from the membrane and from the sap were glycoproteins probably of the high-mannose type characteristic of lysosomal enzymes that have undergone several stages of posttranslational modification.

Vacuoles are by far the largest membrane-bounded organelles of most mature plant cells. They play a role in osmotic regulation, space filling, deposition of metabolic products, and the compartmentation of hydrolytic enzymes (see references 24 and 25 for reviews). Many of these properties depend on the vacuole membrane to compartmentalize the exoplasmic space, to act as attachment sites for enzymes, or to perform specialized functions such as transport, binding, or release of ions and macromolecules. Because the molecular organization of the membrane is basic to these mechanisms, we have analyzed the major components of the vacuole periphery. This paper reports our initial results.

MATERIALS AND METHODS

Fractionation of Vacuoles

Vacuoles from the root storage tissue of red beets (*Beta vulgaris* L. var. *esculenta* Gürke) were prepared according to a procedure modified from Leigh and Branton (17). The modified procedure yielded more vacuoles, and involved changes in the number of cuts, volume of collection medium, temperature, method of filtration, and the final centrifugation conditions. In this modification, 500 g of fresh, minced tissue were sliced into 500 ml of ice-cold collection medium (1.0 M sorbitol, 5 mM EDTA, 25 mM β-mercaptobenzothiazole, 50 mM Tris-HCl buffer, pH 7.6), using a motor-driven tissue slicer (17) operated at 90 rpm. All subsequent operations were at 4°C. The collection medium containing vacuoles was filtered through a stainless steel sieve (570-μm holes, 240-μm wire diameter), and the tissue retained by the sieve was resliced at 90 rpm, using 500 ml of fresh collection medium. After filtration, the slices were cut a third time, using 500 ml of fresh medium, and filtered. The trice-sliced tissue was discarded and the three 500-ml portions of filtered brei were pooled and divided among six tubes and centrifuged at 2,000 g for 10 min at 4°C in a Sorvall GS3 rotor (Du Pont Co., Sorvall Biomedical Div., Wilmington, Del. The supernatant solutions were discarded and the pellets containing the vacuoles were each resuspended in 2.5 ml of 15% (wt/vol) Metrizamide (Accurate Chemical & Scientific Corp., Hicksville, N. Y.) in isolation medium (1.5 M sorbitol, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6) and pooled together before filtration through one layer of Miracloth (Chicopee Mills, Inc., Milltown, N.J.) premoistened with 15% (wt/vol) Metrizamide in isolation medium. The filtrate (16 ml) was divided into four SW40 rotor tubes (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and overlayered first with 5 ml of isolation medium containing 9% (wt/vol) Metrizamide and then 4 ml of isolation medium without Metrizamide. The discontinuous gradients were centrifuged for 35 min at 100,000 g in a slowly accelerated and very slowly decelerated Spinco SW40 rotor in a Sorvall OTD-65 ultracentrifuge operated on C-slow acceleration and reograd deceleration modes. This centrifugation procedure yielded a larger proportion of small vacuoles than the slower centrifugation (15 min at 650 g) used in the original Leigh and Branton (17) method. After either centrifugation the vacuoles were found as a sharp band at the interface of the 0% and 9% Metrizamide layers. The purity of the final vacuole fractions was assessed according to the criteria of Leigh and Branton (17). Because the preparation obtained by our modified procedure was of equal

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or better purity but higher yield than that obtained by the Leigh and Branton (17) procedure, it was used for the experiments reported in this paper. Vacuoles (1.1 mg protein/ml) were either used directly or resuspended in isolation medium and washed once by sedimentation at 3,500 *g* in a Sorvall SS-34 rotor for 10 min.

Subfractionation of Vacuoles into Membrane and Sap Components

Freshly prepared vacuoles (440 μg of protein in 400 μl) were lysed by hypotonic treatment in 10 ml of 10 mM Tris-HCl, pH 7.6, buffer containing 1 mM EDTA, followed by one cycle of freezing and thawing. The volume was finally brought to 35 ml with the hypotonic buffer and the vacuole membranes were sedimented at 39,000 *g* for 30 min in a Sorvall SS-34 rotor without braking. Pink supernates containing the released vacuole sap were removed, dialyzed against deionized water, lyophilized, and finally solubilized for SDS-polyacrylamide gel electrophoresis. The pellets of vacuole membrane were extensively drained and directly solubilized for electrophoresis or resuspended by vigorous vortexing in 700 μl of the appropriate elution solution for extraction.

Elution Treatment

Extractions included the treatment of the vacuole membranes for 30 min at 37°C in a reciprocal shaking bath with deionized water, 5 mM phosphate buffer, pH 8.0, containing 25 mM NaCl, 10 mM Tris-HCl buffer, pH 6.5, containing 1 mM EDTA and either KCl (0–1.5 M) or urea (0–8 M). Membranes were also extracted for 20 min with ice-cold 0.5% (wt/vol) Triton X-100 buffered at low and moderate ionic strength. DFP[1] (0.1 mM) was included when specified. The extracted membranes were recovered by sedimentation at 39,000 *g* for 30 min in a Sorvall SS-34 rotor without braking. The pellets were directly solubilized for SDS-polyacrylamide gel electrophoresis. Membranes were also extracted with ice-cold 10 mM Tris-HCl containing 1 mM EDTA, pH 7.6, or with water adjusted with NaOH to pH 9–13. The membranes were pelleted immediately after extraction and the pellets treated as above. All supernates were centrifuged at 135,000 *g* for 60 min in a Spinco SW 50.1 rotor to clear them of any possible membrane remnants; pellets were never discernible. The resulting supernates (700 μl) were dialyzed overnight against 5 mM Tris-HCl, pH 6.8 containing 0.02% (vol/vol) β-mercaptoethanol, and then lyophilized before solubilization for gel electrophoresis.

Extraction and Determination of Main Lipid Classes

Packed vacuole membranes (200–400 μl) were extracted at room temperature with 4 ml chloroform-methanol (2:1, vol/vol) for 10–30 min with intermittent mixing. The chloroform-methanol was partitioned with 0.8 ml of 50 mM NaCl according to Folch et al. (10). After vortexing, the tube was left for at least 15 min and then centrifuged at 1,500 rpm for 20 min in a clinical centrifuge. The lower phase was collected, transferred to a new tube, and dried under a stream of nitrogen gas at 30°C. The residual lipids were immediately dissolved in chloroform, made up to 500 μl, and analyzed. Total lipid phosphate was assayed by the procedure of Bartlett (2).

The lipids were separated and identified by two-dimensional thin-layer chromatography on 20- × 20-cm glass plates precoated with silica gel G, 250 μm thick, and activated at 110°C for 30 min before use. For the detection of polar lipids, chloroform-methanol-ammonia (65:25:5, vol/vol) was used for chromatography in the first dimension, and chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5, vol/vol) was used in the second dimension. The less polar lipids, which migrated with or near the front in this system were subfractionated using petroleum ether (bp, 40°–60°C)-diethyl ether-acetic acid (75:25:1, vol/vol) as the developing solvent. Because it was impossible to accomplish complete separation by using this single solvent, the chromatograms were developed to the midline in chloroform, then in hexane-ether (85:15, vol/vol) to the top; in chloroform-methanol-ammonia (65:25:5, vol/vol) to the midline, then in chloroform to the top or in chloroform-methanol-ammonia (65:25:5, vol/vol) to the first third, then in chloroform to the second third, and finally in hexane-ether (85:15, vol/vol) to the top (21).

The following nondestructive procedures were used to detect lipid classes on TLC plates: (*a*) The plate was sprayed with 0.003% (wt/vol) rhodamine 6G in 1 N sodium hydroxide and viewed while wet under UV (predominantly 366 nm) light; the individual lipid classes appeared as fluorescent areas. (*b*) The plate was placed in a closed container of saturated iodine vapor; the lipid stained yellow after a few minutes. After either procedure the lipid could be scraped from the plate and recovered for further analysis. For destructive detection of lipids, the plates were sprayed with 5% (vol/vol) sulfuric acid in 95% (vol/vol) ethanol and then heated at 180°C for 30 min. Lipids as well as other nonvolatile organic compounds formed dark brown spots.

Individual lipid classes were tentatively identified by comparison of their R_f values with those of authentic standard lipids (Supelco Inc., Bellefonte, Pa.) chromatographed in the same conditions (13) and by tests for phosphate (38), choline (Dragendorff stain [4]), NH-group (13), sterols, and sterol esters (18); the OsO_4 test for unsaturated double bonds; and the ninhydrin test for amino-lipids (22, 28). Schiff reagent was used to test for aldehydes either directly or after mild $HgCl_2$-oxidation (31), or after sodium metaperiodate oxidation (29). Glycolipids were characterized by the periodate-Schiff stain and by the orcinol (33), diphenylamine (39), α-naphthol (30) and resorcinol tests (34).

