J. Membrane Biol. 89, 39–51 (1986)

The Journal of
Membrane Biology

Isolation and Characterization of Granules of the Toad Bladder

Sandra K. Masur,† Shulamit Cooper,† Stephen Massardo,† Gloria Gronowicz,† and Meryl S. Rubin‡
†Department of Physiology and Biophysics and ‡Center for Polypeptide and Membrane Research,
Mount Sinai School of Medicine, New York, New York 10029

Summary. The electron-dense granules that lie just below the apical plasma membrane of granular epithelial cells of toad urinary bladder contribute glycoproteins to that apical membrane. Also, exocytosis of granules (and tubules) elicited by antidiuretic hormone potentially doubles that apical surface, during the same period the transport changes characteristic of the hormonal response occur.

Granules separated from other membrane systems of the cells provide the material to assess the importance of the granules as glycocalyx precursors and in hormone action. We used isosmotic media to effect preliminary separations by differential centrifugation. Then granules were isolated by centrifugation on self-forming gradients of Percoll of decreasing hypertonicity.

We find qualitative and quantitative changes in protein composition and enzymic activities in the isolated fractions. The primary criterion for granule purification was electron microscopic morphology. In addition, polypeptide species found in the granule fraction are limited in number and quantity. The granules are enzymically and morphologically not lysosomal in nature. Granules may provide the glycoproteins of the apical glycocalyx but they differ from the isolated plasma membrane fraction enzymically, in protein composition and in proportion of esterified cholesterol.

We conclude that the granules are not "average" plasma membrane precursors. Their role in the membrane properties of the toad urinary bladder may now be evaluated by characterizing permeability and other properties of the isolated organelles.

Key Words toad (*Bufo marinus*) urinary bladder · granules · plasma membrane · antidiuretic hormone · osmotic water permeability · membrane shuttle hypothesis

Introduction

Membrane-bounded granules that lie just below the apical plasma membrane of granule-rich epithelial cells of toad urinary bladder provide the immediate precursors of apical membrane components including the extensive apical glycocalyx (Pisam & Ripoche, 1973, 1976; Strum & Ekblad, 1977; Gronowicz, Masur & Holtzman, 1980). The granule core and apical membrane carbohydrates are cytochemically similar (Pisam, Ripoche & Rambourg, 1970; Gronowicz et al., 1980; Spicer, Baron, Sato & Schulte, 1981). Also, apical membrane glycoproteins are quite unusual in structure (M.S. Rubin, C.F. King and J.A. Dunford-Weissman, *in preparation*).

Toad urinary bladder is the anuran equivalent of collecting duct and the major site of physiological osmoregulation by antidiuretic hormone. Antidiuretic hormone increases the transepithelial flux of water and some small solutes (urea, Na^+) across the rate-limiting apical membrane, and simultaneously induces enough exocytosis of membrane-bounded subapical granules to potentially double the apical membrane surface area (Gronowicz, 1979; Masur, Gronowicz & Holtzman, 1979; Gronowicz et al., 1980). Thus we are interested in determining the role of granule constituents in responsiveness to antidiuretic hormone (vasopressin).

Granules could be general membrane precursors, or provide units specifically involved in ADH-induced permeability changes in the apical membrane. If granules are general apical membrane precursors, most of their components will always be represented in the surface membrane fraction (*and vice versa*). If not, granules may have unique constituents different from those found in surface membranes of cells not stimulated by vasopressin.

We separated granules from other membraneous organelles by differential centrifugation in isotonic medium and Percoll gradient centrifugation. Enzymic assays, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy were used both to assess the quality of the separation and to begin delineating differences between granules and plasma membrane from unstimulated bladders. Granules differ from other membranous organelles in their enzymic, polypeptide and glycoprotein content, in their content of cholesterol and cholesteryl esters, in their density, and in their microscopic appearance.

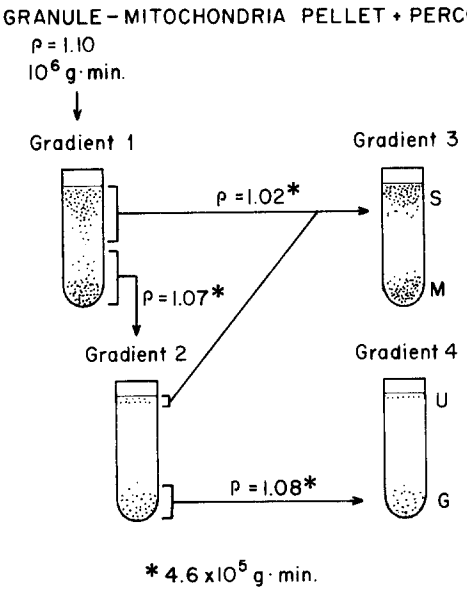


Fig. 1. Scheme for organelle isolation from granule-mitochondria pellet (*GM*) on Percoll density gradients. Percoll, *BR*, and biological material were mixed and subjected to centrifugation as described in Materials and Methods. Approximate locations of fractions in the gradients are shown by shading. Adjacent to each arrow is the starting density (ρ, g/ml) of the next self-forming Percoll gradient

Materials and Methods

Dominican toads (*Bufo marinus*) obtained from National Reagents, Bridgeport, CN., were maintained on damp peat moss. Analytical grade chemicals and biochemicals were obtained from major suppliers or as noted.

Solutions for Cell Fractionation

HEPES-buffered amphibian Ringer's solution (HR) contained 18 mM D-glucose, 3 mM $KHCO_3$, 110 mM NaCl, 10 mM HEPES base (Research Organics, Inc., Cleveland, OH), and 1 mM Na_2HPO_4 (and no added Mg^+ or Ca^+ salts), to pH 7.4–7.6 and ~240 mOsm. BR contained 1 mg BSA/ml, 10 mM NaCl, 132 mM sucrose, 10 mM HEPES and 2 mM EGTA, to pH 7.4–7.6 and ~240 mOsm. Both solutions were freshly prepared for each experiment.

To inhibit serine and sulfhydryl-dependent hydrolases and some glycosidases (Blumenfeld & Adamany, 1978), stock solutions of 0.2 M phenylmethylsulfonyl fluoride in isopropanol and 0.2 M N-ethylmaleimide (stored 0°) and 0.5 M α-methylmannoside (stored 0°) were diluted 100-fold into fresh medium used for each organelle resuspension. Both the lack of added Ca^+ and EGTA inhibit Ca^+-dependent enzymes.

Cell Fractionation (Fig. 1)

Toads (20 to 40) were killed by double pithing. Bladders immediately were excised and inverted over Lucite® forms, 1 × 3 in, with rounded corners. Epithelial cells were scraped off with cover slips into HR (0–4°), and washed twice by centrifugation and resuspension in BR. All further procedures were at 0–4°. Fractions were collected within 6 hr for storage in 0.22 M sucrose. (At each step, 50–100 μl portions were taken for analysis and were operationally described as granule-mitochondria pellet, post-mitochondrial supernate, etc.)

Cells were resuspended in 10 vol BR and triturated 10–40 strokes in a tight glass-Teflon homogenizer (Tri-R, Rockville Center, NY) with a T-line #106 A motor (Talboys Engineering Corp, Emerson, NJ) at 55 in its lower speed range, until >90% of the cells admitted Trypan Blue (1% in saline). The homogenate was diluted with the same vol of BR again, 0.01 final vol of each inhibitor added, and then centrifuged (3000 × *g*, 5 min, Sorval SS-34 rotor).

The first supernate was saved. The pellet, containing unbroken cells, nuclei and other organelles, was rehomogenized and recentrifuged. Then the supernates from these two homogenizations were combined and again subjected to centrifugation (17,370 × *g*, 20 min) to produce a granule-mitochondrial pellet (GM) which was further fractionated on Percoll. The post-mitochondrial supernate was separated (at 100,000 × *g*, 1½ hr) into a post-microsomal supernate and a microsomal pellet (Mi).

Percoll (Pharmacia, Piscataway, NJ) suspensions were prepared just before use from purchased Percoll and fivefold concentrated BR diluted appropriately, for desired density and osmolarity. Fractions were collected from each gradient from above with Pasteur pipettes and weighed to determine their densities. They were then resuspended to the next starting density for recentrifugation by adding either Percoll or BR. The organelle fractions were again weighed and then freed from Percoll by resuspension in 0.22 M sucrose (230–240 mOsm) and centrifugation at 100,000 × *g* for 1½ or 2 hr.

