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Isolation and Compositional Analysis of Secretion Granules and their Membrane Subfraction from the Rat Parotid Gland

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Summary. A secretory granule fraction has been isolated from rat parotid by discontinuous gradient centrifugation using hyperosmotic sucrose-Ficoll solutions of low ionic strength. The secretion granule fraction comprises 25% of the total tissue α-amylase activity and is judged to be of high purity, both morphologically and by its low level of contamination by enzyme activities associated with other organelles.

Secretion granules were lysed by capitalizing on their lability in KCl-containing media, and the low density granule membranes were separated from residual organelle and soluble contaminants by flotation in a sucrose gradient. Residual, poorly extractable secretory contaminants of the granule membrane subfraction were selectively removed by a saponin- (10 μg/ml) Na_2SO_4 (0.3 M) wash, apparently with negligible disruption of granule membrane structure. Based on detailed consideration of the extent of contamination by residual mitochondria and incompletely removed secretory polypeptides, it is possible to estimate that ~95% of the protein associated with the purified secretion granule membrane is bona fide granule membrane protein. Further analyses indicate that γ-glutamyltransferase constitutes a marker enzymatic activity shared by granule membranes and the apical domain of the plasma membrane.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretograms of radio-iodinated granule membrane polypeptides are characterized by 20–25 radioactive bands of which 5–6 are suggested to be glycoproteins by virtue of their binding of concanavalin A. The limited polypeptide composition of the secretion granule membrane (in comparison to membranes of other cellular compartments) and the high phospholipid-protein ratio (4.4 mg/mg) may reflect the functional specialization of this storage container for secretory proteins.

Key Words parotid · secretory granule membranes · exocytosis · secretion

Introduction

Within exocrine acinar cells secretion granules constitute repositories for macromolecules destined for export in response to external stimuli. Individual elements comprising this storage compartment are bounded by unit membranes and arise as a consequence of molecular sorting and packaging processes operating at the level of the Golgi complex. Mobilization from storage and discharge of secretory macromolecules is achieved by exocytosis, a process involving the selective recognition, interaction, and eventual coalescence of granule membrane with the apical domain of the plasma membrane. As in the case of other structurally distinct endomembrane compartments, the molecular composition of the granule membrane is expected, in large part, to reflect specific intracellular functions.

The dimensions of individual secretion granules and the size of the stored granule population vary greatly among cell types. Consequently, certain tissues may be especially favorable as sources for the preparation of secretion granules and their membranes for subsequent compositional and functional analyses.

According to these considerations, the rat parotid salivary gland has been chosen as a tissue source for the preparation and characterization of secretion granules and their membranes. Acinar secretory cells comprise 85–90% of the glandular volume, and the stored granule population occupies approximately one-third of the cytoplasmic volume [7]. Lipolytic and proteolytic activities, which potentially could interfere in analyses of granule membrane composition, have not been detected among the secretory products [28, 64]. The preparation of a highly purified granule fraction is described in this report. Further, the partial characterization of the membrane subfraction has established a comprehensive catalog of tightly associated membrane polypeptides (resolved by one-dimensional SDS polyacrylamide gel electrophoresis) and has indicated that γ-glutamyl transferase (EC-2.3.2.2) constitutes a granule membrane-associated enzyme activity. The considerable effort invested to validate the purity of this fraction makes possible future detailed analysis of potential relationships of selected polypeptides to granule-specific functions and has estab-

lished an unambiguous compositional probe for evaluating quantitatively both the level of polypeptide overlap for plasma membrane isolated from the same tissue [2] and the dynamic behavior of granule membranes during secretory content storage, exocytosis, and compensatory reinternalization events.

Materials and Methods

Preparation of Parotid Secretion Granules

The protocol and rationale for isolation of parotid secretion granules and subsequent preparation of granule membranes and secretory content will be presented in the Results section.

Preparation of Parotid Mitochondria

A mitochondrial fraction was prepared from parotid tissue using as starting material the 0.35 M/1.42 M + 4% Ficoll 400 interface of granule gradient 1 (*see* Fig. 1). This interface suspension was adjusted to a final sucrose concentration of 0.80 M (by refractive index), resuspended by 5 strokes in a tight-fitting Dounce homogenizer, and overlaid on a first sucrose step gradient having underlayers of 1.0 and 1.42 M sucrose, each containing 4% Ficoll 400, 2.0 mM imidazole, 5.0 mM EDTA and 0.20 μg/ml N, N'-diphenyl-*p*-phenylenediamine (DPPD)[1] at pH 6.7. Centrifugation (1.3×10^4 g_{av} $\times$ 10 min in a Beckman SW27 rotor) yielded a crude mitochondrial fraction at the 1.0–1.42 M interface. The majority of microsomal and plasmalemmal elements banded at the load −1.0 M sucrose interface. Subsequent purification of the crude mitochondrial fraction utilized the following steps: sedimentation from 1.0 M sucrose onto an interface of 1.42 M sucrose + 4% Ficoll 400; adjustment of the mitochondrial fraction collected from this interface to 1.58 M sucrose (by refractive index) and flotation through 1.42 M + 4% Ficoll 400 to an overlying interface of 0.80 M sucrose (centrifugation conditions for each step—1.5×10^5 g_{av} $\times$ 90 min in a Beckman SW41 rotor). Sedimentation (1.5×10^5 g_{av} $\times$ 45 min) yielded a purified parotid mitochondrial fraction free of secretion granules, nuclear and plasma membranes.

Processing of Subcellular Fractions for Electron Microscopy

Secretion granules, mitochondria, and secretion granule membranes were fixed in suspension by the slow addition of one volume of 6% glutaraldehyde, 2% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M Na phosphate, pH 7.4. Samples were then pelleted in a microcentrifuge and postfixed in 1% OsO_4 for 2 hr at 0°C. Subsequently, pellets were stained in block with 0.5% uranyl acetate in maleate buffer, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Thin sections were stained in both uranyl acetate and lead citrate. All micrographs were taken on a Siemens Elmiskop 101 or 102.

Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was carried out according to Laemmli [42]. Resolving gels contained a 6–12 (or 14% (wt/vol)) linear gradient of acrylamide and were overlaid with a 4.50 acrylamide stacking gel.

All protein samples were reduced with 40 mM dithiothreitol (5 min at 100°C) and alkylated with 100 mM iodoacetamide (at 37°C for 60 min). After electrophoresis the gels were fixed and stained in 0.08% Coomassie brilliant blue (R-250), 25% (vol/vol) isopropanol + 10% (vol/vol) acetic acid, and then destained sequentially in 10% (vol/vol) isopropanol + 10% (vol/vol) acetic acid, and 10% (vol/vol) acetic acid alone.

For radio-iodinated secretion granule membrane samples, destained and dried gels were autoradiographed by exposure to Kodak X-Omat film at −70°C using an intensifying screen. Silver staining of polyacrylamide gels was performed using a modified procedure of Merril [53].

Method of Iodination

Membrane samples were radioiodinated using either 1, 3, 4, 6-tetrachloro3α, 6α diphenyl glycoluril (Iodogen) [45] or the Bolten-Hunter reagent [9]. Iodogen (1 mg) was dissolved in chloroform (20 ml), and 200 μl of the resulting solution was evaporated to dryness under nitrogen in a borosilicate vial. After rinsing in 50 mM Tris-HCl (pH 7.4), 50 μl of the same buffer was added to the iodination vial, followed by $Na^{125}I$ (500 μCi, 5 μl) and 50 μl of membrane suspension. The iodination was allowed to procede for 10 min at 4°C and terminated by removal of the membrane suspension. Gel filtration on a 5-ml column of Agarose A-0.5 M (in 50 mM Tris-HCl, pH 7.4) served to separate labeled membranes from unincorporated molecular iodine. Where the Bolton-Hunter reagent was used, granule membranes (suspended to a volume of 40 μl in 100 mM potassium phosphate at pH 8.3) were added to the reaction vial (500 μCi), and the mixture was agitated for 60 min at 4°C. Gel filtration on a 5-ml column of Agarose A 0.5 M (in 100 mM potassium phosphate, 200 mM glycine at pH 8.3) allowed separation of labeled membranes from unincorporated reagent quenched with glycine.

Concanavalin A (1 mg dissolved in 200 μl of 50 mM Tris-HCl containing 0.2 M dextrose and 0.5 mM each of $MnCl_2$, $MgCl_2$ and $CaCl_2$ at pH 7.4) was radioiodinated using Chloramine-T (20 μl, 2.5 mg/ml) and 1.0 mCi $Na^{-125}I$. The reaction was allowed to procede for 2.5 min at 4°C and terminated by the addition of sodium metabisulfite (20 μl, 2.5 mg/ml). Labeled Concanavalin A was separated from unincorporated molecular iodine by gel filtration on Biogel P-2.