Because sterols were present in the 100 ng range, microanalysis was carried out by use of quantitative thin-layer chromatography according to Fisher (9), except that the plates were sprayed with 2 mM ferric chloride in 5% H_2SO_4/5% acetic acid in distilled H_2O (vol/vol). Samples and at least six standards, each containing 100–800 ng pure ergosterol, were run on the same plate. Charred spots were scanned with a recording microdensitometer (Joyce-Loebl & Co., Ltd., Gateshead, England) and the optical density peaks were integrated.

Polyacrylamide Gel Electrophoresis

Total protein was determined in duplicate aliquots by the method of Lowry et al. (19), using BSA as a standard. When betalains were present, the protein was first precipitated with ice-cold, 10% (wt/vol) TCA, washed in methanol, and redissolved in 1 N sodium hydroxide before assay. Polypeptides were analyzed by SDS-polyacrylamide gradient gel electrophoresis, using the discontinuous SDS-Tris-glycine buffer system of Laemmli (16) and 1.5-mm-thick slab gels with 12 wells (1.2 × 0.7 cm) in a vertical apparatus. The stacking gel (3 × 16 cm) was 3% (wt/vol) polyacrylamide and the resolving gel (11 × 16 cm) was a 5–15% (wt/vol) linear acrylamide gradient generated by a mixer connected to a peristaltic pump. Solutions were made up according to Maizel (20) with these modifications: the ammonium persulfate concentration was reduced to 0.02% (wt/vol) to slow the polymerization in the resolving gel and increased to 0.3% (wt/vol) in the stacking gel for polymerization within 20 min. TEMED (*N,N,N',N'*-tetramethylethylenediamine) was raised to 0.1% (vol/vol) and Tris-HCl buffer made up to 125 mM in the resolving gel, according to Weber and Osborn (40). Sucrose (10%, wt/vol) was added to the buffer containing the higher concentration of acrylamide used for the gradient. The Tris-HCl electrode buffer was diluted to 25 mM.

To reduce the amount of staining at the top of the resolving gel and the background staining along the electrophoretic path of membrane polypeptides, we tested the following procedures: (*a*) solubilization in sample buffer (10% vol/vol glycerol, 3% wt/vol SDS, 10 mM Tris HCl, 0.025% wt/vol bromophenol blue) in the presence or absence of 3% (wt/vol) DTT, 5% (vol/vol) β-mercaptoethanol or 2 mM EDTA, separately or in combination; incubations at 37°C for 60 min, 60°C for 20 min, or 90°C for 2 min; sonication for 5–10 s at 20 W; (*b*) solubilization in guanidine-hydrochloride followed by alkylation to ensure the complete denaturation of the proteins and rapid inactivation of any proteases (40); (*c*) performic acid oxidation of the sample before lyophilization and solubilization; (*d*) pretreatment of the sample by DNase at 37°C for 30 min. The background staining was never completely abolished when undelipided membranes were used. Background staining was considerably reduced when membrane proteins were precipitated with 10% (wt/vol) TCA before solubilization, but this procedure also caused irregular losses of membrane polypeptides. The following procedure was finally adopted: The sample derived from 0.5 ml of purified vacuoles was solubilized in 100 μl of sample buffer containing 3% (wt/vol) DTT, 5% (vol/vol) β-mercaptoethanol, 2 mM EDTA and sonicated (20 W, 5 s). After 20 min of incubation at 60°C, the mixture was cooled to room temperature for immediate use or stored at −70°C.

Dilute extracts of soluble proteins were mixed with aliquots of SDS buffer, lyophilized, and made up to appropriate volume with deionized water before electrophoresis.

Electrophoresis was carried out at a constant current of 25 mA until the tracking dye was 1 cm above the bottom of the gel (5 h). Gels were fixed and stained in 0.1% Coomassie Brilliant Blue R-250 in 25% (vol/vol) 2-propanol with 10% (vol/vol) glacial acetic acid for 6–8 h followed by a final wash in 10% (vol/vol) acetic acid. Densitometric tracings of stained gels were made through an orange filter by use of the recording microdensitometer. Phosphorylase *a*, BSA, ovalbumin, carbonic anhydrase and cytochrome *c* were used as molecular weight standards, in addition to human erythrocyte ghost membranes prepared according to Dodge et al. (6). The molecular weights of the erythrocyte membrane components were assumed from the data of Steck (32).

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; Con A, concanavalin A; DFP, diisopropylfluorophosphate; DTT, dithiothreitol; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; PAS, periodic acid-Schiff; RCA I and II, *Ricinus communis* agglutinins I and II; TLC, thin-layer chromatography; WGA, wheat germ agglutinin.

Quantification of Glycoconjugates

The vacuole membrane and the TCA-precipitate from the vacuole content were dialyzed overnight against 1 mM Tris-HCl, pH 6.8, containing 0.1 mM EDTA, and the amount of carbohydrate in the glycoconjugates was determined in each subfraction by the colorimetric method of Dubois et al. (8).

Identification of Vacuole Glycoproteins in SDS-Acrylamide Gels

Gels were stained with PAS reagent for the detection of glycoproteins according to Glossman and Neville (11). Stained gels were scanned on the densitometer and photographed through a Kodak no. 61 green filter.

Vacuole glycoproteins containing lectin-binding sites were detected directly in the gels after electrophoresis by a procedure similar to that described by Rodriguez-Boulan et al. (27). Individual lanes from the slab gels containing the separated proteins were fixed overnight in 25% (vol/vol) isopropanol with 15% (vol/vol) glacial acetic acid and thoroughly washed in 50 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$ when appropriate. A final wash (30 min) contained the appropriate inhibitor sugars in the controls. The gel lanes were then incubated for 60–90 min in the same buffers containing one of the following FITC-conjugated lectins (1.66 mg/ml, Vector Laboratories, Inc., Burlingame, Calif.,): Con A with or without 260 mM α-methylmannoside, WGA with or without 226 mM *N*-acetylglucosamine, RCA I or II with or without 280 mM galactose. After incubation, the lanes were extensively washed in the appropriate buffers containing, in the controls, the inhibitor sugars. The rate of destaining was monitored under UV light. When all the free lectins were washed out, the gels were photographed over a UV light box (C-62 Transilluminator, Ultra-Violet Products, Inc., San Gabriel, Calif.) through a Wratten 61 barrier filter (Eastman Kodak Company, Rochester, N. Y.), using Kodak SO-115 emulsion. Photographic negatives were scanned with the microdensitometer.

Electron Microscopy

Approximately 200-μl pellets were fixed for 45 min at 4°C with 3% (vol/vol) glutaraldehyde–1.5% (wt/vol) paraformaldehyde in 50 mM cacodylate buffer, pH 7.3, containing 0.05% (wt/vol) $CaCl_2$. Fixed samples were either sedimented (27,000 *g*, 15 min, Sorvall SM24 rotor) before mixing with an equal volume of 1% (wt/vol) agar or collected on a filter (Millipore Corp., Bedford, Mass.) according to Baudhuin et al. (3). The samples were washed twice in 100 mM cacodylate buffer, pH 7.3, for 10 min total, then washed again in acetate-Veronal buffer for 10 min and postfixed for 1 h with 1% (wt/vol) OsO_4 in acetate-Veronal buffer, pH 7.4, at room temperature. After two washes in the corresponding buffer for 10 min, the samples were stained with 1% (wt/vol) uranyl acetate in acetate-Veronal buffer, pH 5.2, for 1 h in the dark, dehydrated in ethanol, and embedded in an Araldite-Epon mixture. Thin sections were mounted on Formvar-carbon-coated grids, stained for 5 min in alkaline lead citrate, and examined with a Philips 301 electron microscope at 60 or 80 kV.

RESULTS

Vacuole Fraction

Because we used a modification of the Leigh and Branton (17) isolation procedure, we examined the vacuole fraction extensively by phase microscopy and spectrophotometry to ascertain the amount and purity of the isolated vesicles. Microscope observation of the fraction showed a large number of highly pigmented vacuoles ranging from 40 μm in diameter down to the resolution limit of the microscope. Unpigmented particles were also visible but nuclei were rarely seen. Spectrophotometry showed that when alcian blue (0.16%, wt/vol) was added to the collection medium, it remained in the sample layer at the bottom of the gradient and mixed with the soluble betalains released by the vacuoles broken during isolation. Alcian blue did not appear in the vacuole fraction, indicating that the vacuole fraction was not grossly contaminated by broken membranes that had resealed around the collection medium during the slicing and isolation procedures.