To isolate granules and other organelles, the granule-mitochondrial pellet was successively fractionated through a series of four self-forming Percoll gradients. The first gradient contained the granule-mitochondria pellet, resuspended in Percoll and BR to final density of 1.098 g/ml and 307 mOsm, treated for a short time with DNase I (2.5 μm/ml final concentration), and centrifuged (40,000 × *g*, 25 min in the SS 34 rotor). Upper and lower halves of gradient 1 were separated (Fig. 1). The lower half, containing most of the granules was subjected to two further centrifugations to produce the granule fraction. Specifically, it was diluted with Percoll/BR to 1.07 g/ml (290 mOsm) and centrifuged (30,900 × *g*, 15 min) (gradient 2) and yielded a lower band (often diffuse) and an upper band. The lower band from gradient 2 was brought to 1.08 g/ml (301 mOsm) with Percoll and BR and recentrifuged (30,900 × *g*, 15 min) (gradient 4 on Fig. 1) to produce the final very dense "granule" (*G*) fraction. Occasionally gradient 4 also had an upper band (*U*) containing empty membranes or lysosomes by EM criteria.

The final "surface membrane" (*S*) and "mitochondria" (*M*) fractions resulted from combining the upper half of gradient 1[1] with the upper band of gradient 2, diluting them with BR and Percoll to 1.02 g/ml (267 mOsm) and centrifuging at 30,900 × *g* for 15 min (gradient 3). We collected the abundant upper band of surface membrane and the more dense (lower) band of mitochondria.

[1] The upper half of gradient 1 often contains two bands. EM of one preparation showed that the upper band was mainly surface membranes and the more dense (lower) band was mostly mitochondria and lysosomes.

The density of each organelle class in Percoll was determined and then each fraction was diluted with isotonic sucrose and subjected to centrifugation at 100,000 × *g* for 1.5–2 hr to separate biological material and Percoll. Percoll formed a pellet with a pellicle of biological material on its surface. Each pellicle was resuspended in ~1 ml of isotonic sucrose. Fractions were left overnight at 0–4° and/or frozen in liquid nitrogen.

Processing of Fractions for Electron Microscopy (EM)

All fractions were examined by EM. Fifty to 100 μl of each fraction was fixed by adding 1–5 vol 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2–7.4 for ~12 hr (to several weeks) at 0–4° (Masur, 1969). Then each fraction was pelleted in an Eppendorf microfuge, postfixed with reduced OsO_4 (2%, 2hr) (Karnovsky, 1971), dehydrated in a graded series of ethanol/propylene oxide, and embedded in Epon (Luft, 1961). Silvergold sections were cut through the length of the pellet to provide a representative view of the fraction composition and were examined in a JEOL 100 B electron microscope without additional staining or after uranyl acetate and/or lead citrate staining (Watson, 1958; Reynolds, 1963). Quantitation of mitochondria and granules in *GM*, *M* and *G* fractions was performed on electron micrographs (12,000 × final magnification) by standard morphometric techniques (Williams, 1977).

Cytochemistry

Acid β-glycerophosphatase activity, a cytochemical marker for lysosomes, was demonstrated in briefly fixed bladders (30–60 min) and subcellular fractions captured on 0.22 μm GS (Millipore) filters (Baudhuin, Evrard & Berthet, 1967). Bladder pieces (~0.5 mm^2) were briefly frozen in 5% sucrose on a glass slide on dry ice before cytochemical incubation to make them permeable to the medium and substrate (Masur et al., 1971).

Bladder slices or Millipore filters were rinsed three times in 0.1 M cacodylate, twice in 0.1 M acetate and then incubated at room temperature for 60 min in two changes of full medium (0.1 M acetate buffer (pH 5.0), 1 mM β-glycerophosphate (disodium salt, Sigma) and 2 mM $CeCl_3$ (Alfa Products, Danvers, MA)) or medium lacking the substrate as a cytochemical control (Robinson & Karnovsky, 1983). Three changes of 0.1 M acetate terminated the incubation and post-fixation was in 1% OsO_4 in 0.1 M cacodylate buffer.

Biochemical Characterization

Protein content was determined at 750 nm as described by Lowry, Rosebrough, Farr and Randall (1951) in a final volume of 1.2 ml with bovine serum albumin as standard. When residual Percoll interfered with the Lowry reaction, protein content was assessed with a modified Coomassie Blue G-250 assay (Serva Blue G) (Read & Northcote, 1981; Vincent & Nadeau, 1983).

Cytochrome oxidase (EC 1.9.3.1.), an enzymic marker for the inner mitochondrial membrane, was assayed (Cooperstein & Lazarow, 1951) after preincubating 100 μl of each fraction at ~22°C with 10 mM succinate and 25 mM sodium phosphate buffer. The substrate, cytochrome *c* (horse heart type VI, Sigma, St. Louis, MO), was reduced with sodium dithionite and excess reductant removed by gel chromatography on Sephadex G-25 (Horton, 1968). Reduced cytochrome *c* was mixed with 50 mM sodium phosphate buffer, pH 7.0, and the absorbance at 550 nm measured. The reaction was started by adding 5–40 μl of preincubated fraction (10-110 μg membrane protein) and the absorbance decrease at 15 and 45 sec was used to calculate nmol cyt *c* reduced per min per mg protein.

NADPH-cytochrome c reductase (EC 1.6.2.3.) was used as a marker for endoplasmic reticulum and was immediately assayed (Phillips & Langdon, 1962; Sottacasa, Kuylenstierna, Ernster & Bergstrand, 1967) at 30° with 10–40 μg of fraction protein because it did not survive freezing. NADPH (0.1 mM) was added to start the reaction and the rate of cytochrome *c* reduction followed by the increase in optical density at 550 nm.

Alkaline phosphatase (EC 3.1.3.1.), a marker for kidney brush border (apical) membranes (Wilfong & Neville, 1970) and toad urinary bladder apical plasma membrane (Robbie, 1971; M.S. Rubin, *unpublished*), was assayed by the release of *p*-nitrophenol (measured at 410 nm) from *p*-nitrophenyl phosphate (Sigma) (Coleman & Finean, 1966).

β-glucuronidase (EC 3.2.1.31), a lysosomal marker, was assayed at 30° by the release of *p*-nitrophenol from *p*-nitrophenyl-β-glucuronide (Sigma) (Michell, Karnovsky & Karnovsky, 1970).

Na^+, K^+-Mg^{++}ATPase (EC 3.6.1.3.) is a basolateral surface marker in many transporting epithelia (for a recent review, *see* Ernst, Riddle & Karnaky, 1980). Total ATPase was assayed in the presence of 85 mM Na^+, 15 mM K^+ and 3 mM Mg^{++}, and in the presence of specific inhibitors of the Na^+-K^+-ATPase, 1 mM ouabain (Ernst et al., 1980) or 80 μM vanadate (Cantley et al., 1977). The Na^+,K^+-independent activity was measured in the presence only of 3 mM $MgCl_2$. ^{32}P-phosphate from ATP-γ-^{32}P (New England Nuclear, Boston, MA) was extracted (Berenblum & Chain, 1938) for liquid scintillation counting.

Enzyme specific activity is expressed as nmol product formed or substrate lost per min per mg fraction protein, and % recovery compared to total activity in the homogenate.

Assay of Total and Free Cholesterol

Bovine pancreas cholesterol esterase was obtained from Pharmacia P-L Biochemical, Piscataway, N.J. *Nocardia* cholesterol oxidase, horseradish peroxidase type II, cholesterol, cholesteryl arachidonate and *p*-hydroxyphenylacetic acid (the fluorogen) were from Sigma. The standards—cholesterol and cholesteryl arachidonate—were prepared as described by Noel, Dupras and Filion (1983) and assayed essentially according to Gamble, Vaughan, Kruth and Avigan (1978). Standards (0.4–12 nmol) were dissolved in potassium phosphate, pH 7.4, sodium cholate, Triton X-100 and ethanol to final concentrations of 73.5, 2.9 and 1.4 mM and 17% in 150 μl. The biological material was treated with cholate and Triton X-100 at 37% for 10 min before being diluted to 150 μl with potassium phosphate and ethanol. Free cholesterol was assayed by adding 450 μl of a mixture containing 50 mM potassium phosphate, pH 7.4, 1 mM sodium cholate, 45 mU cholesterol oxidase, 4.5 U of horseradish peroxidase and 0.45 mg *p*-hydroxyphenylacetic acid. Total cholesterol was assayed by adding the same solution containing 41 mU cholesterol esterase as well. Triplicates were incubated for 1 hr at 37°C, and then relative fluorescence efficiency (RFE) was measured (λex = 325 nm, λem = 415 nm, slit widths 2 nm each) on a Perkin-Elmer 650-10S fluorescence spectrophotometer. The standards contained equal amounts of cholesterol and cholesteryl arachidonate. Amount of free cholesterol was determined by direct com-