Enzymatic and Chemical Assays

α-Amylase was assayed according to Bernfeld [6]; 1 U of amylase activity is defined as the amount of enzyme which liberates 1 μmol of maltose equivalents per min at 30°C. Glutamyl transferase (transpeptidase) was assayed after Tate [70] using γ-glutamyl-*p*-nitroanilide and glycylglycine as substrates at 25°C; 1 U of activity corresponds to the release of 1 μmol of *p*-nitroaniline per min. Amine oxidase (Type A) was assayed by the procedure

[1] N,N'-diphenyl-*p*-phenylenediamine (DPPD) is an antioxidant agent [31] previously demonstrated to maintain secretion granule intactness during cell fractionation [74].

described by Castro Costa [18] using H^3-tryptamine as the substrate. Enzyme activity is expressed as pmol/min unless indicated otherwise. Cytochrome *c* oxidase was assayed by the method of Peters [57] using the first-order rate constant as a measure of activity [47]. [H^3]-UDP-galactosyl transferase was determined using a modification of the assay described by Fleischer [23] in which 2 mM ATP and 2 mg/ml asialoagalacto-fetuin [38] were included; acid precipitable ^{3}H-label was used as a measure of activity expressed as nmol/min. β-N-acetyl glucosaminidase was assayed according to Findlay [22] by measuring the formation of *p*-nitrophenol (ΔOD_{410}) from *p*-nitrophenyl-N-acetyl-β-glucosaminide at 37°C; 1 U of activity corresponds to the liberation of 1 μmol of substrate per min.

Total protein was assayed according to the modified Lowry protocol of Markell [46] using bovine serum albumin as a standard.

Phospholipids of subcellular fractions were extracted using the procedure of Folch [24] and were ashed in 70% perchloric acid and assayed for phosphate as described by Bartlett [4].

Materials

[^{3}H]tryptamine and iodine-125 were obtained from Amersham (Arlington Heights, IL); Bolton-Hunter reagent and [^{3}H]uridine diphosphogalactose from New England Nuclear (Boston, MA). Iodogen was obtained from Pierce Chemical Co. (Rockford, IL). Bio-Gel A-0.5 M and molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, CA). Eagle's minimal essential medium or Ham's F-12 used for *in vitro* incubations and cleaning of tissue were obtained from GIBCO (Grand Island Biological Co., Grand Island, NY). Acrylamide for gel electrophoresis was obtained from Eastman Organic Chemicals (Rochester, NY) and recrystallized (1×) from acetone before use. Concanavalin A was obtained from Calbiochem (La Jolla, CA). Ficoll 400 was obtained from Pharmacia (Piscataway, NJ). All other chemicals were of reagent grade. Nitex nylon mesh (20 μm^2) was obtained from Tetko, Inc. (Elmsford, NY).

Results

Preparation of a Parotid Secretion Granule Fraction

In order to begin to assess the potential roles of granule membrane components in distal events along the secretory pathway we sought to isolate a secretion granule fraction of high purity and yield. We considered the levels of contamination obtained using previously published procedures for the isolation of rat parotid secretion granules [1, 39, 58] to be unsatisfactory. Our goal was met by devising a different procedure shown schematically in Fig. 1.

The extent to which we have succeeded in isolating a highly purified, homogeneous population of rat parotid secretion granules depends on the use of sucrose media having low ionic strength. Increases in imidazole or EDTA concentration increased organelle aggregation and resulting in two- to three-fold higher mitochondrial enzymic activities in the purified secretion granule fraction. Although Mg^{2+} ions are included in the homogenization medium in order to stabilize nuclei, further elevations in concentration increased secretion granule-mitochondrion aggregation and, consequently, significantly increased the mitochondrial contamination of the purified secretion granule fraction.

Morphology of the Secretion Granule Fraction

A representative, low magnification electron micrograph of the secretion granule fraction is shown in Fig. 2. The majority of components (>99%) are large (1 μm diameter) electron opaque, spherical secretion granules that, at higher magnifications, are found to be bounded by a continuous unit membrane. Small membrane blebs, often continuous with the granule membrane, and small, smooth surfaced vesicles are variably present in individual preparations. At present we feel that the generation of membrane blisters is an artifact arising as a consequence of aldehyde fixation. Occasional partially vesiculated and extracted mitochondria located throughout the granule pellet and even fewer nuclear membranes found at the top of the pellet constitute the only visible organelle contaminants.

Enzymatic Analyses for Contaminating Organelles

Each of the sucrose layers and sucrose interfaces generated in the parotid secretion granule isolation protocol was assayed for the presence of enzymatic activities considered to mark contaminating organelles. For the sake of clarity, the data for individual fractions other than that containing the bulk of the granules have been combined and reported as a single value for each step (Table 1). Recoveries of enzymatic activities are based on the values obtained for the preceding fraction and % distributions are relative to the total homogenate activity (*H*) which is the sum of (*NS*) and (*NP*) (defined in the legend of Fig. 1).

Mitochondria constitute the only significant contaminant of the secretion granule fraction detectable biochemically; 0.8–0.9% of the cytochrome *c* oxidase and 0.2–0.3% of the amine oxidase activities present in the homogenate are recovered in the final fraction. The discrepancy in the % contaminant contribution for the two mitochondrial membrane activities reflects the susceptibility of the outer mitochondrial membrane to vesiculation and

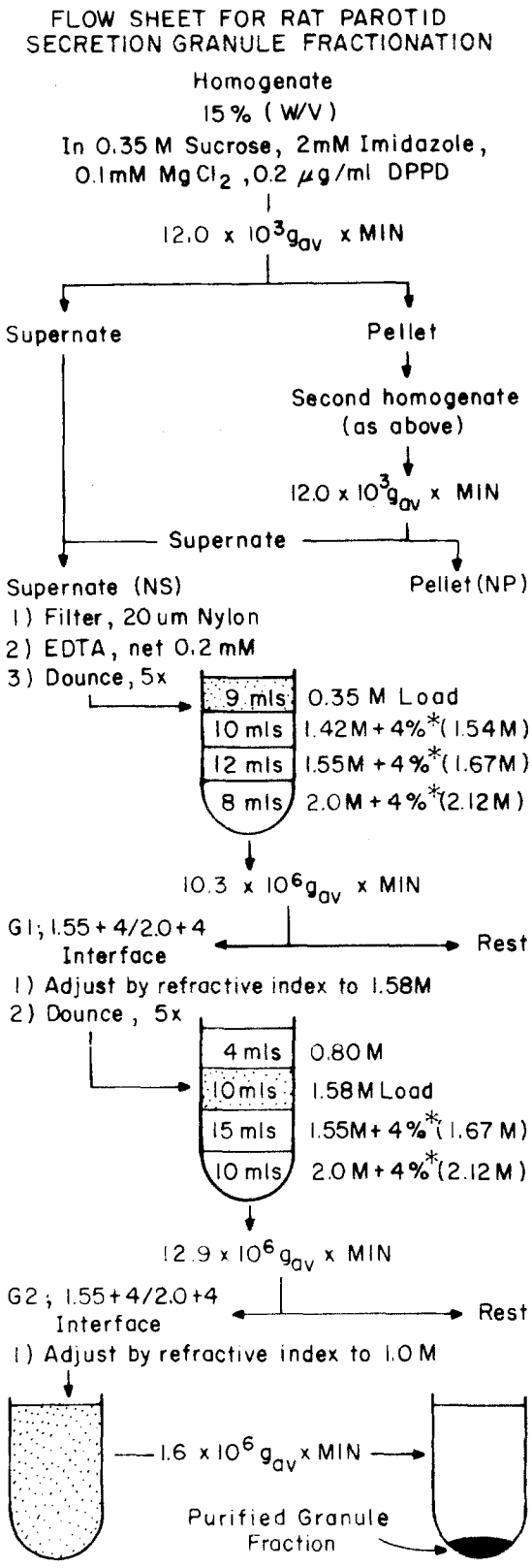

Fig. 1. Isolation protocol for purification of rat parotid secretion granules from 10–12 male Sprague-Dawley rats (100–125 g). For initial homogenization, cleaned tissue (4.0–4.5 g) was subjected in sequence to mincing with razor blades and disruption using a Tissuemizer® (Tekmar; Cincinnati, Ohio) for 10 sec at 1900 rpm followed by 5 passes at 1300 rpm in a Brendler cylindrical glass homogenizer with serrated Teflon pestle. The second homogenization was performed with the Brendler homogenizer only. Low speed centrifugation (used to prepare the post-nuclear supernate (*NS*) and nuclear pellet (*NP*)) was performed in an IEC PR6000 centrifuge. Subsequent ultracentrifugation, which yielded, first, a crude granule fraction freed of the majority of lower density mitochondria, plasma membranes, and smaller organelles and, second, a purified granule fraction was carried out in a Beckman SW 28 rotor. All high density sucrose solutions contained 4% Ficoll 400 in addition to 2.0 mM imidazole, 0.2 mM EDTA, and 0.20 μg/ml DPPD (pH 6.7). *The concentrations shown in parentheses are determined by refractive index where 4% Ficoll contributes an increment corresponding to 0.12 M sucrose

breakage during density gradient centrifugation[2] [8]. The final cytochrome *c* oxidase specific activity is one-tenth that of the homogenate, while amine oxidase is 0.03 of the homogenate specific activity.