The pellet obtained from the band at the 0–9% Metrizamide interface (Fig. 1 *a*), consisted primarily of vesicles ranging in size from 20 to 0.2 μm, limited by a smooth, unit membrane ~7 nm thick (Fig. 1 *a*, *inset*). Their content was practically clear although betalains could be seen in the light microscope just before glutaraldehyde fixation. These pigments were released during fixation. Many of the large spherical vacuoles enclosed smaller vesicles. Ring-shaped and more complicated profiles were sometimes encountered. Thus, the isolated vesicles resembled the vacuoles seen in intact cells, although large spherical vacuoles were much less frequent than in the original tissue, regardless of whether we used the original Leigh and Branton (17) method or our modified procedure. The vacuole fraction obtained by either procedure included few contaminants: (*a*) some partially swollen mitochondria, (*b*) a very few bodies with a dense, angular core (presumably microbodies), (*c*) small vesicles with a thick electron-dense limiting membrane perhaps derived from intracellular, membrane-bound phenolics, (*d*) a few vesicles derived from rough ER, and (*e*) a few large vesicles with a thick unit membrane enclosing cytoplasmic remnants, possibly fragments of plasmalemma resealed around bits of cytoplasm. The vacuole membrane fraction (Fig. 1 b and *inset*) obtained by lysing the vacuoles in buffer contained similar contaminants in roughly the same amounts as in the vacuole fraction. Conversely, the pellet at the bottom of the Metrizamide gradient contained cell wall debris, starch grains, nuclei, plastids, mitochondria, microbodies, and membranes probably derived from plasmalemma, ER, and Golgi apparatus (Fig. 1 *c*). These findings corroborate the enzymatic assays of Leigh and Branton (17), which we also confirmed for our preparations. For example, in the vacuole fraction we found relative specific activities of 8.5, 1.2, and 0.6 for betanin, NADH cytochrome *c* oxidoreductase, and cytochrome *c* oxidase, respectively, using the Leigh and Branton method and 8.2, 0.9, and 0.5 respectively for these same markers, using our modified method. Because preliminary experiments showed that the vacuole membrane fraction obtained by lysing the vacuoles equilibrated as a single band when centrifuged in a variety of sucrose gradients, such additional density gradient centrifugation was not used. We conclude that the preparation obtained by our modified procedure was a reasonably representative and pure vacuole fraction.

The composition of the vacuole fraction is given in Table I. The intravacuolar distribution of materials was investigated by osmotically disrupting the vacuole into its sedimentable membrane and its soluble, nonsedimentable content. About 62% of vacuole proteins, 70% of nondialyzable carbohydrates, and almost all of the phospholipids and sterols were recovered in the membrane fraction, which had a phospholipid:protein ratio of 0.68 and a sterol:phospholipid ratio of 0.21 and thus shared some of the known characteristics of other intracellular membranes.

Lipid Composition

Two-dimensional thin-layer chromatography of lipid extracts of the vacuole membrane fraction and analyses of Rf values and staining behavior led to the tentative identification of 17 lipid classes (Table II). Complex polar lipids included phosphatides and glycolipids. Phosphatidylcholine (54%) and phosphatidylethanolamine (24%) were the major phospholipids besides phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid (1, 4, 5, 12%, respectively). Spots *12*, *14*, and *16* in Table II are minor unidentified phosphatidyl compounds, spots *12* and *16* being present in some, but not all, preparations. Five classes of glycolipids were detected, including two major ceramide glycoside–like lipids

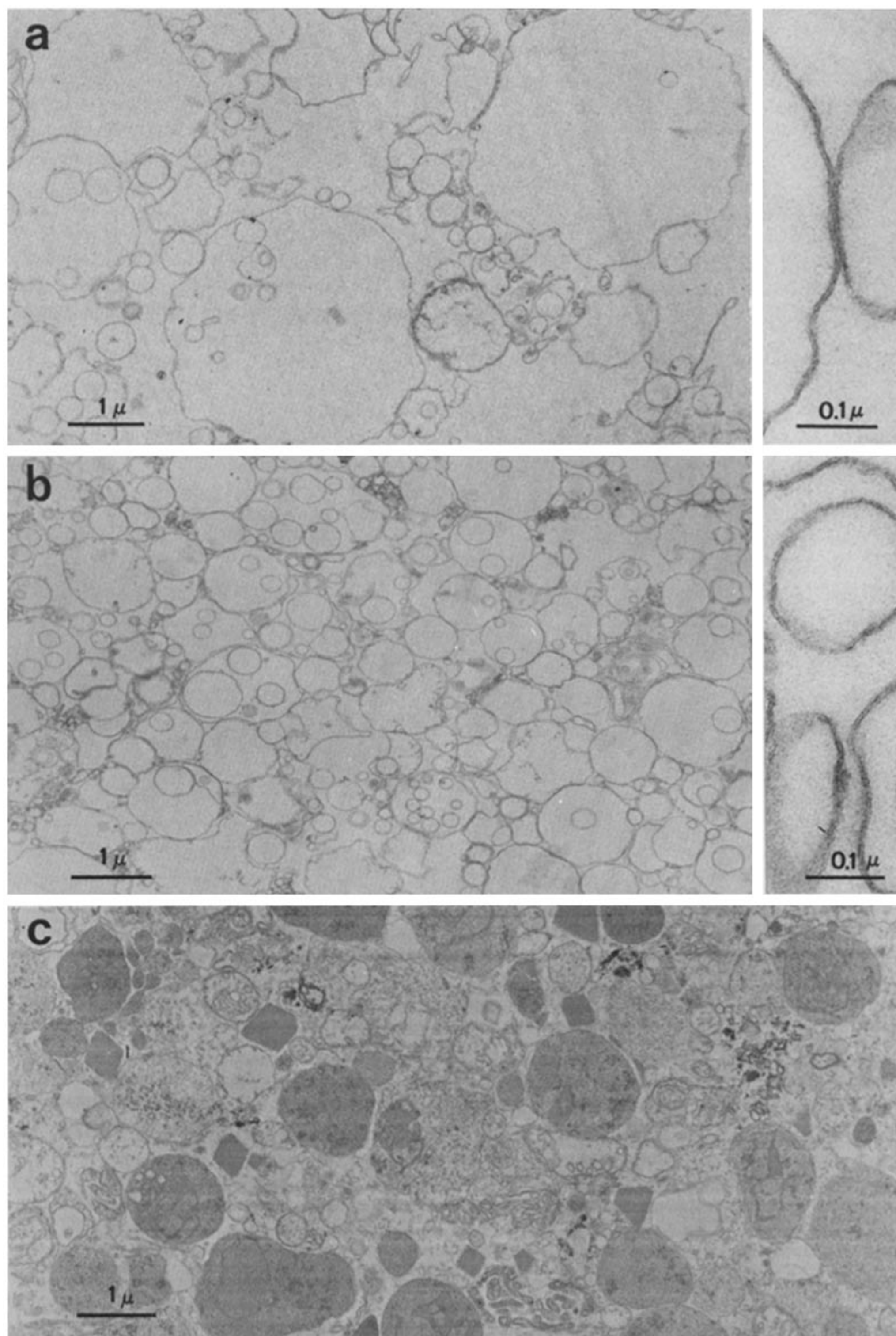


FIGURE 1 Fractions isolated from beetroot. *a*, Vacuole fraction; *b*, membrane fraction obtained by lysing the vacuoles in hypotonic buffer; *c*, pellet fraction, at the bottom of the discontinuous gradient. *a–c*, × 11,250; *insets*, × 119,000.

(spots *9* and *10*) and a major glycoside comigrating with sulfoglycosides in the solvent system used (spot *5*).

The less polar lipids that migrated at, or near, the front in the polar solvent systems were separated and tentatively identified into classes of differing polarities (Fig. 2) by adsorption TLC. Neutral lipids were a minor component of the vacuole membrane compared to the complex polar lipids. Sterols, monoglycerides, and diglycerides were the prominent components of the neutral fraction besides triglycerides, steryl esters, and fatty acids.

Protein Composition

Electrophoretic analysis of the vacuole fraction showed a large number of polypeptides (Fig. 3*A*). Densitometric scans of the electrophoretic gels (Fig. 4) facilitated systematic com-

parison. They were used to analyze all of the electrophoretic data presented in this report although only one set of densitometric scans is actually shown. The numerous bands were grouped into nine domains, each consisting of one to five polypeptides distinguished by their relative mobility and, hence, their molecular weight. Accordingly, 15 major bands and at least 15 minor bands were defined and their molecular weights assigned (Figs. 3 and 4). Except for certain minor variations in the four and six domains, the patterns were remarkably reproducible. A high-mobility band (8.3, designated *M* in Fig. 3) displayed metachromatic staining with Coomassie Brilliant Blue.