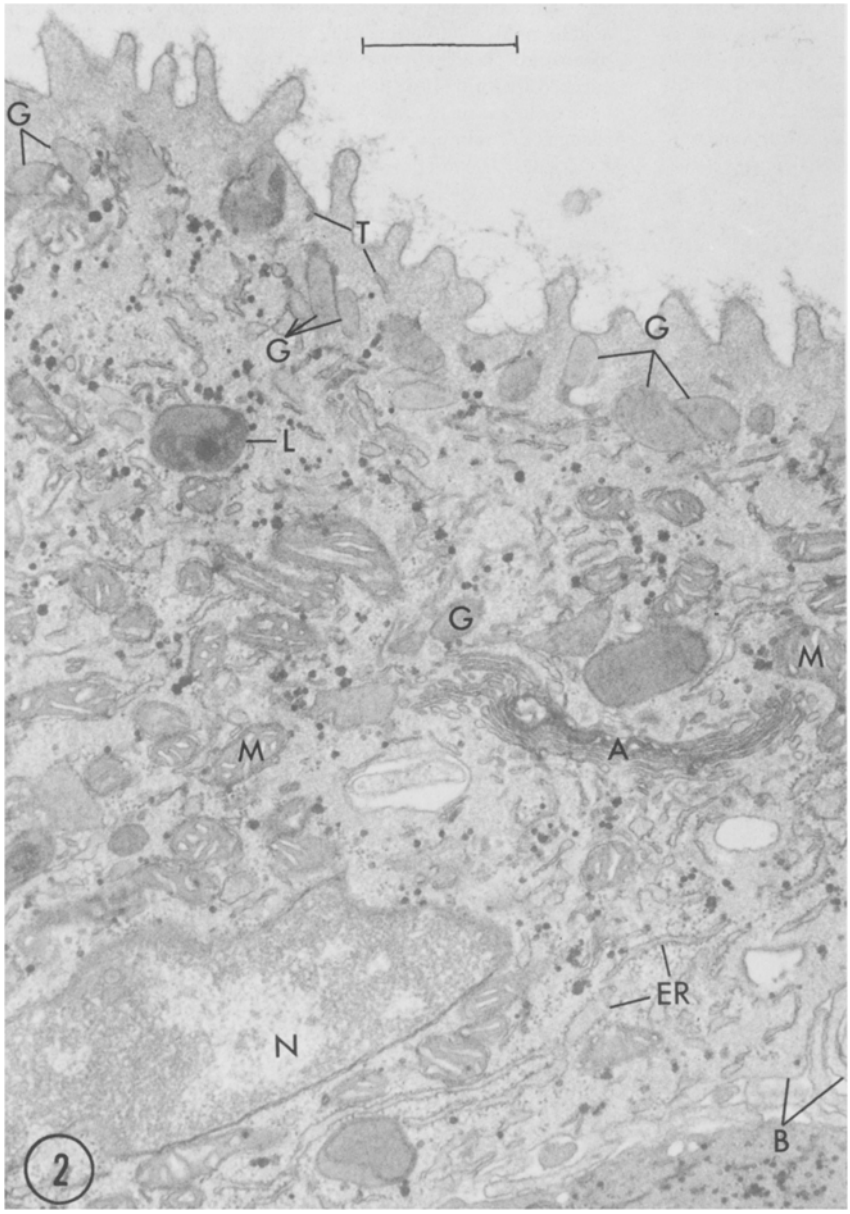


Fig. 2. Portion of a granule-rich epithelial cell in the toad urinary bladder. Membrane-bounded granules (*G*) of a granule-rich cell are mostly in the apical cytoplasm adjacent to the luminal (apical) membrane with a few near the Golgi apparatus (*A*). In the apical region smooth endoplasmic reticulum (*ER*) and other membrane-bounded tubules (*T*) are seen. Rough *ER* and mitochondria (*M*) are mainly in the Golgi region and basal part of the cell. The basolateral membrane (*B*) delimits cytoplasmic interdigitating lateral projections between neighboring cells. Tentative identification of membrane-bounded structures of appropriate size and heterogeneity of electron-dense contents as lysosomes (*L*) is confirmed in other sections by cytochemical means (*see* Fig. 9). Section stained with uranyl acetate and lead citrate. × 19,000. Each bar represents 1 μm

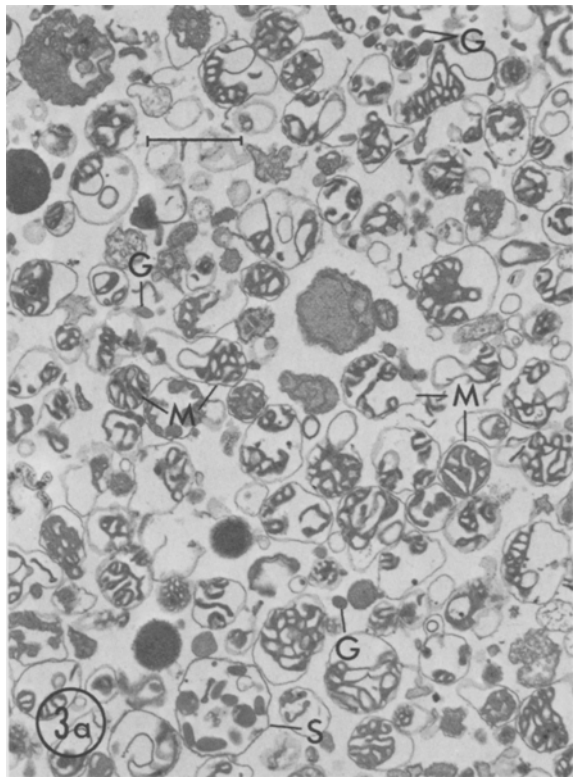
parison to the standards for unesterified cholesterol (no esterase present). Cholesteryl ester content was determined by comparison to the differences in relative fluorescence efficiency between the total cholesterol and free cholesterol standards.

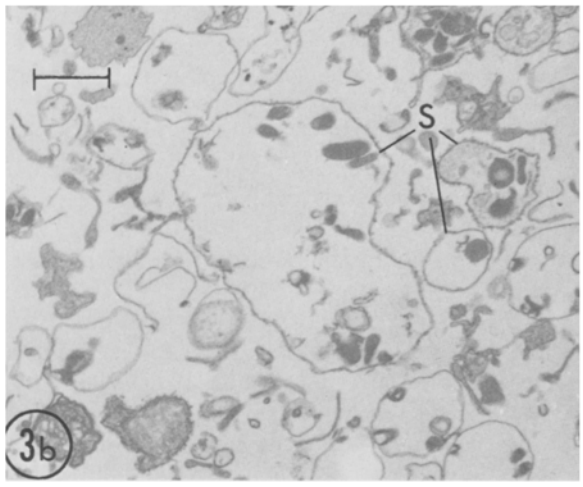
Polyacrylamide gel electrophoresis of detergent-solubilized organelle fractions was performed according to Maizel (1971) on 7.5% acrylamide slabs with 3% acrylamide sieving stack gels. Samples (2–10 μg of protein) were dissolved in glycerol/SDS/2-mercaptoethanol/TRIS-HCl (pH 6.7) to final concentrations of 6%, 5% and 5% and 20 mM. Gels were stained with silver (Merril, Goldman & VanKeuren, 1984). Coomassie blue R-250 (Maizel, 1971) did not stain most components of granules or surface membranes even with 50 μg protein per lane, perhaps because of their high carbohydrate contents.

Results

We obtain 0.17 ± 0.02 ml cells with 4.99 ± 1.16 mg protein (n = 6) per toad by scraping off the epithelial cells (Fig. 2) or about 29 mg protein per ml epithelial cells.

Divalent cations (Ca^{++} and Mg^{++}) are omitted





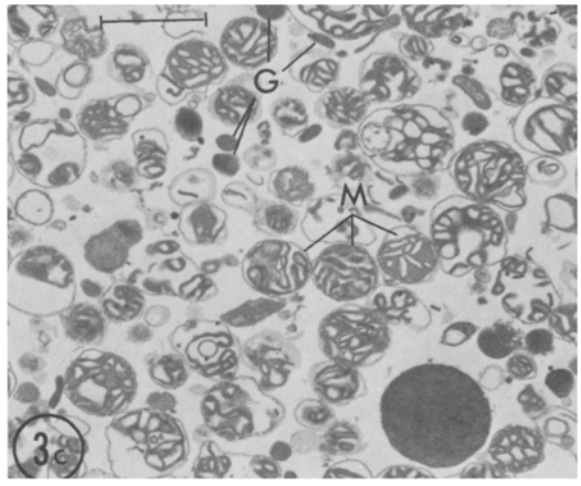


Fig. 3. Granule-mitochondria pellet (*GM*) obtained by recentrifugation after removal of whole cells and nuclei from homogenate by low speed centrifugation (*see* Materials and Methods). (*a*) *GM* has abundant intact mitochondria (*M*) and membrane-bounded granules (*G*). The third major class of membrane-bounded organelles in *GM* is referred to as *S*, "surface membrane." *S* are various diameter, membrane-bounded vacuoles, which we presume are derived from surface membranes, usually containing identifiable cytoplasmic structure, such as *G* and *M* (*see* vacuole labeled *S* and also Fig. 10). × 12,500. (*b*, *c*) A gradient of organelles forms when *GM* is resuspended in glutaraldehyde and spun for 15 min in an Eppendorf table centrifuge to collect the fixed material. (*b*) Membranous vacuoles predominant in the upper part of the pellet. × 13,500. (*c*) Granules (*G*) and mitochondria (*M*) predominate in the lower part of the pellet. The large dense granule could be several fused *Gs* or a mucus drop in cross-section. × 12,500. Uranyl acetate and lead citrate stained sections

from the media and EGTA is added to inhibit aggregation of organelles and calcium activation of hydrolases as both decrease organelle yield and alter membrane characteristics.