Lysosomal contamination as estimated by the activity of β-N-acetyl glucosaminidase is on the order of 1% of the homogenate activity with a specific activity one-tenth that of the homogenate. Contamination by UDP-galactosyl transferase, an enzyme associated predominantly with the Golgi complex, is approximately 0.10% of the homogenate activity with the specific activity in the granule fraction being 1/100 that of the homogenate. Finally, although not shown in Table 1, the purified granule fraction has been found to contain <0.7% of the total rotenone-insensitive NADH-cytochrome *c* reductase activity. Since in some cell types this activity marks rough endoplasmic reticulum [37], Golgi, outer mitochondrial membranes [11], and even plasma membranes [37], the negligible levels found in the granule fraction signify a generally low level of contamination by other cellular organelles. Thus, limited marker enzyme analyses corroborate the morphological observations that mitochondria constitute the principal low-level contamination of the purified secretion granule fraction.

Marker Enzymes of the Secretion Granule

Assuming α-amylase approximates an exclusive and homogeneous marker enzyme for rat parotid

[2] The alternative hypothesis that amine oxidase activity associated with the purified secretion granule fraction is underestimated is considered less likely because the activity is quantitatively recovered in subsequent steps during granule subfractionation.

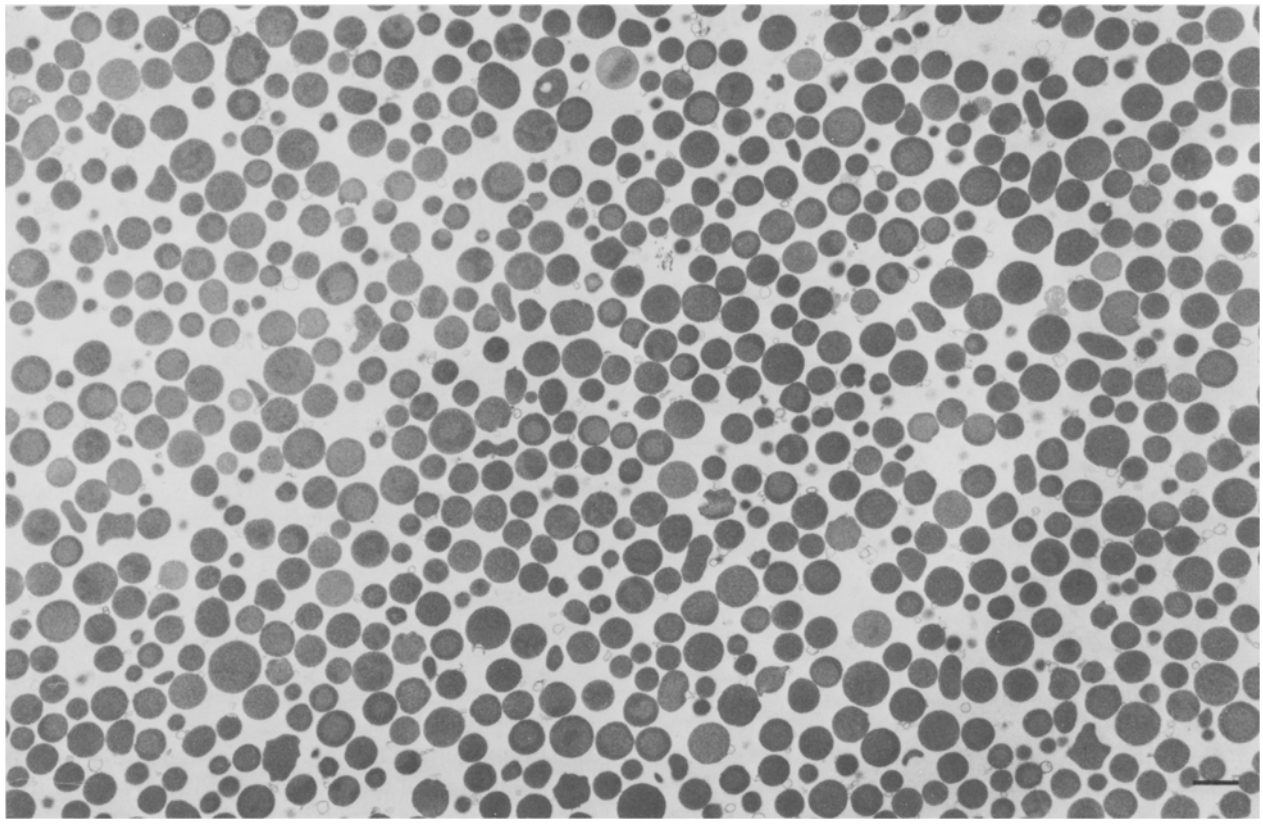

Fig. 2. Representative electron micrograph of the purified secretion granule fraction. Bar, 1 μm × 6,300

secretion granules, we routinely recover 25–27% of the total homogenate activity in the purified secretion granule fraction (Table 2). The amylase specific activity of the granule fraction, normalized to protein, averaged 2.85 times that of the homogenate[3]. Analogous to results previously reported by Wallach [74], the inclusion of the antioxidant DPPD in all isolation media approximately doubled the recoveries of α-amylase in the purified granule fraction. 25–30% of the total amylase activity of the homogenate remains in the 0.35 M load following the first discontinuous gradient centrifugation and is presumed to represent amylase solubilized from granules broken during homogenization. The 25% amylase recovery in the purified granule fraction therefore represents 60% of the intact granules present in the parent fraction (*NS*); barring the possible existence of a special granule subpopulation, which exhibits poor stability during homogenization, we consider our granule fraction to be representative of the total parotid secretion granule population.

γ-glutamyl transferase (γGT), an enzyme characteristically associated with the mucosal surface membranes of a variety of polarized epithelia, is present in our parotid homogenates at a low level of 0.015 μmol/min/mg protein, a value comparable to that reported in the literature—0.016 μmol/min/mg protein [29]. As shown in Table 2, we have found that the purified secretion granule fraction contains 3–4% of the γGT activity of the homogenate. According to the assumption that this enzyme is a bona fide granule membrane protein, we estimate,

[3] Immunocytochemical studies indicate that all secretion granules present in rat parotid acinar cells contain much higher concentrations of α-amylase than do other organelles comprising the biosynthetic pathway for secretory proteins [68]. Secretion granules occupy ~35% of the parotid acinar cell volume in fasted animals [7], which in turn represents 85–90% of the gland volume. If amylase is taken to be an exclusive granule marker and if protein is considered to be distributed homogeneously in proportion to volume, then the expected purification for granule amylase is: $\frac{\%\text{ total amylase in granules}}{\%\text{ total protein in granules}} / \frac{\%\text{ total amylase in homogenate}}{\%\text{ total protein in homogenate}} = 100/(0.9 \times 35)/(100/100) = 3.2$. Values less than 3.2 can be rationalized according to the observation that amylase is not an exclusive granule marker and that the concentration of protein within granules is higher than in other tissue compartments (and thus is disproportionately high with respect to volume occupied).

Table 1. Marker enzyme analysis of contaminating organelles in secretion granule preparation

Fraction	Protein		β-N-acetyl glucosaminidase		Cytochrome *c* oxidase		Amine oxidase type A		UDP-galactosyl transferase	
	mg	% *H*	% *H*	RSA[a]	% *H*	RSA	% *H*	RSA	% *H*	RSA
Homogenate (*H*)[b]	397	100	100	1 (4.62)[c]	100	1 (100.23)	100	1 (104.61)	100	1 (3.62)
NS	265.5	66.9	64.3	0.93	80	1.10	74.9	1.09	79.3	1.13
NP	131.7	33.1	35.7	1.14	20	0.73	25.1	0.80	20.7	0.65
*G*1	46.6	12.0	2.4	0.18	2.4	0.21	2.8	0.20	0.26	0.02
Gradient recovery %		99.8	97		90.6		97.4		90.8	
*G*2	31.5	8.2	1.1	0.12	0.89	0.10	0.30	0.03	0.12	0.01
Gradient recovery %		104.5	92.4		90.1		64.1		97.9	

[a] RSA = sp act (per mg protein) relative to that of the homogenate (*H*).

[b] Abbreviations for isolated fractions are as in Fig. 1.

[c] Numbers in parentheses represent total homogenate activities. Enzyme activity units are: μmol/min, β-N-acetylglucosaminidase; nmol/min, monoamine oxidase and UDP-galactosyl transferase. Cytochrome *c* oxidase is expressed in units corresponding to the first rate constant [47]. Recoveries in each fraction represent the mean value from a minimum of 3 experiments.

based on the ~25% yield of secretion granules discussed previously, that ~14–15% of the total rat parotid γGT activity could be granule associated. As secretion granules comprise a minimum of 30–35% of the total parotid protein (since their fractional contribution to acinar cell volume is probably less than their contribution to total parotid protein), granule purification should lead to a depurification (decreased specific activity) of γGT. Table 2 shows that the specific activity of γGT in the purified granule fraction is 0.43 times the homogenate value.