To determine whether proteolysis was occurring during vacuole preparation, we harvested vacuoles in the presence of protease inhibitors. Either 0.1 mM phenylmethylsulfonyl fluoride presolubilized in dimethyl sulfoxide or 0.5 mM *N*-carbobenzoxy-α-L-glutamyl-L-tyrosine in 0.1 N sodium hydroxide was added to the collection medium and all subsequent buffers until the vacuoles were finally mixed with the SDS sample buffer, boiled 2 min and immediately loaded for gel electrophoresis (*N*-carbobenzoxy-α-L-glutamyl-L-tyrosine was partially insoluble in collection medium but soluble in all other buffers). The resulting electrophoretic patterns (not shown) were similar to those from vacuoles prepared without protease inhibitors. Because all the polypeptides of the whole vacuole preparations entered the stacking and resolving gels, we believe that the pattern observed is comprehensive.

As expected, the vacuole luminal polypeptide content (sap, Figs. 3 *B* and 4 *a*) was a subset of the entire vacuole (Figs. 3 *A* and 4 *d*). The doublets 1.2–1.3, 3.2–4.1, and 5.1–5.2 were easily identified in the sap, whereas bands 6, 7.1, 7.3, and 8 were also present but in much smaller amounts. The distribution between membrane and sap of some polypeptides, namely 4.3, 5.3, 5.4, 7.2, and 9.2 varied from experiment to experiment but in general the electrophoretic patterns observed for vacuole membrane (Figs. 3 *C* and 4 *b*) were complementary to those of the sap (Figs. 3 *B* and 4 *a*) and the arithmetic addition of the membrane and sap components (Fig. 4 *c*) reproduced the pattern of intact vacuoles (Fig. 4 *d*). The relative abundance of the major polypeptide groups recovered in the luminal content and the membrane fractions is given in Table III.

TABLE I

Gross Composition of Isolated Vacuoles

Constituents	Weight in the membrane*	Fractional distribution in membrane:sap
	mg	
Protein	100	1.70
Phospholipids	58	11.50
Sterols	17.5	15.70
Carbohydrates (nondialyzable)	79	2.20
Betanin	Trace ($<10^{-3}$)	<0.01

* Normalized to 100 mg of protein determined by the method of Lowry et al. (19). Other constituents were measured as described in Materials and Methods.

Extraction at Low Ionic Strength and Increasing pH

Packed membranes derived from 500 μl of purified vacuoles were diluted with 700 μl of ice-cold deionized water adjusted or not with NaOH to pH 9.0, 10.0, 11.0, 12.0, and 13.0 and immediately centrifuged. The pellets obtained after extraction at pH 9.0–11.0 were slightly pink, as were those from membranes washed with the Tris buffer or extracted with H_2O alone. The pellets from membranes extracted at pH 12.0 and 13.0 had a gelatinous colorless aspect. The supernatant fractions were optically clear.

TABLE II

Chromatographic Analysis of Polar Lipids of Vacuole Membrane

Spot no.	R_f values in solvents* (1)	(2)	Staining behavior‡ A	B	C	D	E	F	G	H	I	P	Tentative identity of components
	%											% of total	
1	4.3	37.5	+	–	–	–	–	–	–	–	–	12	Phosphatidic acid
2	7.4	4.4	+	–	±	–	–	–	–	–	–	1	Lysophosphatidylcholine
3	9.9	23.8	+	–	–	–	–	+§	–	–	–	5	Phosphatidylinositol
4	12.4	13.1	+	±	±	–	–	–	–	–	–	1	Phosphatidylserine
5	20.5	48.1	–	–	–	+‖	–	+	+	+	+	0	Sulfoglycoside (?)
6	26.7	15	+	–	–	+	–	–	–	–	–	5	Phosphatidylcholine
7	33.5	36.2	+	+	+	+‖	–	–	–	–	–	2	Phosphatidylethanolamine
8a¶	37.9	38.7	+	–	?	–	–	+	–	–	–	4	Phosphatidylglycerol
b¶	34.2	43.1											
9	42.9	70	–	–	±	–	–	+	+	+	+	0	Ceramide glycoside
10	47.8	67.5	–	–	±	–	±	+	+	+	+	0	Ceramide glycoside
11	52.8	78	–	–	–	–	–	+	+	+	–	0	Unidentified glycolipid
12	60.2	69.4	±	–	–	–	–	–	–	–	–	<1	Unidentified phosphatide
13	64.6	82.5	–	–	–	+‖	–	+	+	+	–	0	Unidentified glycolipid
14	69.6	76.9	±	–	–	–	–	–	–	–	–	<1	Unidentified phosphatide
15	74.6	86.9	–	–	–	+‖	+	+	+	+	+	0	Steryl glycoside
16	83.9	81.9	±	–	–	–	–	–	–	–	–	<1	Unidentified phosphatide
17	85.7	96.2	–	–	–	+‖	+	±	–	–	–	0	Unidentified compound

* Solvents *(1)* chloroform-methanol-ammonia (65:25:5, vol/vol) and *(2)* chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5, vol/vol).

‡ Letters: *A*, phosphate stain; *B*, ninhydrin stain; *C*, NH-group stain; *D*, choline stain; *E*, sterol stain; *F*, PAS stain; *G*, orcinol stain; *H*, α-naphthol stain; *I*, diphenylamine stain; *P*, phosphate content.

§ Phosphatidylinositol gives a yellow color characteristic of compounds giving a malondialdehyde residue on periodate oxidation.

‖ The positive choline test might be attributable to the presence of highly unsaturated fatty acids in this compound.

¶ *a* and *b* refer to subspots on the chromatogram.