Differential Centrifugation

The pellet from the low-speed centrifugation contains both intact and leaky cells, large cytoplasmic fragments with and without plasma membranes, stripped nuclei, and those membranous organelles large and/or dense enough to pellet under those conditions. Many organelles seem to be "captured" in large fragments of surface membrane. The biochemical composition of the low-speed pellet reflects this heterogeneity—it has one-third the total protein, two-fifths the surface membrane and endoplastic reticulum markers (alkaline phosphatase and NADPH-cytochrome *c* reductase), but only 10% of

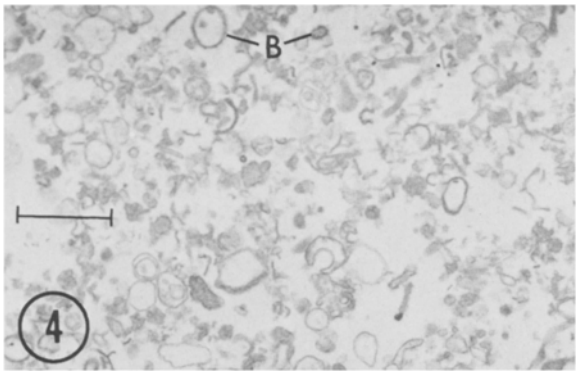


Fig. 4. The microsomal fraction is predominantly small vesicles (0.08–0.2 μm diameter) which may have bristle-like borders (*B*) or ribosomes associated with the membrane. The largest membrane-enclosed vesicles are closer to 0.5 μm diameter. Although the diameter of the majority of the vesicles is very different from that of the surface membrane fraction vacuoles, the two fractions share many biochemical characteristics. Uranyl acetate and lead citrate stained section. × 12,500

the mitochondrial enzyme, cytochrome oxidase. This low-speed pellet was not used routinely for organelle isolation because granules isolated from this pellet were contaminated.

The combined supernates from the low-speed spin are centrifuged to provide a granule-mitochondria pellet (Fig. 3) that then is fractioned further on Percoll. We chose Percoll as the gradient medium because granules and mitochondria were not separated on sucrose or Ficoll gradients (Gronowicz, 1979), whose osmolarity and viscosity increased with their density. Starting with a hypertonic Percoll suspension and decreasing the osmolarity of subsequent gradients protected the granules during fractionation and Percoll removal.

The post-granule mitochondria supernate is fractionated further into a microsomal pellet (Fig. 4) and a post-microsomal supernate.

Density of Organelles in Percoll

Granules, mitochondria and surface membranes band at characteristic Percoll densities. For seven experiments, granules were at 1.119 ± 0.009 g/ml, mitochondria at 1.050 ± 0.004 and surface membranes at 1.022 ± 0.002 g/ml.

Characteristics of the Final Fractions

Granules are not enriched in the organelle-specific enzymes we assayed: specific activities of apical surface and endoplasmic reticulum enzymes are less than in the homogenate (Fig. 5, Table) and one-eighth and one-fourth those in the surface membrane and microsome fractions. Cytochrome oxidase activity is comparable to the homogenate, but only one-fifth that in the mitochondria. Lysosomal β-glucuronidase was retained in the granule fraction (but also in the mitochondrial fraction, *see* below). Granules retained 1.1 ± 0.3% (4) of the protein in the homogenate.

Granule fractions are predominantly granules by EM criteria (Figs. 6–8), between 3 and 20 granules for each intact mitochondrion. The granules are 75–95% of the formed elements in the granule fraction (Fig. 6*c*) and at most 12% of the granule-mitochondria pellet (Fig. 3). This represents a 30- to 60-fold enrichment over the GM fraction. The granules generally have a homogenous and moderately electron-dense content bounded by a single membrane (Figs. 7 and 8). Their long dimension *in situ* or as isolated organelles is ~0.8–1 μm. Because they are discoid (Wade, DiScala & Karnovsky, 1975), they appear as small or large circles or as rods, depending on the angle of section (Figs. 6*c* and 7).

Granules are morphologically distinct from the other organelles. For example, mitochondria have a double membrane structure; mucous drops are ~1 μm diameter and often remain clustered even after homogenization and centrifugation. Lysosomes vary greatly in size and contents but are identifiable by their cytochemically demonstrable acid phosphatase activity (Figs. 9 and 10). Secondary lysosomes also have membranous or dense inclusions. Lysosomes, mucous droplets, small vesicles and Percoll particles remaining after washing in sucrose are minor contaminants of the granule fraction (*see* Fig. 6*c*).

Percoll separates by exploiting density differences between organelles, especially granules and mitochondria. Granules, the densest organelles, separate and are enriched but not freed of other organelles or other large and/or dense biological material (bacteria, epithelial fragments, or muscle cells) carried over from the homogenate. The latter contaminants can be minimized by using fresh media and by passing the resuspended granule-mitochondria pellet through a 100-μm nylon mesh filter before Percoll gradient centrifugation.

Since the lysosomal enzyme β-glucuronidase is present in both granule and mitochondrial fractions, cytochemical demonstration of acid phosphatase in neither granules nor mitochondria but rather in other membrane-bounded organelles was essential (Figs. 9 and 10). The strength of cytochemistry lies in its ability to distinguish the average activity of a fraction from the specific organellar localization. In fact, granules (*in situ* or isolated) have no acid phos-