In order to evaluate the alternative possibility that γGT activity associated with the purified secretion granule fraction reflects a low level of contamination by plasmalemmal elements (in which case apparent depurification would also be expected), we have compared the distribution of α-amylase and γGT for *NS* fractions of parotid tissue that had been subjected to centrifugation on linear sucrose gradients supplemented with Ficoll 400 (5% wt/vol). Figure 3 shows that there are principally two peaks of both α-amylase and γGT activity. The lower density amylase peak is found in the region of the original load (fractions 2 to 4) and is presumed to represent soluble enzyme released from damaged organelles during homogenization. The lower density γGT peak (fractions 3 to 9) contains most of the *NS* γGT activity and is located within the first half of the gradient. Both low density enzyme peaks are well separated from the high density coincident peaks of α-amylase and γGT activity that characterize the sedimented secretion granules. The constant activity ratio found for the two enzymes across this peak (*see* inset in Fig. 3) argues that both enzymes mark the same high density organelle. We view unlikely the possibility that plasmalemmal elements (which characteristically exhibit densities <1.20) cosediment in completely parallel fashion with the secretion granules. Since purified parotid mitochondria (Fig. 7) and rough microsomes [2] do not contain significant levels of γGT activity and as lysosomes constitute a minor compartment (0.4% of cell volume) in parotid tissue [19] (and are thus not a significant source of membrane), suggest that γGT is a bona fide granule membrane activity.

Preparation of Secretion Granule Membranes

The procedure developed for lysis takes advantage of the known lability of parotid secretion granules in KCl-containing media [63]. Soluble secretory protein and granule membranes were obtained by the steps shown in Fig. 4.

This protocol has several characteristics which should be clarified. First, isotonic conditions are used for lysis, whereas other published procedures rely on hypo-osmotic conditions [1, 16, 58]. Second, most loosely bound and soluble contaminants are removed by initial centrifugation. Then a more drastic treatment, the combined presence of saponin and high salt, has been applied to partially perturb the membrane in order to facilitate the removal of residual, poorly extractable contaminants. As in the case of secretion granule membranes of the rabbit parotid [17], both agents must be present simultaneously to achieve effective decontamina-

Table 2. Distribution of secretion granule marker enzymes in cell fractionation of rat parotid

Fraction	α-Amylase		γ-Glutamyl transferase	
	% *H*	RSA[a]	% *H*	RSA
Homogenate	100	1 (166,000)[b]	100	1 (5.40)
NS	69.3	0.94	53.7	0.76
NP	30.7	1.19	46.3	1.55
G1	28.0	2.70	4.7	0.44
Gradient recovery %	96.8		93.6	
G2	25.3	2.85	3.7	0.43
Gradient recovery %	97.7		93.9	

[a] RSA = sp act (per mg protein) relative to that of the homogenate.

[b] Numbers in parenthesis represent total homogenate activities. Enzyme activity units are (μmol/min).

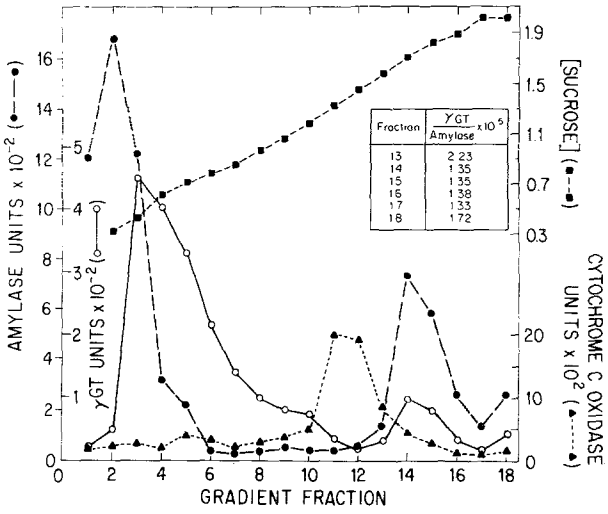

Fig. 3. Enzymatic profiles for a rat parotid nuclear supernate during purification of a secretion granule fraction on linear sucrose-Ficoll (5% wt/vol) gradients

tion. Third, a decontamination technique that causes subtle changes in membrane bilayer integrity can potentially displace peripheral membrane proteins. Therefore, the catalog of proteins established for secretion granule membranes isolated by use of this protocol should be regarded as a subset containing integral membrane polypeptides but possibly excluding more loosely associated species. Finally, it seems unlikely that the use of saponin (at low concentrations) will affect the recovery of integral membrane polypeptides in the final membrane fraction. Saponin apparently does not affect the distribution of integral proteins of the erythrocyte membranes [65] or phospholipids and presumed membrane proteins of rabbit parotid granule membranes [17]. Further, although 0.1% saponin induces bilayer discontinuities in electroplax synaptosomal membranes, the polypeptide composition observed by SDS polyacrylamide gel electrophoresis and the distribution of intramembranous particles observed in freeze fractured preparations appear to be unperturbed [66].

Morphology of the Secretion Granule Membrane Subfraction

Electron microscopic observations of thin sections of secretion granule membrane pellets reveal a homogeneous population of apparently sealed, smooth surfaced vesicles of ~0.6–0.9 μm in diameter (Fig. 5). Regardless of whether or not saponin and sulfate have been used, there is no evidence of fibrillar material (presumed residual granule content) adhering to the inner membrane surface and leading to collapsed vesicular profiles. This appearance should be contrasted with that shown for secretion granule membranes isolated from rabbit parotid [16] and rat parotid [58] following granule lysis in hypotonic media and possibly reflects the prolonged presence of electrolytes during granule lysis. Higher magnification shows that the trilaminar organization of the membrane is preserved with no discernable discontinuities following saponin treatment (10 μg/ml). However, the large size (1.5–2.0 μm) of some of the membrane vesicles (rat parotid secretion granules are 1 μm in diameter) argues that the treatment has included a limited structural rearrangement. Since membrane yields are presently higher than in previous studies of rabbit parotid granules, the extent of structural preservation may reflect the relative amounts of saponin used and membranes treated.

Contamination Analysis of Secretion Granule Membrane Fraction

Since secretion granule membranes are characteristically protein poor (reflected by their low bouyant density during centrifugation), we have paid special attention to the possible contribution to total protein of the secretion granule membrane fraction by soluble and organelle contaminants.

Residual Secretory Proteins

Contamination of secretion granule membranes by residual, poorly extractable secretory proteins has

been estimated by two means: (1) distribution of α-amylase, the enzymatic marker for granule content used throughout this study; and (2) determination of the extent to which exogenous radioactively-labeled parotid secretory proteins added to isolated granules prior to lysis are removed during subsequent membrane purification.

The distribution of α-amylase activity during granule subfractionation is shown in Table 3. The final membrane fraction contains <0.01% of the initial lysate activity; the sub-microgram quantities of residual enzyme constitute <1% of the total protein associated with the membrane.

Previous studies have indicated that secretory proteins other than α-amylase constitute problematic contaminants of both rabbit [16] and rat [58, 74] parotid secretion granule membranes. In order to examine the extent of residual secretory contamination more comprehensively, we added rat parotid secretory proteins that had been biosynthetically radiolabeled to the isolated granules prior to lysis. Subsequently, we checked the purified membrane for the presence of radioactive protein that had relocated (by adsorption or entrapment) from this exogeneously added physiological standard of secretory proteins. As determined by liquid scintillation counting, the membranes were found to be free of >99.99% of the initial radioactivity. A fluorogram [43] of this final membrane preparation shows a single radioactive band having a mobility corresponding to that for α-amylase (Fig. 6); however, prolonged exposure (>30 days) reveals two additional low molecular weight bands. Based on these findings, we can conclude that contamination of secretion granule membranes by residual amylase and by other secretory proteins relocated during granule lysis is minimal. Overall, we feel that the current evidence suggests compellingly that the final mem-