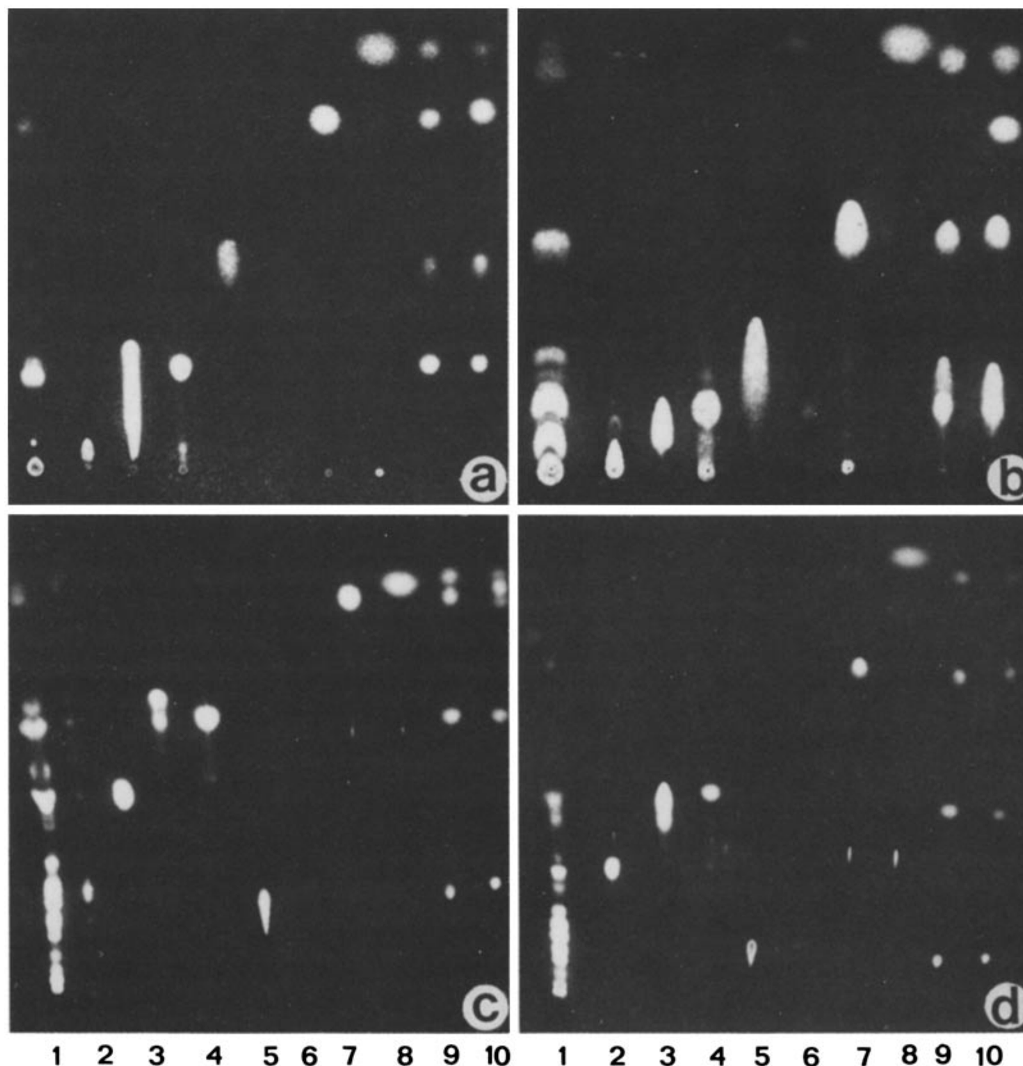


FIGURE 2 Silica gel thin-layer chromatograms for the identification of the less polar lipids from the vacuole fraction. Extract was developed (*a*) in petroleum ether-diethyl ether-acetic acid (75:25:1, vol/vol); (*b*) halfway to the midline in chloroform, then in hexane-ether (85:15, vol/vol) to the top; (*c*) in chloroform-methanol-ammonia (65:25:5, vol/vol) to the midline, followed by chloroform to the top; (*d*) in chloroform-methanol-ammonia (65:25:5, vol/vol) to the first third, then chloroform to the second third, and finally hexane-ether (85:15, vol/vol) to the top. For each chromatogram, the extract was loaded in lane *1*, and commercial standards were run in lanes *2–10* as follows: *2*, monoglyceride (rac-glyceryl-1-monopalmitin); *3*, diglyceride (rac-glyceryl-1,3-dipalmitin); *4*, sterols (plant sterol mixture including brassicasterol, campesterol, stigmasterol, and β-sitosterol); *5*, fatty acid (palmitic acid); *6*, α-tocopherol; *7*, triglyceride (tripalmitin); *8*, hydrocarbon (*n*-hexadecane); *9*, commercial sample A (cholesteryl palmitate, tripalmitin, palmitic acid, and cholesterol); *10*, commercial sample B (cholesteryl oleate, methyl oleate, triolein, oleic acid, and cholesterol).

Although no polypeptides were extracted by washing membranes with 5 mM phosphate buffer, pH 8.0, containing 25 mM NaCl for 30 min at 37°C (Fig. 5 *D* and *E*) or with ice-cold 10 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA (Fig. 6 *A* and *G*), a set of polypeptides was selectively eluted by treating the membranes with deionized water at 37°C for 30 min (Fig. 5 *B* and *C*). Increasing pH at low ionic strength eluted a set of polypeptides similar to those eluted by H_2O (Fig. 6 *B–F* and *H–L*). At pH 11, band 8.3 was eluted but bands 2.2, 3.2, and 6.2 were still not completely released (Fig. 6 *D* and *J*).

High Ionic Strength Elution

Packed membranes derived from 500 μl of purified vacuoles were diluted with 700 μl of KCl (0–1.5 M) or urea (0–8 M) adjusted to pH 6.5 by use of 10 mM Tris-HCl buffer containing 1 mM EDTA and 0.1 mM DFP. After incubation for 30 min at 37°C, the suspensions were centrifuged and the membrane pellets separated from the clear supernatant extracts.

In contrast to effects noted at ice temperatures (Fig. 6), washing the membranes in Tris buffer with EDTA at 37°C for 30 min released some polypeptides, especially in bands 2.2, 3.2, and 6.2 (Fig. 7 *A* and *E*). In addition, band 5.4, two doublets in the region 7, and bands 8.1, 8.3, and 9.3 were partially extracted by treatment with 0.3 M KCl (Fig. 7 *B* and *F*). Exposure to 0.75 M KCl increased the elution of bands 2.2, 3.2, and 9.3 and extracted band 5.1 (Fig. 7 *C* and *G*). No additional bands were extracted by incubation with 1.5 M KCl. Bands 4.3, 6.3, 7.3, and most of 8.3 were retained in the residual pellet (Fig. 7 *D* and *H*). In 5 and 8 M urea, polypeptides 2.2, 3.2, 4.3, 6.2, and 9.1, as well as several other minor species, were dissociated from the membrane (Fig. 7 *I* and *J*).

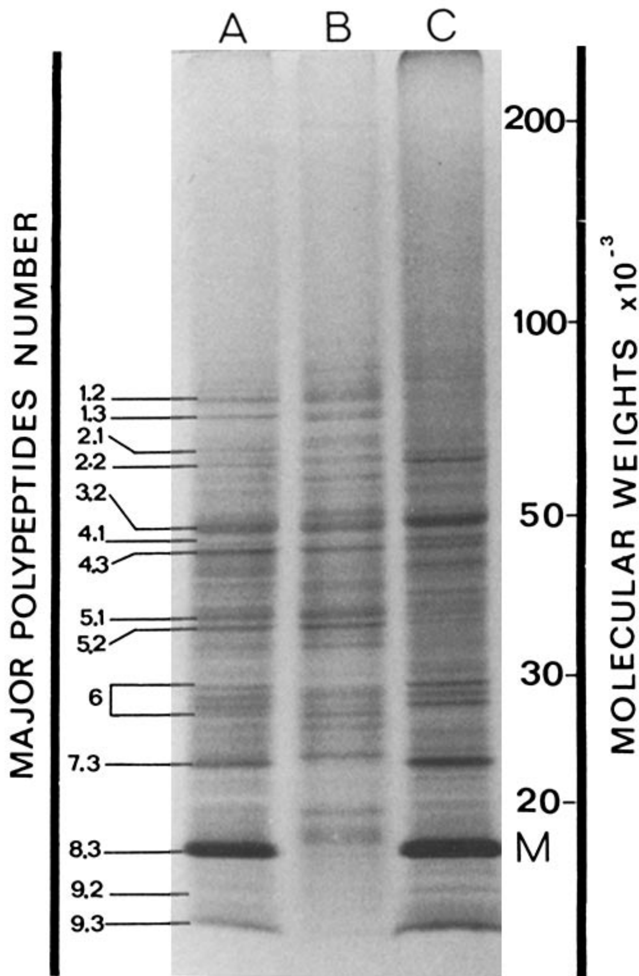


FIGURE 3 SDS-polyacrylamide gel electrophoretic patterns of the polypeptides from the vacuole fractions. Freshly isolated vacuoles (*A*) were osmotically disrupted into the sap (*B*) and membrane components (*C*). The amount of protein loaded in each well was derived from equal amounts of vacuoles. Polypeptide bands were numbered according to an arbitrary scheme (left side) and their apparent molecular weights estimated by comparison with standards (see Materials and Methods). *M* designates a band metachromatically stained with Coomassie Blue.

Treatment with Triton X-100

Membranes suspended in cold 0.5% (vol/vol) Triton X-100 in 56 mM sodium borate buffer, pH 8.0 (ionic strength [I], ~0.008), or in 5 mM phosphate buffer, pH 8.0, containing 25 mM NaCl (I, ~0.04) dispersed when incubated for 20 min at 4°C. Translucent pellets representing the residue of unextracted material were spun down by centrifuging the suspensions at 39,000 *g* for 30 min in a Sorvall SS-34 rotor. The electrophoretic patterns of the extracts and residues obtained after Triton extraction indicate that bands 1.1, 2.2, 3.2, 6, 7.3, and 9.3 were selectively released by 0.5% (vol/vol) Triton X-100 in either of the buffers (Fig. 8 *C* and *E*). Conversely, bands 1.2, 2.1, and 4.3, as well as part of band 8.3 and a broad band in region 3.2, were retained in the pellets (Fig. 8 *B* and *D*). The results revealed that band 8.3 is composed at least of three polypeptides, a doublet being extracted by Triton whereas the third polypeptide, of intermediary size, was not (Fig. 8 *B–E*).

Glycoproteins

Carbohydrate-containing polypeptides from vacuoles, membranes, and sap fractions have been detected in SDS gels using PAS reagent and FITC-labeled lectins of defined specificity. The results indicated that the electrophoretic mobility of these glycoproteins is coincident with that of most of the Coomassie Blue–stained polypeptides (Fig. 9 *A* and *B*).

A number of polypeptides tentatively identified as 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, 4.1, 5.1, 5.2, and 8.1 were weakly PAS-positive, whereas bands 3.2, 7.3, and 8.3 stained slightly more intensely. Because the PAS staining was too faint for an accurate estimate, we routinely used the lectin labeling technique. The presence of glycoproteins was confirmed with fluorescent Con A (Fig. 9 *C* and *D*), the binding of FITC–Con A being in good agreement with the PAS staining pattern. Most of the glycoproteins stained by Con A were in the membrane fraction. Some of the most intensely fluorescent bands, including 2.1, 3.1, and 5.5, were relatively minor components of the membrane as judged by Coomassie Blue staining, and several high molecular weight bands (labeled *1α* in Fig. 9) were not even visible after Coomassie Blue staining. On the other hand, some of the major components seen after Coomassie Blue staining, including 3.2, 5.1, 4.3, 7.3, 8.3, and 9.3, were only weakly labeled by Con A.