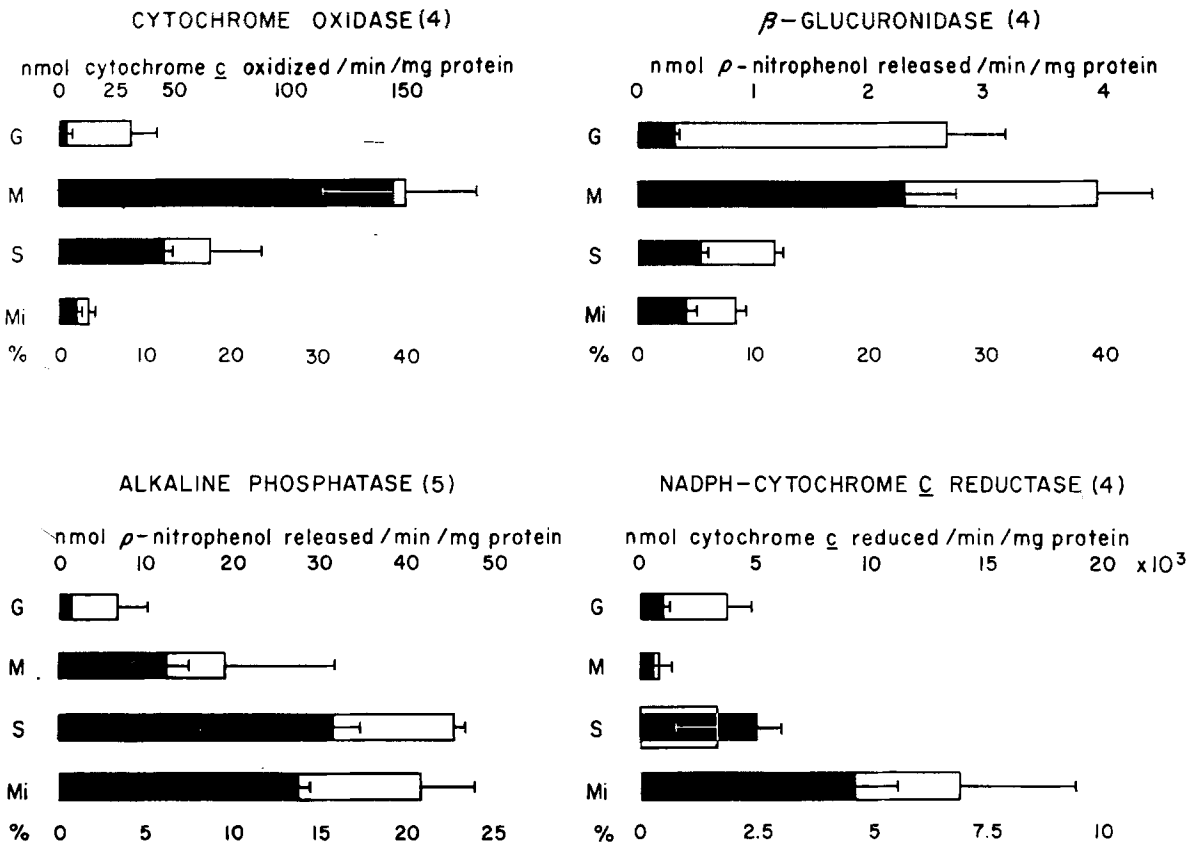


Fig. 5. Specific activities and recoveries of marker enzymes in organelle fractions. In each panel, the upper scale shows specific activity as nmol substrate altered/min/mg protein ($x \pm$ SE, □ – 1) and the lower scale shows that % recovery of that enzyme in a fraction as compared to homogenate ($x \pm$ SE, ■ – 1). Scales are different for each panel. Total recoveries (sum of all fractions compared to homogenate) were, in several experiments: protein, 82 ± 8% (4); alkaline phosphatase, 105 ± 8% (5) β-glucuronidase, 73 ± 12% (3); and NADPH-cytochrome *c* reductase, 52 ± 16% (4)

Table. Fold enrichment of marker enzymes

Enzyme activity	Percoll fractions			Microsomes
	Granules	Mitochondria	Surface membrane	
Cytochrome oxidase (5)	0.95 ± 0.30	5.28 ± 1.40	2.10 ± 0.13	0.41 ± 0.08
β-glucoronidase (3)	2.23 ± 0.24	3.07 ± 0.22	0.86 ± 0.03	0.90 ± 0.09
Alkaline phosphatase (6)	0.53 ± 0.07	1.10 ± 0.30	3.90 ± 0.90	4.30 ± 1.20
NADPH-cyt *c* reductase (4)	0.61 ± 0.22	0.41 ± 0.26	0.44 ± 0.12	2.50 ± 0.75

Enrichments ($x \pm$ SE) were calculated from the total activity in fraction/total activity in homogenate for the number of experiments shown in parenthesis.

phatase reaction product (Masur et al., 1971; Gronowicz, 1979) (Figs. 9, 10).

Mitochondria have a fivefold increase above the homogenate of the specific activity of the inner membrane constituent cytochrome oxidase (Table, Fig. 5). The lysosomal marker β-glucuronidase is increased threefold in the mitochondria fraction. Alkaline phosphatase and NADPH-cytochrome *c* reductase are removed from this fraction quite effectively with the endoplasmic reticulum marker activity less than half the homogenate activity, and retention of 6.6 ± 0.8% (4) of the homogenate protein.

Again, micrographic and biochemical data concur. The mitochondrial outer membranes appear intact and the inner membranes are often in the very

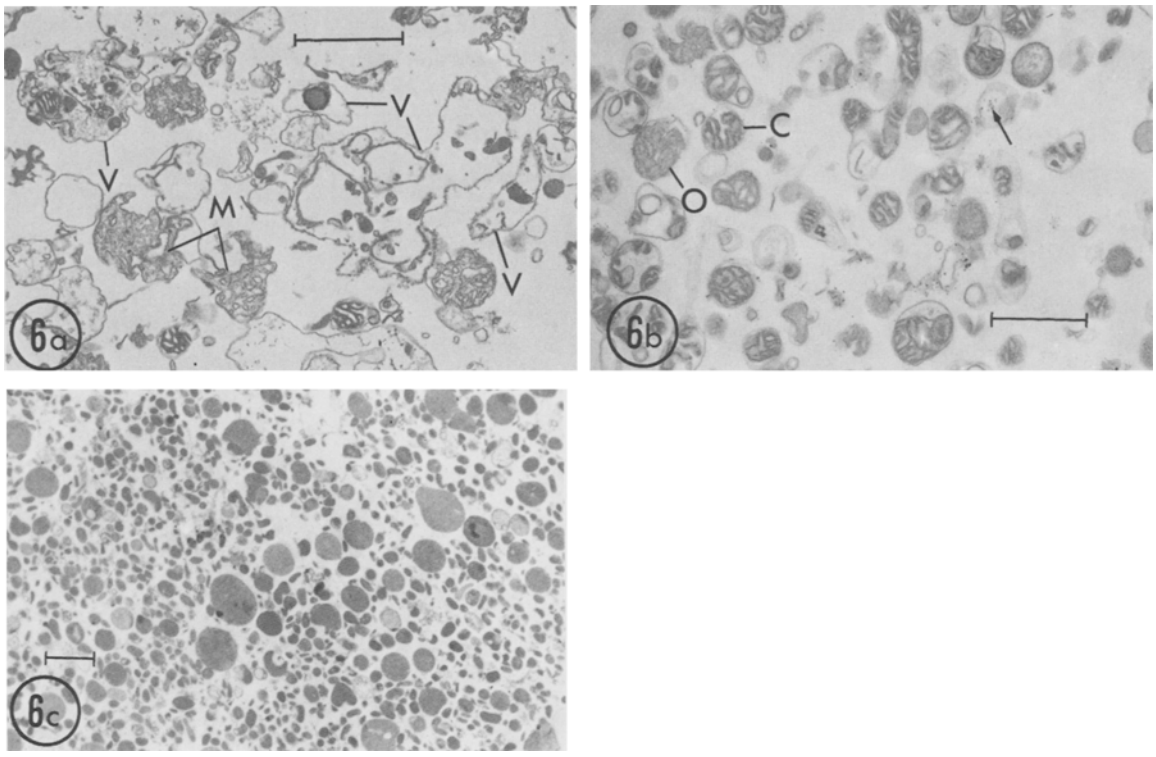


Fig. 6. Final gradient fractions after Percoll removal. (*a*) Surface membranes collected from Percoll gradient 3 at ρ = 1.02 g/ml. The membrane-bounded structures are mainly vacuoles (*V*) of heterogeneous diameter and contents (recognizable organelles, electron-dense material or nothing). Vacuole diameter ranges from 0.1 to 2.5 μm, usually ~0.8 μm. Occasionally amorphous material is associated with the outer surface of the vacuole membrane. *M* indicates mitochondrial fragments apparently lacking outer membranes. Uranyl acetate and lead citrate staining. × 12,250. (*b*) Mitochondria from Percoll gradient 3 at ρ = 1.06 g/ml. Mitochondria are the main components and are present in both orthodox (*O*) and condensed (*C*) forms. Some granules and other membranes are also present to a lesser extent. Percoll (arrow) within a membrane (sometimes a mitochondrion) suggests at least a transient opening in the membrane. Section stained with uranyl acetate and lead citrate. × 12,000. (*c*) Granules predominate in fractions of ρ = 1.12 g/ml from Percoll gradient 4. Note that the magnification is approximately half that of *a* and *b* in order to demonstrate the homogeneity of the fraction. Isolated granules are seen as rods or circular electron-dense structures after Percoll removal. The granules' major dimension (rod length or sphere diameter) ranges between 0.8 and 1.5 μm and the cross-section rod diameter is 0.2–0.5 μm. The granules are morphologically comparable to granules seen *in situ* (*see* Figs. 1 and 9) with 0.3 μm diameter (Choi, 1963) and up to 0.9 μm long. The circular profiles of 1.0–1.5 μm diameter seen here may be swollen granules and/or granules that fused during the isolation procedure and/or secretion droplets of mucous-secreting cells. Stained with uranyl acetate and lead citrate. × 6,500

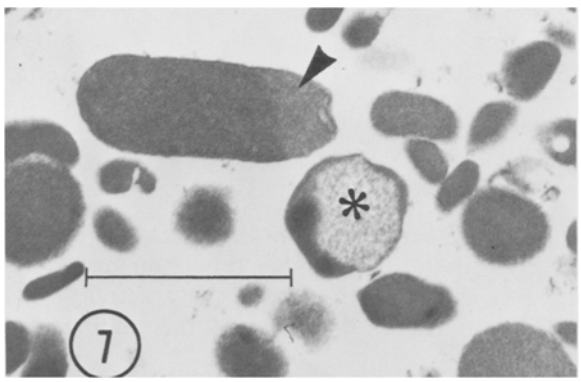


Fig. 7. Isolated granules. Where granule membrane is disrupted, contents are less electron dense (arrow) or fibrillar (*). Section stained with uranyl acetate and lead citrate. × 27,000

convoluted active configuration (Fig. 6*b*), with a few small (contaminating) vesicles or vacuoles (which may contain Percoll). Some lysosomes and granules are present. As noted above, reaction product from acid phosphatase cytochemistry does not stain the mitochondria but is seen in many of the membrane-bounded electron-dense bodies (Fig. 10).