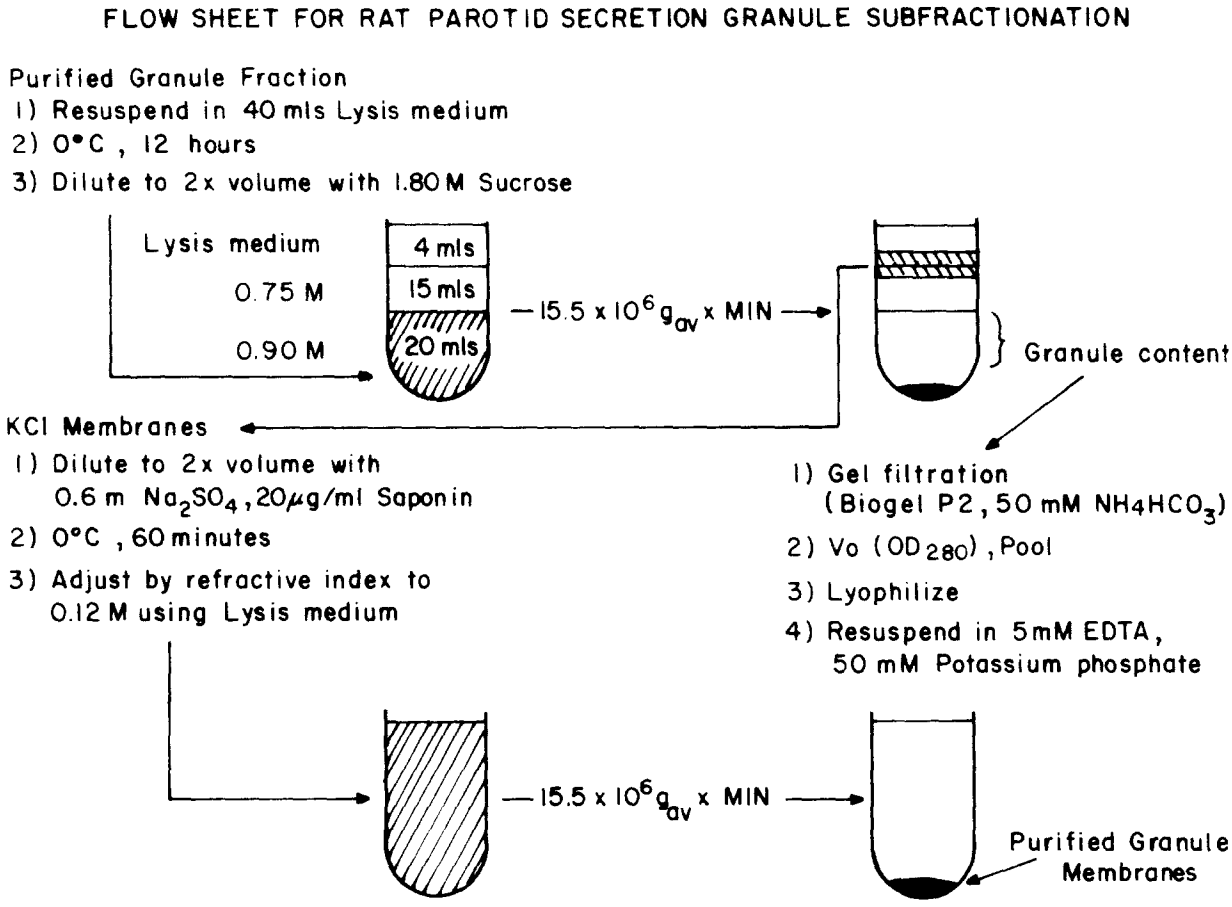

Fig. 4. The procedure for purification of membrane and content subfractions from secretion granule lysates. *Lysis medium* is 190 mM KCl, 2.0 mM imidazole, 5.0 mM EDTA, 1 μg/ml DPPD (pH 6.7). Low density granule membranes are initially obtained by flotation (in a Beckman SW 28 rotor) away from both soluble secretory protein and contaminating membrane. Subsequently, membranes are treated to remove residual secretory proteins and sedimented by centrifugation, whereas content is freed of sucrose and electrolytes by gel filtration

brane preparation is contaminated negligibly by residual content species.

Mitochondrial Contamination of the Secretion Granule Membrane Fraction

Traditionally mitochondria have been the most problematic organelle contaminants in attempts to purify membranes of exocrine secretory granules. Since one of our major goals is to establish a catalog of bona fide granule membrane proteins, we have examined in detail the extent to which mitochondrial elements constitute significant contaminants of the granule membrane fraction. Of necessity, these considerations are qualitative because, on the one hand, granule membranes contain so little protein and, on the other, mitochondrial membranes are especially protein rich. Our analysis, presented in detail in Table 4, has included markers for both the mitochondrial inner (cytochrome *c* oxidase) and outer (amine oxidase) membranes. With 90–95% recovery of the total activity of each enzyme present in the secretion granule lysate suspension, 4% of the amine oxidase and 1% of the cytochrome *c* oxidase activities are recovered in the purified secretion granule membrane fraction.

In order to transpose these levels of contamina-

Table 3. Marker enzyme analysis of contaminating content in secretion granule subfractions

Fraction	% Lysate	α-Amylase RSA[a]	mg α-amylase[b]	% contribution to protein of fraction
Lysate	100	1 (26,000)[c]	8.84	46.0
Buffer overlay	—	—	—	—
Membrane interface	0.26	0.12	0.02	5.4
0.75M layer	2.58	0.71	0.23	32.7
0.75/0.90M interface	8.74	1.33	0.77	61.4
0.90M—content extract	87.02	1.10	7.70	50.6
Pellet	0.75	0.33	0.07	15.0
Gradient recovery % =	101.8			
Membranes	0.007	0.016	0.0007	0.73

[a] RSA = sp act (per mg protein) relative to that of the lysate.
[b] Calculation based on a sp act for purified rat parotid α-amylase, 2780 μmol/min/mg protein [62].
[c] Number in parenthesis represents total lysate activity (μmol/min).

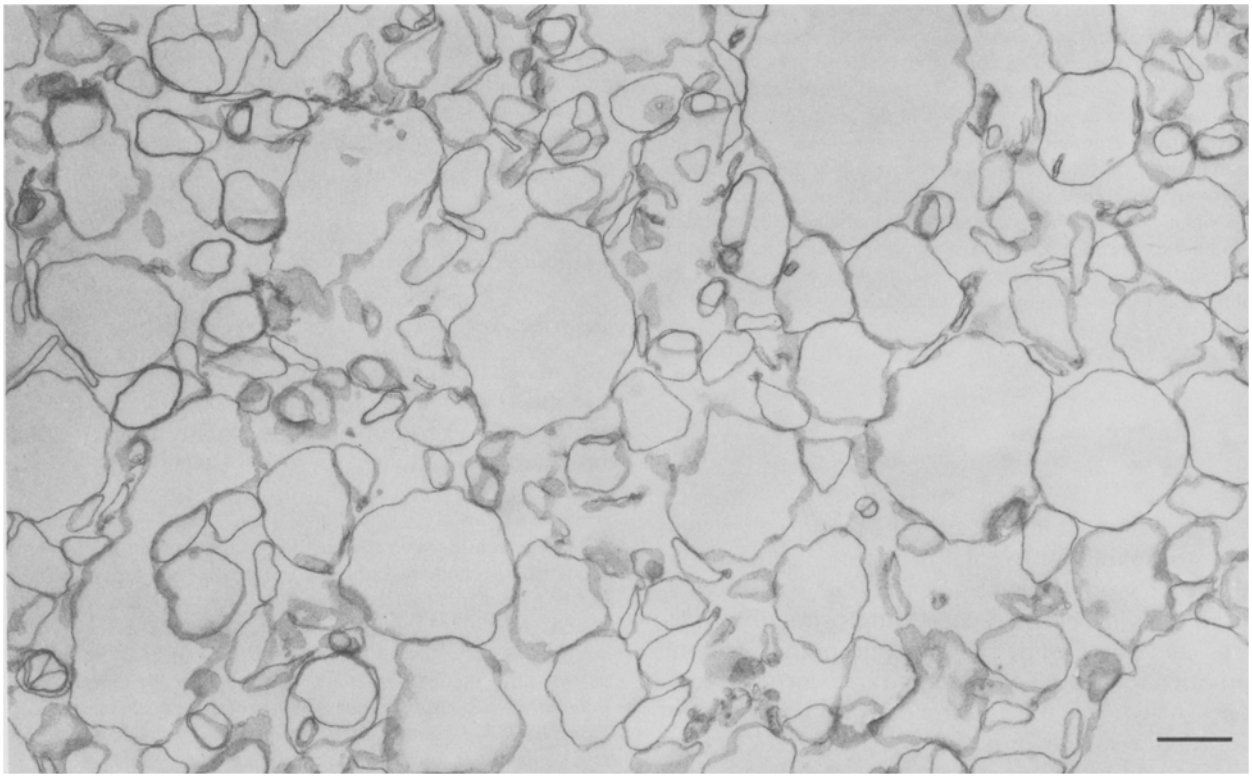

Fig. 5. Electron micrograph of the granule membrane subfraction. The fraction consists of smooth-surfaced, predominantly sealed vesicles, which in some cases appear multivesicular due to resealing of larger membrane pieces around smaller membrane fragments. Bar, 0.5 μm × 21,000

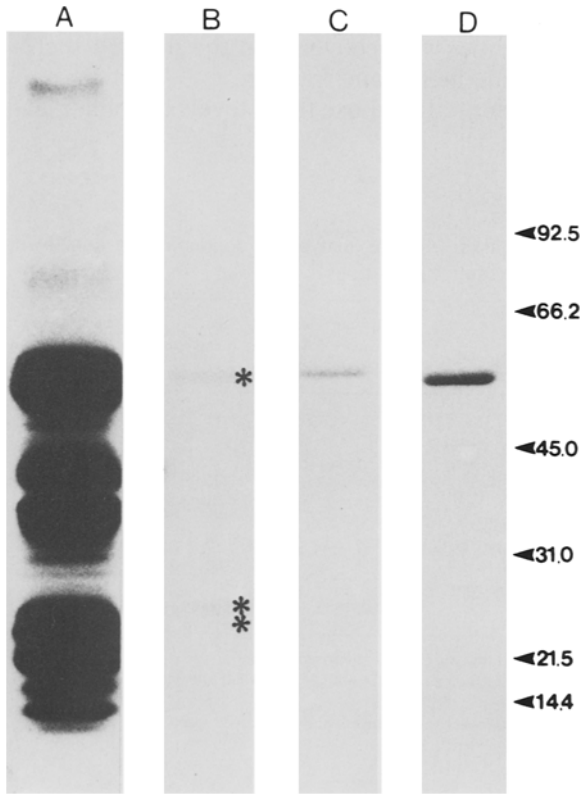

Fig. 6. Fluorogram of a purified membrane subfraction from a secretion granule fraction that was lysed in the presence of biosynthetically labeled (^{3}H-amino acids) secretory polypeptides. Lane *A* is a fluorographic profile for total labeled secretory proteins and serves as a physiologic reference. Lane *B* represents the purified membrane subfraction profile while *C* and *D* are fluorographic profiles for purified α-amylase at 1 and 3 μg, respectively. By comparison it is clear that only α-amylase remains as a significant adsorbed secretory contaminant to the secretion granule membrane subfraction. Prolonged fluorogram exposure indicates two additional low M_r contaminants of the membrane subfraction as indicated by the lower two asterisks in lane *B*. Mol wt, × 10^3

tion into contributions of mitochondrial protein (and phospholipid) to total protein (and phospholipid) of the granule membrane fraction, we have prepared a highly purified parotid mitochondrial fraction and determined the appropriate specific activities for amine oxidase and cytochrome *c* oxidase. A low magnification electron micrograph of the fraction is shown in Fig. 7. Using the enzyme-specific activities determined, we estimate that mitochondrial membrane contaminants of the granule membrane fraction contribute a maximum of 3–4% of the total protein and 0.3–0.5% of the total phospholipid[4]. Thus, as in the case of soluble secretory contaminants, mitochondrial contamination of the purified granule membrane fraction, though detectable, can be viewed as practically negligible.