Many of the bands labeled by Con A were also labeled by FITC-WGA (Fig. 9, lanes *E* and *F*) and FITC–RCA I or II

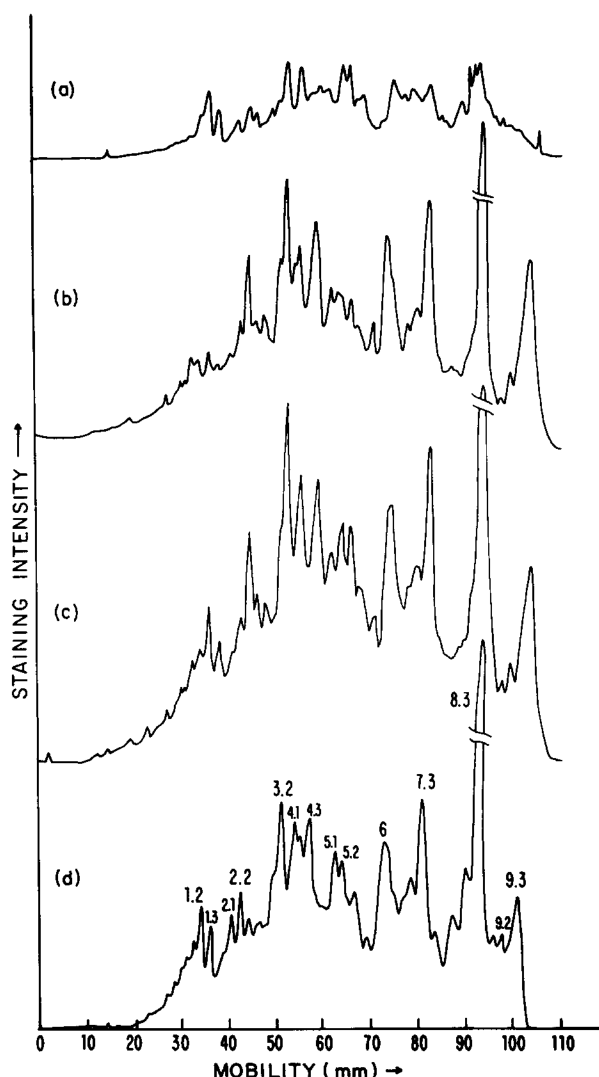


FIGURE 4 Densitometric scans of the gel electrophoretic patterns from (*a*) the sap and (*b*) the membrane. Scan *c* is the arithmetic addition of scans (*a*) and (*b*). Scan *d* is the entire vacuole before separation of sap from membrane. Samples used in *a*, *b*, and *d* were derived from equal amounts of vacuoles.

Table III

Size and Abundance of the Major Polypeptides of the Vacuole Components

Major polypeptide no.	Mol wt	Mass fraction			Molecular fraction		
		Vacuole	Membrane	Sap	Vacuole	Membrane	Sap
	$\times 10^3$	%					
1.2	91	1.6	1.3	2.6	0.6	0.5	1
1.3	86	1.0	1.1	1.5	0.4	0.4	0.6
2.1	77	1.6	2.0	1.6	0.6	0.8	0.7
2.2	73	2.1	3.1	1.9	0.9	1.3	0.9
3.2	59	6.0	5.8	5.6	3.2	3.1	3.2
4.1	55	5.0	3.1	6.1	2.9	1.8	3.7
4.3	50	6.4	7.6	3.2	4.0	4.8	2.2
5.1	44	4.8	2.3	6.0	3.4	1.6	4.6
5.2	42	2.4	2.0	3.8	1.8	1.5	3.1
6	33.5	9.0	7.5	9.8	8.4	7.5	9.8
7.3	27.5	7.2	7.4	4.8	8.1	8.6	5.9
8.2	21	3.7	1.1	6.9	5.6	1.7	11.1
8.3	17	14.1	13.2	3.7	2.6	24.7	7.3
9.2	13	1.2	0.9	1.3	2.9	2.1	3.9
9.3	12	3.7	6.6	1.1	2.6	17.4	3.1
Total*		69.7	65.3	60	78.3	77.8	60.6

* The total includes only the major polypeptides. All of the polypeptides together total 100%.

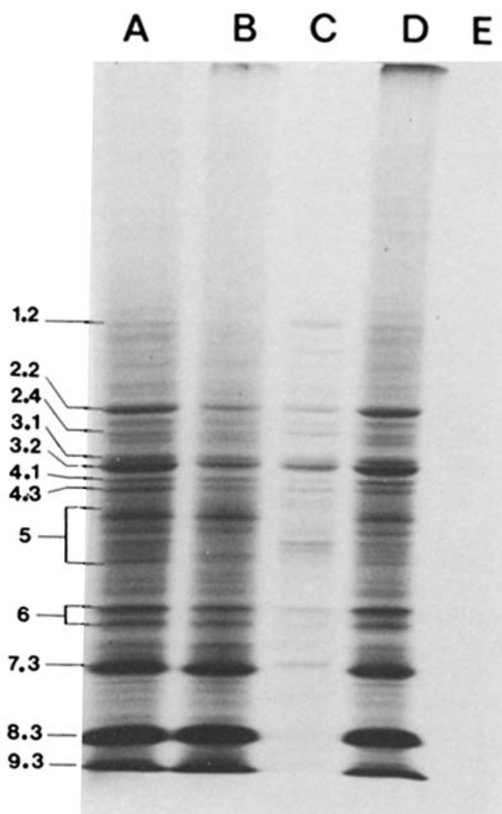


Figure 5 The release of membrane polypeptides at 37°C. Vacuole membranes (*A*) were washed for 30 min at 37°C with deionized water (*B* and *C*) or 5 mM phosphate buffer, pH 8.0, containing 25 mM NaCl (*D* and *E*). The suspensions were centrifuged; pellet (*B* and *D*) and supernatant (*C* and *E*) fractions were sampled for electrophoresis.

(not shown), but the relative fluorescence intensities and overall staining patterns were distinct for each of the lectins. Bands 4.1, 6, and 9.3, which were only faintly labeled by Con A, were strongly labeled by WGA (Fig. 9 *E* and *F*), whereas bands in the 6 and 7 domains were labeled by RCA. Because the fluorescence was abolished in the presence of α-methylmannoside (Fig. 9 *G* and *H*), *N*-acetylglucosamine, and galactose (not shown), the labeled polypeptides were considered to contain specific receptors for Con A, WGA, and RCA.

DISCUSSION

In contrast to the heterogeneity of animal lysosomes, plant vacuoles are a relatively homogeneous population of organelles suitable for analytical characterization. As a result of their activity in cellular autophagy (23), vacuoles, like animal lysosomes, may contain materials derived from other subcellular organelles, but, in contrast to animal lysosomes, it is likely that vacuoles contain few, if any, extracellular components.

The results of this study show that the lipid composition of the vacuole membrane is analogous to that of many other intracellular membranes, at least with respect to phospholipids. The two phosphoglycerides, choline (PC) and ethanolamine (PE), are the most prominent components, together composing more than 75% of the phospholipids. The ratio of PC to PE (~2:1) in the vacuole membrane from beetroot resembles that in the tonoplast of yeast (15, 37), in several plasma membranes (5, 12, 15, 35, 37), and in the ER and glyoxysomes of castor bean endosperm (7, 26). This ratio is considerably smaller in the mitochondria, the proplastids, and the protein body membrane (7, 26). Sphingomyelin, which accounts for 15% of the total phospholipids in lysosomes and for 25–33% in the plasma membrane of animal cell (see reference 35 for review) has not been detected in the vacuole membrane. Phosphatidylinositol,