Surface membranes (Fig. 6*a*) have about four-fold greater specific activity of the apical marker alkaline phosphatase than the homogenate. The fraction has relatively little NADPH-cytochrome *c* reductase but is significantly contaminated with cytochrome oxidase at an activity about 40% that of the mitochondrial fraction (Table, Fig. 5), suggesting that inner membrane fragments of damaged mi-

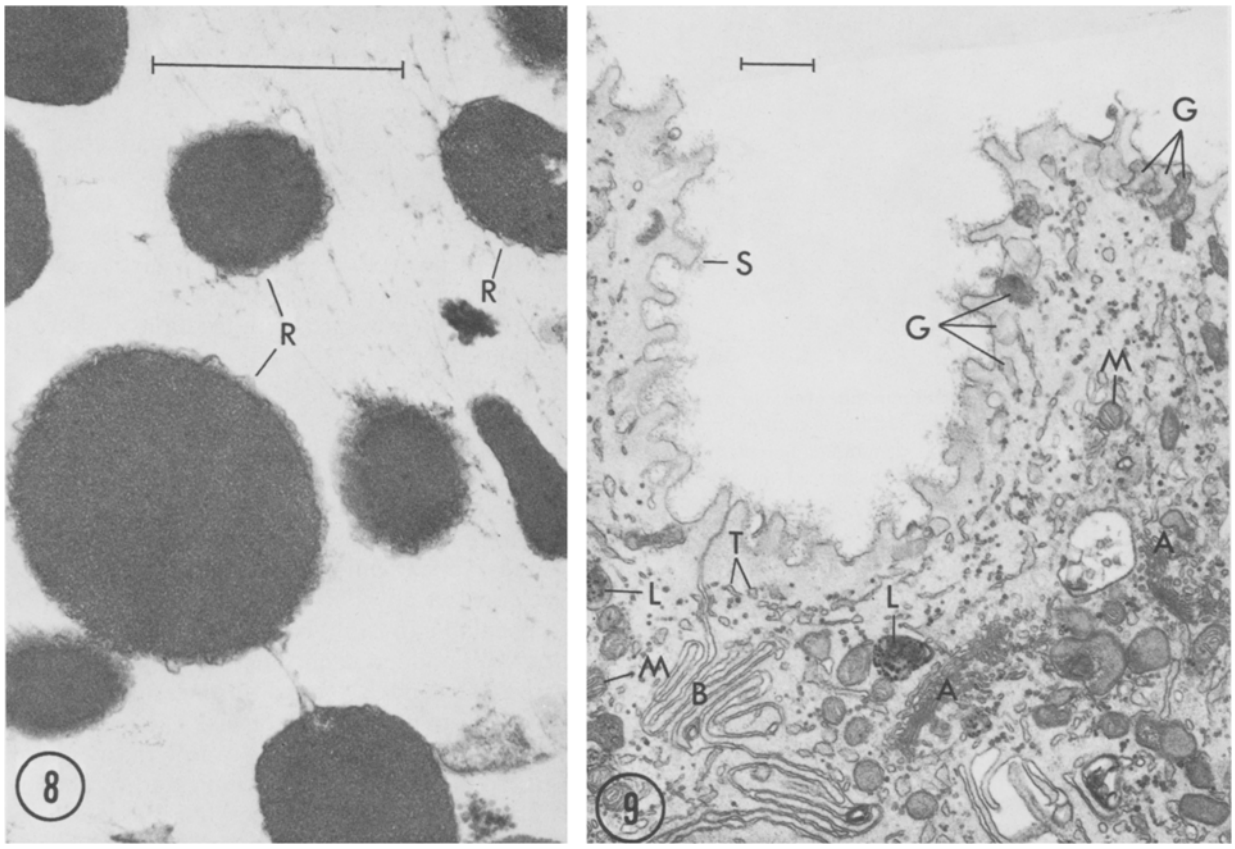


Fig. 8. Isolated granules. The limiting membrane of isolated granules is generally difficult to discern, but in some preparations is easily seen, may be ruffled, and may have attached microfilaments. Section stained with uranyl acetate and lead citrate. × 32,000

Fig. 9. Portions of adjacent G cells after cytochemical demonstration of acid phosphatase activity with β-glycerophosphate as substrate. β-glycerophosphatase activity identifies various membrane-bounded structures as lysosomes (*L*). Reaction product is absent from the apical surface (*S*), granules (*G*), membrane-bounded tubules (*T*), Golgi membranes (*A*), mitochondria (*M*) and a multivesicular body (*B*). Lead citrate stained section. × 9,100

tochondria are of low density. Surface membranes retain 6.4 ± 0.7% (4) of the homogenate protein.

Micrographs of the surface fraction show various membrane profiles. The long dimension of the large vacuoles ranges between 0.6 and 1.6 μm, and occasional vacuoles enclose mitochondria, granules, membrane-bounded electron-dense sausage or cup-shaped bodies, vesicles (~.09 by 1 μm), microfilaments, or small amounts of Percoll. Some of the long membrane sheets appear "fuzzy" on one surface, probably the glycocalyx. The presence of fragments of mitochondria, presumably with intact inner membrane, supports the biochemical finding of cytochrome oxidase activity (Fig. 6*a*).

Microsomes (Fig. 4) have as great a purification of the apical marker as do surface membranes, but the microsomal NADPH-cytochrome *c* reductase level is 2.5-fold more than in the homogenate, and fivefold greater than in the surface membranes (Fig. 5). The β-glucuronidase activity of the microsomes is about that in the homogenate (Table). This fraction contains 6.7 ± 1.2% (4) of the homogenate protein.

The microsomal fraction consists mainly of small vesicles, an average of 0.08 μm, some large membranous vacuoles (0.5 μm diameter) and few, if any, other intact organelles (Fig. 4).

ATPase Assays

As in previous studies of whole cells (Cortas & Walser, 1971), the ATPase in each of our final membrane fractions is only slightly inhibited by lack of K^+ (or Na^+) or by ouabain addition, even when contaminating mitochondrial ATPases are specifically inhibited (Rodriquez, 1977; M.S. Rubin, *unpublished observation*). Vanadate inhibits total

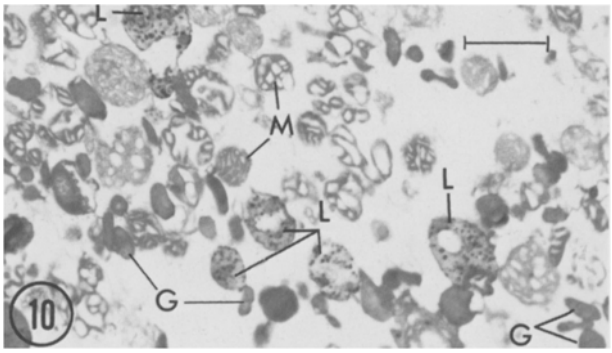


Fig. 10. GM pellet trapped on a Millipore filter for acid phosphatase demonstration. β-glycerophosphatase reaction product identifies as lysosomes (*L*) several membrane-delimited structures of varying electron density and is absent from mitochondria (*M*) and granules (*G*). Lead citrate stained section. × 11,250

ATPase by 50–60% in each fraction from toad urinary bladder epithelium. Although decreased sensitivity to or decreased specificity for the inhibitors is not unique to toad bladders (*see*, e.g., Werden, Bauriedel, Krawietz & Erdmann, 1984; Willis & Ellory, 1984), the lack of specific inhibitability in our fractions could be an artifact of the preparative techniques.

Cholesterol and Cholesteryl Ester Content

Granules contained much less free cholesterol than surface membranes, 82.6 ± 23.9 (5) nmol/mg protein vs. 252.1 ± 57.9 (6) ($P < 0.001$ by t test for unpaired samples). However, esterified cholesterol in granules, 44.1 ± 19.3 nmol/mg protein (5), did not differ from that in surface membranes, 31.1 ± 16.6 nmol/mg protein (6). Still, 35% of total cholesterol in granules is esterified, and only 11% in surface membranes. When compared to bovine pancreas cholesterol esterase, several microbial cholesterol esterases (from Miles, Sigma, US Biochemicals or Boehringer) released only small amounts of cholesterol in our fractions.

Polyacrylamide Gel Electrophoresis

Each organelle displays a distinctive banding pattern of membrane polypeptides (Fig. 11). Solubilization with 1% SDS and 1 to 2% 2-mercaptoethanol was incomplete. Granule and surface membrane fractions especially left silver-stainable material at the top of both stacking and separating gels and within the stacking gel. Earlier studies of surface membrane protein and glycoprotein structure in this epithelium had presaged such difficult solubilization (Rubin, 1977; M.S. Rubin, J.A.D. Weissman and C.F. King, *in preparation*). Solubilization is complete with ≥5% SDS and ≥5% 2-mercaptoethanol (final concentrations).