Composition of the Secretory Granule Membrane of Rat Parotid

Biochemical Analyses

We routinely recover in the purified secretion granule membrane fraction approximately 0.4–0.5% of the protein of the original granule fraction or 0.02% of the starting homogenate protein (Table 5). The average protein yield for a 12-rat parotid preparation is 80–90 μg. Additionally, we obtain for the rat parotid secretion granule membrane a phospholipid/protein weight ratio of 4.4 (mg/mg), which is considerably higher than that of a previously reported value for the same type of membrane—0.58 (mg/mg) [49] or for the secretion granules of bovine adrenal medulla, 2.2 mg/mg [76], but comparable to the electroplax synaptic vesicle, 5.0 mg/mg [73][5] and to rat pancreatic zymogen granule membranes—2.56 (mg/mg) [13] *versus* that previously reported, 0.83 (mg/mg) [60]. Consequently, a high phospholipid-protein ratio appears to be a common feature among membranes constituting a storage compartment for secretory molecules that are discharged in response to cell stimulation. Evidently the absolute values obtained are variable even for the same tissue. In part this may reflect residual secretory or organelle contamination but also possibly the content of rapidly biosynthesized membrane proteins (e.g., GP-2 in rat pancreatic zymogen granule membranes [13]), and possibly secretory component [67].

Enzyme Activities

As discussed previously, preliminary evidence identified γGT as a putative marker enzyme for the rat parotid secretion granule membrane. Detailed biochemical analysis for γGT distribution during

[4] We consider these values to represent upper limits to the predicted contamination since the enzyme specific activities of the purified mitochondrial fraction have probably been underestimated. The fold purification observed for both mitochondrial enzymes is lower than that determined by others (as well as by us) for mitochondrial fractions of lower morphological purity than our own. This discrepancy is currently under investigation. However, if we use a theoretical fold purification, then the protein contribution by mitochondrial membrane contamination is 1–1.5% of the total secretion granule membrane protein.

[5] The value obtained for electroplax synaptic vesicles is not directly comparable because a different protein assay (Bio-rad) was used.

Table 4. Distribution of mitochondrial membrane marker enzymes in secretion granule subfractions

Fraction	Cytochrome *c* oxidase		Amine oxidase	
	% Lysate	RSA[a]	% Lysate	RSA
Lysate	100	1 (.960)[b]	100	1 (337)[b]
Buffer overlay	1.4	1.09	1.4	1.13
Membrane interface	5.6	2.54	4.9	2.25
0.75M layer	4.5	1.23	3.7	1.03
0.75/0.90M interface	3.1	0.47	0.2	0.03
0.90M—content extract	18.3	0.23	1.2	0.02
Pellet	58.2	25.23	82.4	35.81
Gradient recovery %	91.0		93.9	
Membranes	1.3	3.04	3.9	9.18
	[c]Percent contribution to final membrane protein			
	5.6		3.7	
	[c]Percent contribution to final membrane phospholipid			
	0.46		0.23	

[a] RSA = sp act (per mg protein) relative to that of homogenate.
[b] Number in parenthesis represent total lysate activities. Cytochrome *c* oxidase is expressed in units corresponding to the first order rate constant. Amine oxidase enzyme activity units are pmol/min.
[c] Calculations based on sp act (per mg protein and per μmol phospholipid) for our purified rat parotid mitochondrial fraction.

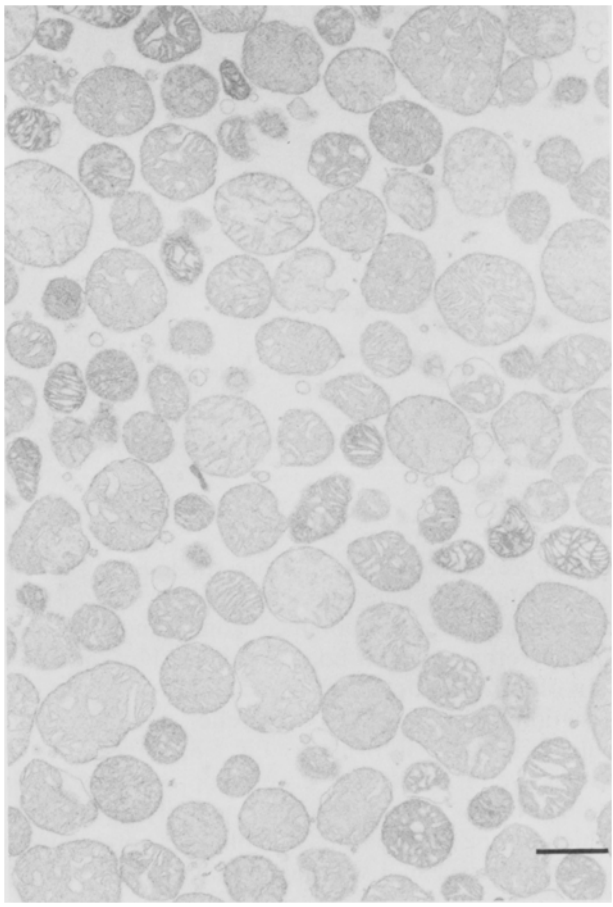

Fig. 7. Electron micrograph of a rat parotid mitochondria fraction. Rough microsomal elements constitute the principal organelle contaminant in a homogeneous appearing pellet

granule subfractionation is presented in Table 6. The recovery of γGT activity following granule lysis is 95–100% of the initial lysate γGT activity. Typically, 70–85% of the lysate γGT activity is recovered in the granule membrane interface of the discontinuous gradient, and 90–100% of this activity is recovered in the purified membrane fraction after saponin-sulfate treatment and subsequent centrifugation. The nearly quantitative recovery of γGT in the final membrane pellet suggests that saponin-sulfate treatment apparently does not dislocate this membrane protein even though the treatment has a demonstrable effect on membrane structure.

It can be seen in Table 6 that the specific activity of γGT increases >125-fold in purifying granule membranes from the initial lysate supension. By contrast, if enzyme activity is normalized to lipid phosphorus, there is at most a minor increase in γGT specific activity as would be expected for a secretion granule membrane enzyme since an estimated 98–99% of the phospholipid present in the purified secretion granule fraction is granule membrane-associated. A reasonable parallelism between γGT activity with the copurification of the majority of membrane phospholipid further suggests, but does not conclusively demonstrate, the disposition of γGT within the secretion granule membrane. Finally, as γGT specific activity, normalized to lipid phosphate, is quantitatively similar for the initial secretion granule fraction and the KCl lysate suspension, we suspect that the lysis and purification conditions used in this protocol do not alter γGT enzymatic activity.

Comprehensive Examination of Granule Membrane and Content Polypeptide by SDS-Polyacrylamide Gel Electrophoresis

To enable our future examination of compositional, functional, and dynamic relationships between granule membranes and the membranes of other compartments forming the secretory pathway, we have sought to characterize the spectrum of granule membrane polypeptides resolved by SDS polyacrylamide gel electrophoresis and simultaneously to evaluate to what extent this composition reflects the presence of residual secretory proteins not detected in our contamination analyses.

Table 5. Distribution of membrane during granule subfractionation—chemical analysis

Fraction	Protein		Phospholipid	
	mg	% Lysate	μmoles	% Lysate
Lysate	20.0	100	1.40	100
Buffer overlay	0.24	1.28	0.05	3.54
Membrane interface	0.42	2.19	0.76	56.29
0.75M layer	0.70	3.63	0.15	11.44
0.5/0.90M interface	1.26	6.56	0.04	2.92
0.90M—content extract	15.20	79.17	0.15	11.25
Pellet	0.44	2.30	0.17	12.50
Gradient recovery %		95.1		97.9
Membranes	.082	0.43	0.49	38.18
		Phosphodlipid-protein 4.42 mg/mg[a]		

[a] Calculation according to the assumption that 750 represents an average mol wt for phospholipid.