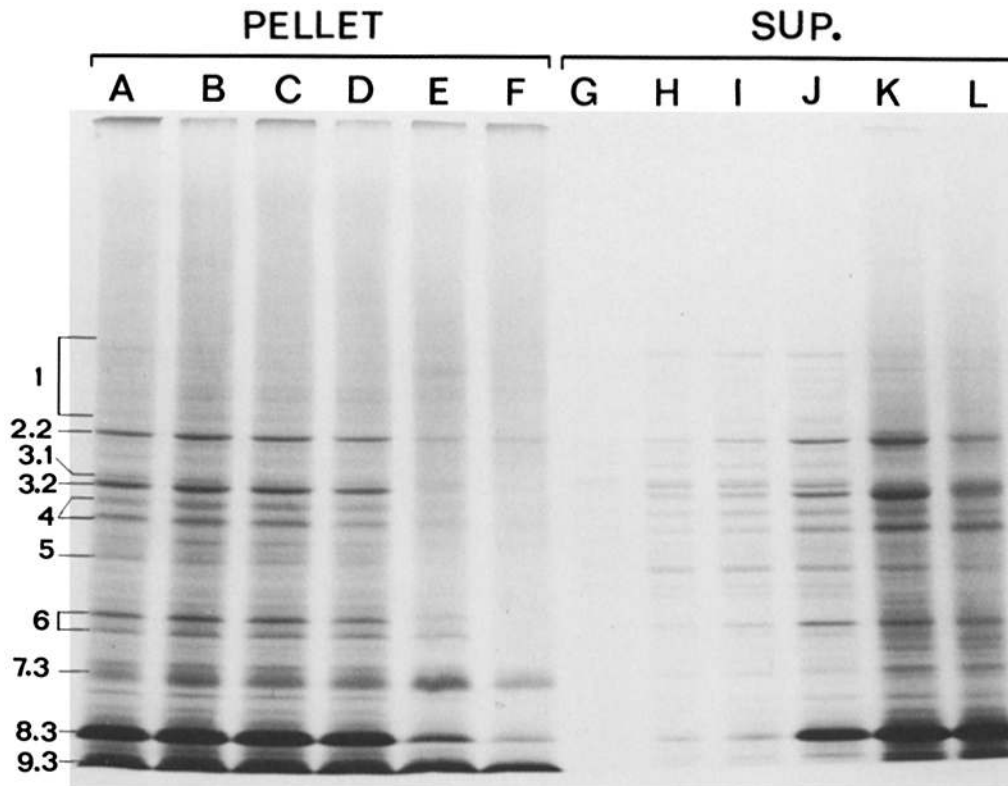


FIGURE 6 The release of membrane polypeptides at alkaline pH. Packed membranes were diluted with ice-cold 10 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA (*A* and *G*), or ice-cold deionized water adjusted with NaOH to pH 9.0 (*B* and *H*), 10.0 (*C* and *I*), 11.0 (*D* and *J*), 12.0 (*E* and *K*), and 13.0 (*F* and *L*), and immediately centrifuged. Pellet (*A–F*) and supernatant (*G–L*) fractions were sampled for electrophoresis.

which is another abundant phospholipid in the ER, glyoxysome, and protein body membranes of castor bean endosperm (7) as well as in the tonoplast and plasma membrane of yeast (15, 37), is not a major class in the tonoplast of beetroot. Conversely, phosphatidic acid, which is absent from the yeast tonoplast but present in the plasma membrane of the same cell (15, 37), is present in the tonoplast of beetroot. However, we found no significant phospholipase D activity to account for its presence. Lysolecithin has been reported in the tonoplast of yeast (15, 37) in greater amount than we found in the vacuole membrane of beetroot, where the amount of free fatty acids was also very small. We could not positively identify phosphatidylglycerol, an abundant phospholipid in plastids, or diphosphatidylglycerol (cardiolipin), a specific component of mitochondria, although minor species migrating in the same area were sometimes detected. Both of these phospholipids have been reported in the ER and glyoxysomes of the castor bean endosperm (7) and in the tonoplast and the plasmalemma of yeast (15, 37). The vacuole membrane of beetroot with a high ratio of sterol, relative to protein and phospholipids, is similar to the vacuole membrane of yeast (15, 37). Other intracellular membranes, such as the ER, the glyoxysome membrane, and the mitochondria of endosperm cells, contain minor amounts of sterols (7), whereas the plasma membrane from yeast (15, 37), soybean roots (36), and mammalian cells (12) contain the highest ratio of sterol to phospholipids. It is noteworthy that both the plasmalemma and the tonoplast, which are the most sterol-rich membranes of the cell, are thought to be terminal membranes produced through sequential but antiparallel differentiation steps (23).

Glycolipids were detected in high concentration and diversity in the vacuole membrane. Ceramide glycoside-like lipids, resembling those of animal lysosomes, were detected by thin-layer chromatography, but their identification remains to be firmly established, as is also the case for the putative major sulfoglycoside.

Our results show that the protein to phospholipid ratio for the vacuole membrane of beetroot is similar to that of yeast tonoplast (15, 37) and soybean plasma membrane (36), but higher than that of the ER of endosperm cells (7) and lower than that of the plasma membrane of yeast (15, 37) and the protein body membrane of endosperm (26).

The intravacuolar organization of proteins is difficult to interpret because several polypeptides were found in both the membrane and the vacuole sap. The dual location of some of the polypeptides was not completely overcome by higher dilution (>150) of the purified vacuoles in the hypotonic buffer. This dual location is probably not the result of incomplete washing of the membrane because if the membranes had been incompletely washed all of the sap components would have been included in the membrane preparations in the same proportion as found in the sap. This is clearly not the case (Figs. 3 and 4). Rather, the variable partitioning of individual polypeptides between the membrane and the soluble phase suggests that some of the polypeptides found in the sap can bind to the membrane. Further binding studies will be required to determine if binding is specific and if the associations, be they protein-protein or protein-lipid, have any physiological significance.

Our initial approach to such questions has been patterned

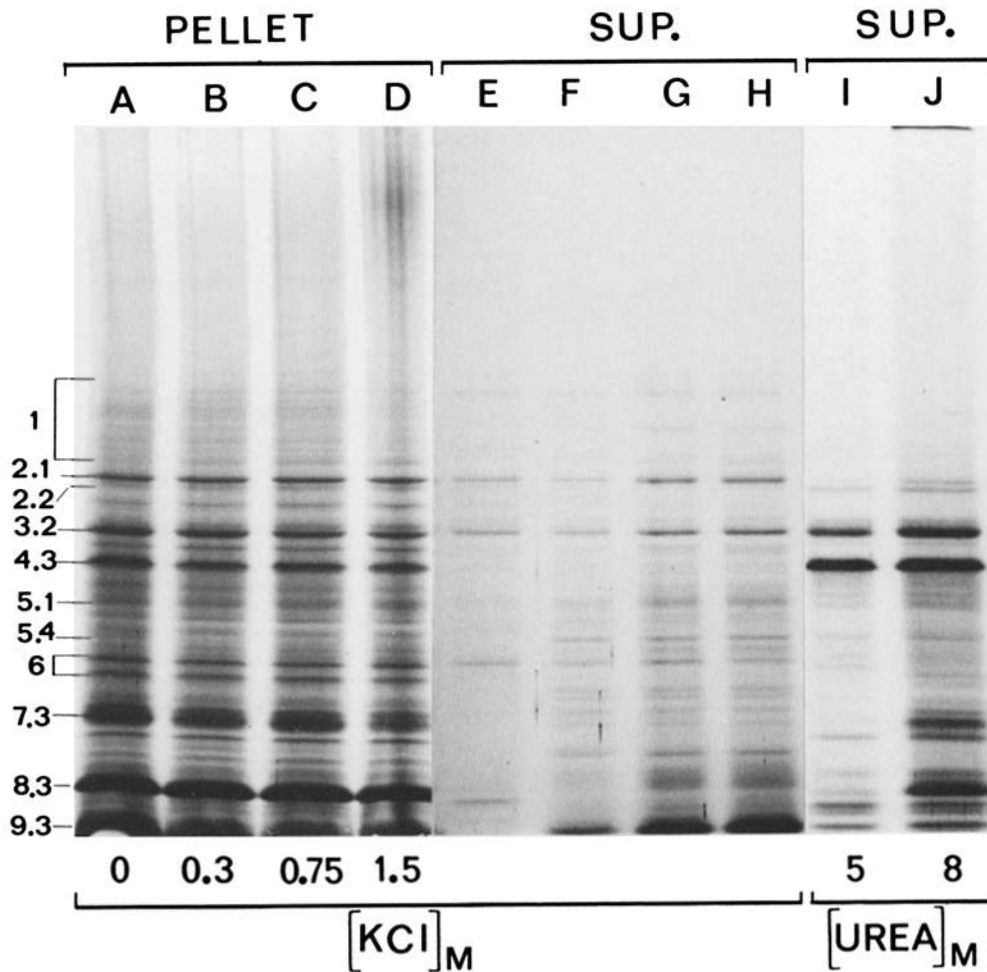
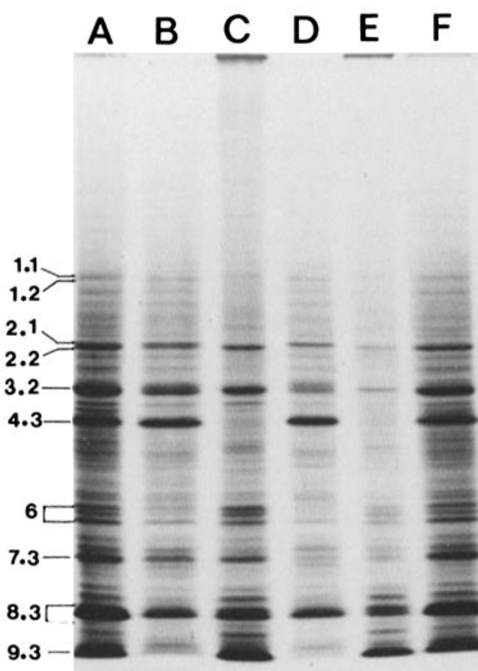