Bovine serum albumin usually was included in the isolation media to protect organelles against proteases and decrease aggregation. However, washing organelles with sucrose at low ionic strength did not remove albumin, which appeared as clear (overloaded) bands on silver-stained gels. The gels shown here are from fractions isolated in albumin-free media.

Granules contain relatively few silver-stained polypeptides (Fig. 11, lane *G*), and those are less sharp, more diffuse and almost blurry, as compared to other lanes. At least five times more Lowry-measured protein is needed to reach a staining intensity similar to that of other organelles. Several transparent bands appear during the silver-staining procedure (Merril et al., 1984) and develop very slowly with repeated exposure to silver. Since protease inhibitors were added each time the biological material was resuspended, band diffuseness probably did not result from partial proteolysis. The presence of polysaccharide chains bound to protein or lipid or free in the membrane may be indicated by the low apparent protein concentration on the gels, the clear areas and the diffuseness of the stained bands. In support of this, the apparent protein content of protein standards in the presence of Dextran 4000 (a model polysaccharide) increased >25–30% in the dye-binding assay (Read & Northcote, 1981). This is consonant with, though not to the extent of, the apparently higher levels of protein in granules determined by assay than found on the gel.

The lanes of special interest in Fig. 11 are the granules (*G*) and surface membranes (*S*) and for comparison, microsomes (*Mi*) and mitochondria (*M*). The major polypeptides in the *granule* fraction are at $M \sim 69$ (most evident), 104, 116, 138, 151, and 263 kD, and different preparations usually have some two of the bands of 219, 232 and 243 kD. If protease inhibitors are not added with *every* resuspension in fresh medium, all granule polypeptides of $M > 69$ kD are hydrolyzed, often completely, and major bands appear at 46, 38 and 19 kD, with minor bands at 71–72 kD. The 69-kD polypeptide does not appear to change in intensity after proteolysis during granule isolation.

The molecular weight range for *surface membrane* polypeptides is much wider than for the granules, but several bands are common to both (Fig. 11, *compare S* with *G*). The bands are very sharp, with many doublets (some doublets are indicated by a slash between 2 kD values). Some major bands are 18, 35, 40/41, 44/45, 46/48, 56, 69–70 (but less in-

tensely-stained than for granules), 75, 86, 105, 116, 120/124, 151, 203, 230 and 247 kD. There are many other fine bands, especially the many faint doublets between 116 and 195 kD. The distribution of polypeptides in the granules and surface membranes are quite dissimilar. After proteolysis during preparation, the most apparent stained polypeptide bands are at 105, 94, 79/75, 47, 41, 24 and 18 kD, even then in a much wider size range than present for granules.

The microsomal fraction (Fig. 11, lanes labeled *Mi* from two different preparations) is shown to indicate how different its polypeptides are from those of granules or of surface membranes, and how consistent from experiment to experiment. The most strongly stained microsomal polypeptides are at less than 46 kD, though there are many minor but sharply defined polypeptides of larger molecular weight.

The mitochondrial fraction (Fig. 6*b*, lanes labeled *M* from two different preparations) displays a pattern of polypeptide molecular weights quite unlike any of the other fractions.

Thus, polyacrylamide gel electrophoresis in dissociating solvent clearly differentiates among the various fractions, and shows that at this level of purification the granules display unique bands, at 138, 219 and 263 kD (as well as others) and have no polypeptides at lower molecular weights.

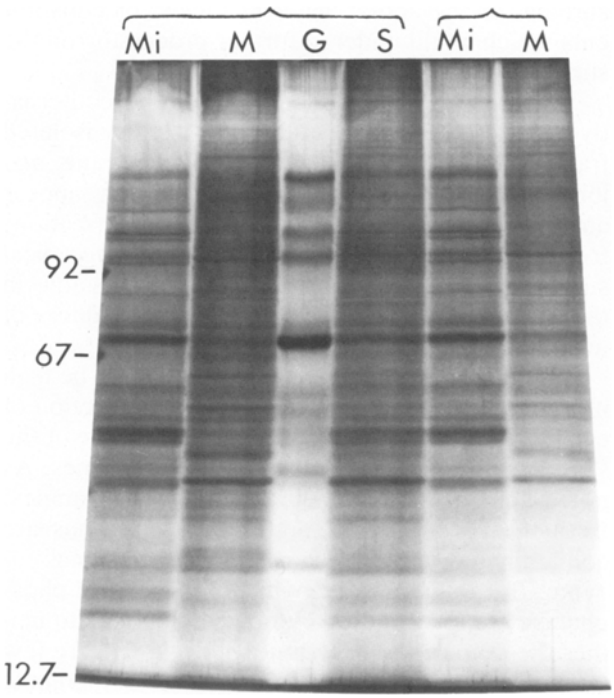


Fig. 11. Silver-stained SDS-polyacrylamide gel of urinary bladder organelle fractions. The brackets indicate four lanes of different fractions from one experiment and two lanes of organelle fractions from another experiment. The organelle fractions are microsomes (*Mi*), mitochondria (*M*), surface membranes (*S*) and granules (*G*). Migration positions of molecular weight standards phosphorylase *a*, bovine serum albumin, and horse heart cytochrome *c* are marked on the left (92, 67 and 12.7 kD)

Discussion

We designed this preparative scheme to separate and distinguish epithelial cell granules from the other membranous organelles, especially the surface membranes and fractions rich in both mitochondria and lysosomes, by taking advantage of density differences among organelles of similar size. We relied on morphologic observation (*EM*) and fraction density to develop the procedure, and then assayed characteristic enzymes to show the extent of removal of other organelles from the granules. In addition, gel electropherograms clearly show that the polypeptide composition of the granules is quantitatively and qualitatively different from those of fractions which are predominantly mitochondria, surface membranes, or microsomes. *In toto,* the data mutually indicate that isolated granules are a distinct, fairly pure organelle as yet without a specific biochemical marker.

Knowledge of granules has been limited to their fine structure and to cytochemical characterization of their glycoconjugates, which are similar to those of the apical surface (Choi, 1963; Gronowicz et al., 1980; Spicer et al., 1981) and appear to be their precursors (Pisam & Ripoche, 1973; Strum & Ekblad, 1977). Though the physiological role of the granules is unknown, morphological or (indirect) biochemical data indicate their involvement in the apical surface characteristics. Current models suggest ADH-stimulated hydroosmosis is modulated by ADH-induced exocytosis at the apical membrane of intracellular membranes (granules and/or tubules). Recovery of the rather impermeable state of the epithelium may well depend on subsequent endocytic retrieval of membrane from that surface.

As intracellular precursors to the apical surface, granules could provide apical glycoconjugates or store (glyco) proteins. Or, they could provide "average" apical membrane, in which case we would expect many common constituents in granules and surface membranes. (It is important to note that apical and basolateral surface membranes have not been separated.) Or, granules entering the surface in response to antidiuretic hormone could provide specific constituents necessary for the hydroosmotic response, e.g., membrane enhancing the placement of "water channels" into the apical

surface, or the actual water channels, or constituents which could undergo further processing on the surface, or be released into the urine.

Our findings distinguish between these alternatives only to the extent that granule in our isolated fraction are not "average" surface membrane; major polypeptides of the granules do not appear among those seen in surface membrane fractions from unstimulated toads. Furthermore, the apical surface membrane alkaline phosphatase is low in the granules in fractions isolated from bladders of unstimulated toads.

The surface enzymic marker also could be high if the granules were stored for reuse/destruction of endocytosed material, and then at least some of the "granules" might contain degradative enzymes. As noted above, the apical marker is low in granules. Both the mitochondrial and granule fractions are contaminated with lysosomes and a lysosomal enzyme. But the structures which possess acid phosphatase are localized by cytochemical assay to neither the granules nor the mitochondria and are few in number compared to the granules. Our granule fraction is quite homogenously made up of granules—with a small amount of contamination by mitochondria as determined by EM. In fact, the small degree of mitochondrial contamination of the granule fraction is borne out by the relatively low levels of mitochondrial inner membrane enzyme in the granule fraction and by the very dissimilar SDS-PAGE bond patterns in the granule and mitochondrial fractions.

Granules contain significantly less cholesterol than the surface membranes, but a higher proportion of cholesteryl ester. The effectiveness of mammalian, rather than microbial, esterase in releasing cholesterol may indicate that the fatty acid is polyunsaturated (Noël et al., 1983) and perhaps is a precursor to prostaglandins which act to terminate the ADH-dependent osmotic water flow.

We are still looking for other specific biochemical markers of the granule. On the other hand, we can now use these fractions to study the dynamic relationships of these membranous organelles.