Table 6. Distribution of secretion granule membrane marker during granule subfractionation

Fraction	γ-Glutamyl transferase		RSA-L[b]	ng γGTase[c]
	% Lysate	RSA-P[a]		
Lysate	100	1 (.072)[d]	1	98
Buffer overlay	—	—	—	—
Membrane interface	69.7	31.62	1.24	68
0.75M layer	5.8	1.60	0.51	6
0.75/0.90M interface	3.6	0.55	1.25	4
0.90M—content extract	8.0	0.10	0.71	8
Pellet	8.2	3.52	0.65	8
Gradient recovery % =	95.3			
Membranes	61.8	133.33	1.60	60.6

[a] RSA-P = sp act (per mg protein) relative to that of the lysate.
[b] RSA-L = sp act (per μmol phospholipid) relative to that of the lysate.
[c] Calculation based on a sp act for γ-glutamyl transferase 700 μmol/min/mg protein [71].
[d] Number in parenthesis represents total lysate activity; (μmol/min).

Representative silver nitrate-stained 6–14% polyacylamide gradient gel electrophoretograms of content polypeptides obtained from granule lysates and secretory proteins both as collected by cannulation of parotid ducts and as recovered from the incubation medium of isoproterenol-stimulated lobules [2] are shown in Fig. 8, lanes *A–C*, respectively. In each case, approximately 20 stained bands can be resolved, identifying species which for the most part have mobilities corresponding to apparent mol wts in the range of 14K–65K. One rather diffuse, light-stained band having an apparent M_r > 100K is also consistently observed. The near identity of these patterns, especially those for granule content and parotid saliva collected by cannulation, indicates, on the one hand, that there is little or no selective loss of polypeptides during processing of content extracts for electrophoresis and on the other, that the composition of granule content nearly quantitatively reflects the polypeptide composition of parotid secretion. Among the secretory polypeptides resolved by electrophoresis, only α-amylase has been identified. It coelectrophoreses with a purified α-amylase standard, has an estimated mol wt of 55K–58K (equivalent to that previously reported [62]), and represents a prominent stained band in agreement with estimates that it represents 35–40% of the total secretory protein (e.g., Table 3).

Visualization of granule membrane polypeptides in gel electrophoretograms stained with either Coomassie Blue or silver nitrate required such large samples that membrane phospholipids generally caused distortion of the polypeptide patterns, especially in the low mol wt range. Since extraction of phospholipids using organic solvents led to irreversible protein aggregation, we resorted to the use of more sensitive radiolabeling procedures for detecting membrane polypeptides which would enable us to subject smaller samples to electrophoretic analysis.

Autoradiograms of radioiodinated secretion granule membranes, resolved on 6–14% linear polyacrylamide gradients are shown in Fig. 8. The polypeptide profile observed using oxidative radioiodination by Iodogen (lane *D*) contains ~15 distinguishable species with mobilities corresponding to apparent mol wts ranging between 20K–95K and three or four poorly visible species > 100K. This pattern is very reproducible, and comparative examination with the pattern obtained for secretory polypeptides which were electrophoresed as a parallel sample indicated that only α-amylase migrated with a mobility equivalent to that of one of the labeled polypeptides (indicated by an asterisk in lane *D*).

In an effort to improve the sharpness of individual bands visualized in the autoradiogram, we have also carried out membrane radioiodination by a nonoxidative procedure using the Bolton-Hunter reagent. Since this reagent covalently modifies primary amines, this approach simultaneously served as a way to detect polypeptides not previously identified either because their tyrosyl residues were inaccessible to modification or because they lack tyrosine altogether. The latter consideration is especially appropriate for parotid secretion granule membranes where proline-rich secretory proteins lacking tyrosyl residues [55] persist as membrane-associated species during granule subfractionation and membrane purification [59, 74]. Figure 8 (lane *E*) shows the autoradiogram obtained using this approach. As expected, individual, labeled bands are

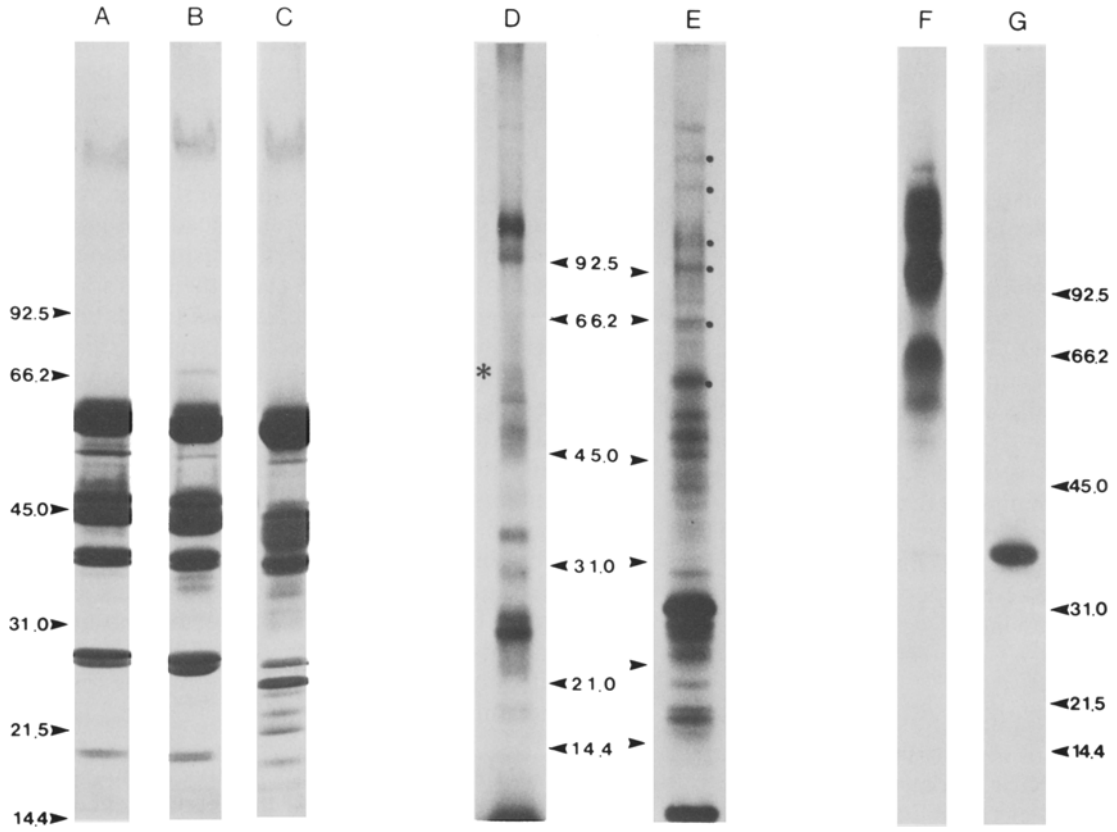

Fig. 8. SDS-polyacrylamide gel electrophoretograms: (1) silver nitrate-stained profiles of secretory polypeptides obtained from granule lysates (lane *A*), cannulation of parotid ducts (lane *B*) and from incubation medium of isoprenaline-stimulated lobules *in vitro* (lane *C*). (2) Autoradiograms of secretion granule membranes radioiodinated by Iodogen (lane *D*) and Bolton-Hunter reagent (lane *E*). Asterisk in lane *D* marks electrophoretic mobility expected for α-amylase. (3) Radioiodinated Concanavalin A overlay of secretion granule membranes (lane *F*) and secretory content obtained from granule lysates (lane *G*). Dots on lane *E* correspond to glycoprotein species as indicated in lane *F*. Numbers indicate $M_r \times 10^3$

generally more sharply focused. Although the spectrum of membrane polypeptides is more complex (up to 25 species can be identified), the pattern agrees quite well with that obtained using Iodogen. Especially notable in Fig. 8*E* are several relatively prominent species having apparent mol wts in the range of 17–30K. Generally, such a profile contrasts with those characterizing the membranes of other cellular compartments where polypeptides having apparent mol wts >40K are prevalent [69]. Nevertheless, for several reasons we currently feel that the profile we have obtained accurately characterizes parotid granule membrane polypeptides. First, all of the species appear to associate and sediment with micelles of Triton X-114 at the detergent's cloud point (data not shown), which suggests that they are integral membrane proteins having a phospholipid binding domain [10]. Second, the inclusion of a battery of protease inhibitors (phenymethylsulfonylfluoride, trasylol, antipain, pepstatin, and EDTA) at all stages of the fractionation yields a polypeptide profile identical to that observed in Fig. 8*D* and *E*. Consequently, endoproteolytic cleavage does not appear to enrich the profile with lower mol wt degradation products. Third, although a number of secretory proteins exhibit similar electrophoretic mobilities to these low mol wt membrane-associated polypeptides, in most cases their final positions in the gel do not exactly correspond. Thus, when considered together with our other contamination analyses and with the absence of detectable interaction between secretory polypeptides and micelles of Triton X-114 (not shown), the data strongly suggest that enrichment of the membrane profile does not reflect residual secretory protein. Finally, in another instance where a granule fraction of high purity has been resolved adequately into membrane and content subfractions, the membranes exhibit a complex polypeptide pattern with several species of apparent low mol wt [13].