FIGURE 7 The release of membrane polypeptides by KCl or urea. Packed membranes were treated for 30 min at 37°C with 10 mM Tris buffer, pH 6.5 (*A* and *E*), or Tris-buffered KCl 0.3 M (*B* and *F*), 0.75 M (*C* and *G*), 1.5 M (*D* and *H*), or urea 5 M (*I*), 8 M (*J*), each containing 1 mM EDTA and 0.1 mM DFP, then centrifuged. Pellet (*A–D*) and supernatant (*E–J*) fractions were sampled for electrophoresis.



on the selective elution studies of erythrocyte membranes (32). For example, the elution pattern observed after washing the membrane with ice-cold water or after treatment with weak alkali (pH 9–10) delineates the same polypeptides. Thus, these polypeptides are tenuously bound to the membrane and their elution properties indicate that they are peripheral membrane proteins or sap proteins adsorbed to the membrane. In addition, other polypeptides are differentially eluted by KCl or urea. For instance, band 3 is eluted with both reagents, whereas band 2.1 is preferentially released with KCl, and band 4.3 is only extracted with urea. Therefore, we conclude that these polypeptides are more tightly bound to the membrane and their behavior in the presence of KCl and urea suggests an electro-

FIGURE 8 The release of membrane polypeptides by nonionic detergent at low (*B* and *C*) and moderate (*D* and *E*) ionic strength. Packed membranes (*A* and *F*) were incubated at 4°C for 20 min in 0.5% (vol/vol) Triton X-100 buffered with 56 mM sodium borate, pH 8.0, I, $\sim 8 \times 10^{-3}$ (*B* and *C*) or with 5 mM sodium phosphate pH 8.0, containing 25 mM NaCl, I, $\sim 4 \times 10^{-2}$ (*D* and *E*). After centrifugation, pellet (*B* and *D*) and supernatant (*C* and *E*) fractions were sampled for electrophoresis.

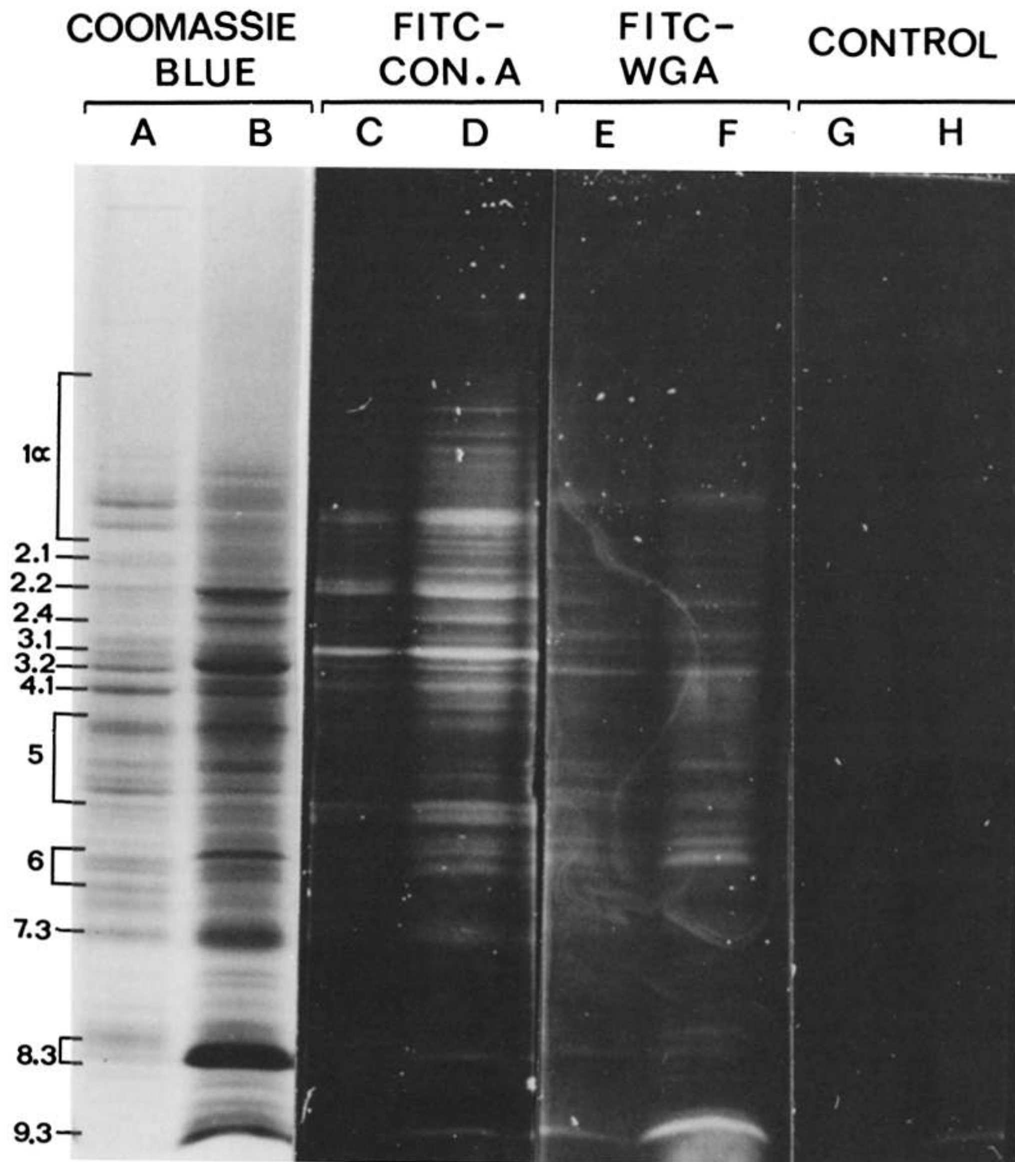


FIGURE 9 Identification of glycoproteins from the vacuole membrane and sap fractions. Polypeptides from the sap (*A*, *C*, *E*, and *G*) and from the membrane (*B*, *D*, *F*, and *H*) were electrophoresed in SDS-polyacrylamide gels, fixed, and stained either with Coomassie Blue (*A* and *B*) or with fluorescent lectins (*C*–*H*). The lectins used were Con A (*C*, *D*, *G*, and *H*) and WGA (*E* and *F*) at 1.66 mg/ml. A representative control is shown (*G* and *H*) where 260 mM α-methyl-D-mannoside was included as an inhibitor in the incubation medium (containing Con A) as well as in the prior and subsequent washes.

static or hydrophobic interaction with the membrane core components. On the other hand, polypeptides like 8.3, one of the major polypeptides, are liberated by none of these disruptive agents but are dislodged only when the hydrophobic continuum is disassembled by the mild, nonionic detergent Triton X-100. We conclude that these polypeptides exhibit hydrophobic interactions with the lipid core. Because a sedimentable insoluble residue was released after mild treatment of the vacuole membrane with Triton X-100, interactions between the polar peripheral proteins leading to large oligomeric complexes appear likely.

Finally, the present results strongly suggest that most of the polypeptides from the membrane and from the sap are glycoproteins. These findings agree with the current idea that vacuoles are plant lysosomes (23, 25) because most of the enzymes that have now been thoroughly purified from animal lysosomes are also glycoproteins (1). Based on their relative affinities for Con A, WGA, and RCA, vacuole glycoproteins must possess mainly terminal mannose/glucose residues, some *N*-acetylglucosaminyl terminals, and a few galactose residues. Therefore, the carbohydrate chains of the vacuole glycoproteins resemble the oligosaccharides of the high-mannose type (14) characteristic of several lyosomal enzymes. Their content of *N*-acetylglucosamine, galactosamine, and galactose indicate that these proteins have undergone several stages of posttranslational processing.

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