We thank Drs. Victor Sapirstein, Eric Holtzman and William A. Brodsky, for advice and encouragement and Tana Ross, John Petrie, Daneen Rivero and James Norman, III, for help with organelle isolation procedures. We appreciate the accuracy of Cristina Matons' ATPase assays, and Maria Rodriguez' expert typing. This work was supported by U.S. Public Health Services, NIH Grant AM-25110 (SKM) and AM-18128 (MSR). SKM was a Research Career Development Awardee.

References

Baudhuin, P., Evrard, P., Berthet, J. 1967. Electron-microscopic examination of subcellular fractions. *J. Cell Biol.* **32:**181–191

Berenblum, I., Chain, E. 1938. An improved method for the colorimetric determination of phosphate. *Biochem. J.* **32:**205–298

Blumenfeld, O.O., Adamany, A.M. 1978. Structural polymorphism within the amino terminal region of MM, NN and NM glycoproteins (glycophorins) of the human erythrocyte membrane. *Proc. Natl. Acad. Sci. USA* **75:**2727–2731

Cantley, L.C., Josephson, L., Warner, R., Yanigisawa, M., Lechene, C., Guidotti, G. 1977. Vanadate is a potent Na^+, K^+-ATPase inhibitor found in ATP derived from muscle. *J. Biol. Chem.* **252:**7421–7423

Choi, J.K. 1963. The fine structure of the urinary bladder of the toad, *Bufo marinus*. *J. Cell Biol.* **16:**53–72

Coleman, R., Finean, J.B. 1966. Preparation and properties of isolated plasma membranes from guinea-pig tissues. *Biochim. Biophys. Acta* **125:**197–206

Cooperstein, S.J., Lazarow, A. 1951. A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.* **189:**665–670

Cortas, N., Walser, M. 1971. Na^+,K^+-activated ATPase in isolated mucosal cells of toad bladder. *Biochim. Biophys. Acta* **249:**181–187

Ernst, S.A., Riddle, C.V., Karnaky, K.A., Jr. 1980. Relationship between localization of Na^+,K^+-ATPase, cellular fine structure, and reabsorptive and secretory electrolyte transport. *Curr. Topics Membr. Transp.* **13:**355–385

Gamble, W., Vaughan, M., Kruth, H.S., Avigan, J. 1978. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. *J. Lipid Res.* **19:**1068–1070

Gronowicz, G.A. 1979. The quantitation of exocytosis and endocytosis during the hydroosmotic response in the urinary bladder of the toad. Ph.D. Thesis, Columbia University

Gronowicz, G., Masur, S.K., Holtzman, E. 1980. Quantitative analysis of exocytosis and endocytosis in the hydroosmotic response of toad bladder. *J. Membrane Biol.* **52:**221–235

Holtzman, E. 1975. Lysosomes: A Survey. Springer-Verlag, Vienna—New York

Horton, A.A. 1968. A simple procedure for the preparation of reduced cytochrome. *Anal. Biochem.* **23:**334–335

Karnovsky, M.J. 1971. Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. American Society for Cell Biology. Abstracts. 11th Annual Meeting, New Orleans

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275

Luft, J.M. 1961. Improvements in epoxy resin embedding methods. *J. Biochem. Biophys. Cytol.* **9:**409–414

Maizel, J.V., Jr. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Meth. Virol.* **5:**179–246

Masur, S.K. 1969. Fine structure of the autotransplanted pituitary in the red eft, *Notophthalmus viridescens*. *Gen. Comp. Endocrinol.* 12:12–32.

Masur, S.K., Gronowicz, G., Holtzman, E. 1979. Bulk membrane movement and hydroosmosis in the toad urinary bladder. *In*: Hormonal control of epithelial transport. *INSERM* **85:**159–166

Masur, S.K., Holtzman, E., Schwartz, I.L., Walter, R. 1971. Correlation between pinocytosis and hydroosmosis induced by neurohypophyseal hormones and mediated by adenosine 3',5'-cyclic monophosphate. *J. Cell Biol.* **49:**582–594

Merril, C.R., Goldman, D., VanKeuren, M.L. 1984. Gel protein stains: Silver stain. *Meth. Enzymol.* **104:**441–447

Michell, R.H., Karnovsky, M.J., Karnovsky, M.L. 1970. The distribution of some granule-associated enzymes in guinea-pig polymorphonuclear leucocytes. *Biochem. J.* **116:**207–216

Noel, S.P., Dupras, R., Filion, A.M. 1983. The activity of cholesterol ester hydrolase in the enzymatic determination of cholesterol: Comparison of 5 enzymes obtained commercially. *Anal. Biochem.* **129:**464–471

Percoll: Methodology and Applications. 1982. pp. 1–64. Pharmacia Fine Chemicals, Uppsala

Phillips, A.H., Langdon, R.G. 1962. Hepatic triphosphopyridine nucleotidecytochrome *c* reductase: Isolation, characterization and kinetic studies. *J. Biol. Chem.* **237:**2652–2660

Pisam, M., Ripoche, P. 1973. Renouvellement des glycoproteins de surface: Etude radioautographque de l'epithelium de la vessie de grenouille. *J. Microsc.* **17:**261–282

Pisam, M., Ripoche, P. 1976. Redistribution of surface macromolecules in dissociated epithelial cells. *J. Cell Biol.* **71:**907–920

Pisam, M., Ripoche, P., Rambourg, A. 1970. Etude ultrastructurale des charges negatives de la surface apical de l'epithelium vesical de la grenoville: leur modifications sous l'action de l'oxytocine. *Comptes Rend. Acad. Sci. (Paris)* **D271:**105–107

Read, S.M., Northcote, D.H. 1981. Minimization of variation in the response to different proteins of the Coomassie Blue G dye-binding assay for proteins. *Anal. Biochem.* **116:**53–64

Reynolds, E.S. 1963. The use of lead-citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17:**208–212

Robbie, D.E. 1971. Ph.D. Thesis, Harvard University

Robinson, J.M., Karnovsky, M.J. 1983. Ultrastructural localization of 5′-nucleotidase in guinea pig neutrophils based upon the use of cerium as capturing agent. *J. Histochem. Cytochem.* **31:**1190–1196

Rodriguez, H.J. 1977. Covalent Labeling and Isolation of Apical and Basal-Lateral Plasma Membranes of Toad Bladder Epithelium. Ph.D. Thesis, University of California

Rubin, M.S. 1977. Control of urea transport across toad urinary bladder by vasopressin. *J. Membrane Biol.* **36:**33–54

Rubin, M.S., Gronowicz, G., Cooper, S., Masur, S.K. 1982. Comparison of isolated dense sub-apical granules and surface membrane of toad urinary bladder cells. *J. Cell Biol.* **95:**263*a*

Sottacasa, G.L., Kuylenstierna, B., Ernster, L., Bergstrand, A. 1967. An electron-transport system associated with the outer membrane of liver mitocondria. *J. Cell Biol.* **32:**415–438

Spicer, S.S., Baron, D.A., Sato, A., Schulte, B.A. 1981. Variability of cell surface glycoconjugates—relation to differences in cell function. *J. Histochem. Cytochem.* **29:**994–1002

Strum, J.M., Ekblad, E.B.M. 1977. ^{3}H-fucose incorporation into glycoproteins of toad bladder epithelial cells. *Am. J. Anat.* **148:**33–48

Vincent, R., Nadeau, D. 1983. A micromethod for the quantitation of cellular proteins in Percoll with the Coomassie brilliant blue dye-binding assay. *Anal. Biochem.* **135:**355–362

Wade, J.B., DiScala, V.A., Karnovsky, M.J. 1975. Membrane structural specialization of the toad urinary bladder revealed by the freeze-fracture technique: I. The granular cell. *J. Membrane Biol.* **22:**385–402

Watson, M.L. 1958. Staining of tissue sections for EM with heavy metals. *J. Biophys. Biochem. Cytol.* **4:**475–478

Werdan, K., Bauriedel, G., Krawietz, W., Erdmann, E. 1984. Inhibitory and stimulatory effects of vanadate on sodium pump of cultured heart cells from different species. *Curr. Topics Membr. Transp.* **19:**939–943

Wilfong, R.F., Neville, D.M., Jr. 1970. The isolation of a brush border membrane fraction from rat kidney. *J. Biol. Chem.* **245:**6106–6112

Williams, M.A. 1977. Quantitative methods in Biology. *In:* Practical Methods of Electron Microscopy. Vol. 6, pp. 1–234. A.M. Glauert, editor. North-Holland, New York

Willis, J.S., Ellory, J.C. 1984. Ouabain sensitivity: Diversity and disparities. *Curr. Topics Membr. Transp.* **19:**277–280

Received 22 August 1985