An initial identification of membrane and content polypeptides that are glycosylated was carried out by overlaying gel electrophoretograms with radioactive concanavalin A [15]. The results are shown in Fig. 8, lanes *F* and *G*. Relatively few of the membrane polypeptides (5–6) are detected as being glycosylated using this technique, and nearly all exhibit mobilities corresponding to mol wts >70K. By contrast, granule content polypeptides contain one major glycosylated species of apparent mol wt 37,000 similar to that reported for one of the proline-rich proteins [74, 77]. As seen in Fig. 8*F*, this species may occur as a minor contaminant in the membrane profile where it is the sole labeled species of mol wt <45K. It should also be noted that one of the glycosylated membrane species has a mobility corresponding to that of rat parotid amylase which itself is not glycosylated (Fig. 8*G*). Thus, the asterisk placed on lane *D* may mark in large part a bona fide membrane polypeptide and not a residual content contaminant.

Discussion

Rationale for the Compositional Characterization of Secretion Granule Membranes

The intracellular transport pathway for secretory proteins comprises a series of structurally distinct membrane-bounded compartments that form a functional continuum for polypeptide processing [56]. Intermediary vesicular carriers that form from one compartment and subsequently coalesce with the membrane of the successive compartment operate at a minimum of two levels to establish indirect continuity of the pathway: (1) between the transitional regions of the rough endoplasmic reticulum and *cis* elements of the Golgi complex; and (2) between *trans* elements of the Golgi complex and the cell surface. In the latter case, the population of carriers may be amplified and to a large extent, depending on the cell type (e.g., those of exocrine and endocrine glands *vs.* immunoglobulin-secreting cells), retained as a storage organelle for eventual discharge of secretory content at the cell surface. Where it has been possible to comparatively examine the membranes of functionally adjacent compartments [32–35, 50–52, 72, 75] qualitative and quantitative differences in protein and lipid composition and enzyme activities are apparent. Therefore, on the time scale under consideration for complete transit of the pathway by secretory polypeptides (≤3 hr), membranes bounding structurally distinct compartments are compositionally distinct. Thus, it may be inferred that intercompartmental movement of secretory polypeptides is not accompanied by net transfer of carrier membrane but that a compensatory retrieval process probably operates in returning carrier membrane to the donor compartment, probably in a selective fashion.

In many cell types, including polarized secretory epithelial cells, the retention of storage vesicles (secretion granules) is so pronounced that granules can be considered as distinct, quasi-permanent organelles. Although in some cases the membranes of secretion granules have been shown to exhibit morphologic and enzymatic specific activities similar to Golgi membranes [12], the current conceptualization, especially where intracellular storage is prolonged and release is stimulus dependent, suggests a compositional refinement to polypeptides related to granule-specific functions operating in the distal portion of the secretory pathway (e.g., terminal chemical modification [14, 44, 75] packaging, and eventual mobilization of macromolecules during exocytosis). In this case, it is expected that negligible amounts of Golgi-associated activities, as demonstrated for galactosyl transferase, will be found in granule membranes ([60], J.D. Castle, *unpublished*).

With the presence of a persisting granule population having limiting membranes of distinct composition, it is reasonable to assume that membrane sorting (selective retrieval) processes operate both during granule formation and following content discharge. Regardless of whether granule formation occurs by progressive filling with net decrease in condensing vacuole volume [36] or by coalescence of small granules to form larger storage forms [21, 27], removal of membrane is anticipated to occur in order to accommodate most efficiently the volume of packaged content and is postulated to be selective in order to establish the specific composition of the granule membrane. This specific composition is apparently retained through multiple rounds of secretory discharge since the rates of synthesis and turnover of granule membrane polypeptides are one to two orders of magnitude lower than those for secretory polypeptides [17, 48, 74, 75]. Consequently, the compensatory reinternalization of membrane following exocytosis, which, at least in part, completes the shuttle process through return of membrane to the level of the Golgi complex [30], is expected to be selective. The apparent nonrandom nature of the reinternalization of granule membrane in continuity with the apical plasma membrane of rat parotid acinar cells has been suggested by electron microscopic analysis of freeze fractured preparations [20]. However, in this and other polarized epithelial cell types the case for selective reinternalization may not be so strong since both func-

tional and compositional overlaps between the granule membrane and the apical surface may be significant. The transport functions customarily associated with apical membranes of epithelial cells may be reduced in exocrine glands where the apical surface is rather attenuated. Under such circumstances, components common to granule membranes, such as those participating in compound exocytosis, would be proportionately more prevalent.

In order to begin to evaluate the extent to which granule membrane composition reflects a refinement to constituents related to granule-specific functions and those that mediate interactions with functionally adjacent Golgi and plasma membrane compartments, the availability of a readily isolated, highly purified and well characterized granule membrane fraction is essential.

Compositional Properties of Rat Parotid Granule Membranes

In the present work, we have reported the preparation and partial compositional analysis of secretion granule membranes of the rat parotid gland. The fraction obtained consists of closed vesicles and is negligibly contaminated by residual secretory protein and by membrane derived from other cellular organelles. We estimate that ≥95% of the protein associated with the membrane subfraction constitutes bona fide granule membrane protein. Thus, the fraction compares favorably with other highly purified membrane preparations derived from similar or different endomembrane compartments [13, 33, 34, 40, 41, 54]. Further, characterization has shown the granule membrane to be unusually enriched in phospholipid (compared to values for other endomembranes) as indicated by both its low buoyant density and its high lipid-to-protein weight ratio. Although the reduced protein concentration as well as the presumed limited functions of the secretion granule membrane suggest that the polypeptide species may be limited in number as well as in individual quantity, we have observed a rather complex polypeptide profile when resolved by one-dimensional SDS-polyacrylamide gel electrophoresis. This complexity, which to some extent reflects the high resolution and sensitivity obtainable by use of nonoxidative radiolabeling techniques, is comparable to, if not greater than, that obtained for electroplax synaptic vesicle membranes [54] but possibly less than that reported preliminarily for the membranes of adrenal chromaffin granules [3, 61]. In relation to the profiles obtained for other membrane compartments that are more functionally diversified (RER, Golgi, plasma membrane), the pattern for granule membranes appears somewhat simplified [41, 69]. However, adequate evaluation of these differences must await a direct comparative analysis with the fractions in question for rat parotid tissue.

We have previously reported that γ-glutamyl transferase (γ-glutamyl transpeptidase) is an enzymatic activity associated with the apical plasma membrane of the parotid acinar cell [2]. Our biochemical data presented herein strongly suggest that γGT is also a bona fide constituent of the secretion granule membrane. Interestingly, however, the specific activity, normalized to phospholipid, is profoundly different in the two cases: 0.08 U/μmol lipid phosphorus for the granule membrane fraction *versus* 0.68 U/μmol lipid phosphorus for the plasma membrane fraction.[6] Consequently, exocytosis establishes continuity between two membranes that possess a marked difference in activity and possibly quantity of a common constituent.

Pertinent to the issue of compositional overlaps between biochemically and structurally distinct cellular membranes is the recent demonstration in a number of laboratories that biosynthesis and delivery of plasma membrane constituents (shown primarily for viral glycoproteins) utilizes the secretory pathway at least as far as the Golgi complex (e.g. [5, 25]). However, according to recent evidence, there may be a divergence of transit pathways for cells that secrete in response to external stimuli [26], such that nascent plasma membrane proteins enroute to the cell surface bypass the secretion granules. In view of these results, γGT constitutes a compositional probe that we anticipate will be useful for beginning to assess to what extent the granule membrane does or does not constitute a pathway for membrane proteins destined to reside at the apical cell surface. This proposition makes necessary a series of studies in which we will evaluate: first, whether γGT is a membrane constituent of the entire granule population; second, whether the difference in concentration of active enzyme associated with the secretion granule relative to that of the plasma membrane reflects the differential content of active and inactive forms of the enzyme (the latter, possibly as a precursor); and third, what is the dynamic behavior of γGT during exocytosis and the ensuing compensatory membrane retrieval. In these

[6] γGT activity associated with the plasmalemmal fraction is likely to be restricted to (or highly enriched in) regions comprising the apical surface. Consequently, the specific activity for the plasma membrane fraction constitutes a lower limit since basolateral membrane contribution of lipid phosphorus exceeds that of γGT activity.

respects, γGT can also be viewed as a prototype against which to compare the behavior of other polypeptides that are present in quantitatively or qualitatively different levels between interacting membrane partners within the secretory pathway.

In summary, we anticipate that further biochemical and immunological examination of the secretion granule membranes will help to clarify the membrane interrelationships encountered within the secretory pathway since these membranes provide not only a major function and reutilization link between the Golgi complex and the plasma membrane but also a possible transit pathway for membrane macromolecules destined to reside at the apical cell surface.

We are very grateful to our colleague, Anne K. Ma, for performing the continuous gradient fractionation of parotid tissue shown in Fig. 3. Further, we thank Dr. George Palade and Peter Arvan for valuable discussions during the course of this work. Finally, we wish to acknowledge the expert assistance of Hans Stukenbrok (preparation of specimens for electron microscopy), Pam Ossorio (photographic work), and Cindy Davis (secretarial assistance in preparing the manuscript). This research was supported by research grants AM 29868 and GM 26524 from the National Institutes of Health.

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