

SECRETION GRANULES OF THE RABBIT PAROTID GLAND

Isolation, Subfractionation, and Characterization of the Membrane and Content Subfractions

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ABSTRACT

A fraction of secretion granules has been isolated from rabbit parotid by a procedure which was found to be especially effective in reducing contamination resulting from aggregation and/or cosedimentation of granules with other cell particulates. The fraction, representing 15% (on the average) of the total tissue amylase activity, was homogeneous as judged by electron microscopy and contaminated to exceedingly low levels by other cellular organelles as judged by marker enzymatic and chemical assays.

Lysis of the granules was achieved by their gradual exposure to hypotonic NaHCO_3 , containing 0.5 mM EDTA. The content and the membranes separated by centrifugation of the granule lysate were characterized primarily by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis which indicated that the content was composed of a limited number of molecular weight classes of polypeptides of which three bands (having approximate mol wt 58,000, 33,000, and 12,000) could be considered major components. The gel profile of the membrane subfraction was characterized by 20–30 Coomassie brilliant blue-staining bands of which a single species of mol wt $\sim 40,000$ was the conspicuous major polypeptide.

Two types of experiments employing gel electrophoretic analysis were carried out for identifying and assessing the extent of residual secretory protein adsorbed to purified granule membranes: (a) examination of staining and radioactivity profiles after mixing of radioactive secretion granule extract with nonradioactively labeled granule membranes and (b) comparison of gel profiles of secretion granule extract and granule membranes with those of unlysed secretion granules and secretory protein discharged from lobules *in vitro* or collected by cannulation of parotid ducts, the last two samples being considered physiologic secretory standards. The results indicated that the membranes were contaminated to a substantial degree by residual, poorly extractable secretory protein even though

assays of membrane fractions for a typical secretory enzyme activity (amylase) indicated quite thorough separation of membranes and content. Hence, detailed examination of membrane subfractions for residual content species by gel electrophoresis points to the general utility and sensitivity of this technique as a means for accurately detecting a defined set of polypeptides occurring as contaminants in cellular fractions or organelle subfractions.

The secretion granule in exocrine cells represents the intracellular storage compartment for protein destined for export. Within the exocrine cell the population of accumulated granules, which can occupy as much as half of the cellular volume, is located in the apical cytoplasm between the Golgi region, where granules originate (2, 13, 28, 29, 54, 55), and the apical cell membrane, where the period of storage is terminated by release of the granule contents into the extracellular space by the process of exocytosis (57).

In our previous studies (13) we undertook a characterization by autoradiography of the secretory process of the acinar cell of the rabbit parotid gland *in vitro* to compare similarities and differences of the kinetics of intracellular transport of secretory protein from its site of synthesis on the rough endoplasmic reticulum to its site of storage in secretion granules in the parotid and in the guinea pig exocrine pancreas (28, 29). The parotid should provide a system in which membrane lipolytic and proteolytic degradation is not expected.

In this report we present (a) the isolation and partial characterization of a fraction of parotid secretion granules; (b) the subfractionation of the granules to obtain the membranes and content; and (c) a rigorous assessment of the extent of separation of the two subfractions. The last aspect indicates, in general, the necessity for the careful consideration of the incidence of adsorbed contaminants to membrane subfractions of cellular organelles.

MATERIALS AND METHODS

Isolation of Parotid Secretion Granules

To isolate secretion granules from rabbit parotid we devised a new procedure by which it is possible to obtain with satisfactory yield a preparation contaminated to an unusually low level by other cellular organelles. The protocol will be presented and discussed in detail in the Results.

Lysis of Secretion Granules and Subsequent Separation of Granule Membranes and Secretory Content

To lyse secretion granules we sought an alternative to the lysis at mildly alkaline pH used for pancreatic zymogen granules (21, 25, 49–51) since rabbit parotid granules were found to be reasonably stable in the pH range 7.5–8.0. Instead, we exploited the instability of rabbit parotid granules to hypotonic media in developing a lysis procedure, which together with the subsequent separation of granule membranes and secretory content is presented in detail in the Results.

Processing of Subcellular Fractions for Microscopy

Secretion granule fractions (collected in 1.9 M sucrose, 40 mM potassium phosphate, 1 mM EDTA¹ were diluted by one of two ways: (a) with 3 vol of a mixture of 2% glutaraldehyde, 2% formaldehyde in 0.08 M potassium phosphate, pH 7.2 or (b) with 1 vol of a mixture of 4% glutaraldehyde in 0.2 M potassium phosphate, pH 7.2. Aldehyde fixation was carried out in suspension at room temperature for 8–16 h after which samples were pelleted in 0.4-ml polypropylene conical tubes in a Beckman 152 microfuge (Beckman Instruments, Spinco Div., Palo Alto, Calif.). Subsequently, the centrifuge tubes were cut away just above the pellet surface and pellet plus residual tube placed in 1% OsO₄ buffered with 0.1 M potassium phosphate, pH 7.2, for postfixation at 4°C. After 2 h osmium fixation, pellets were bisected vertically to aid penetration of the fixative during an additional 2 h of fixation. Subsequently, the pellets were washed in three changes of 1% NaCl (10 min each change) at 4°C, stained in block with 0.5% magnesium uranyl acetate in 1% NaCl for 1 h at 4°C (13),

¹ *Abbreviations used in this paper:* EDTA, disodium ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; F12, Nutrient mixture F12; PAS, periodic acid-Schiff staining technique; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; PCA, perchloric acid; PTA, phosphotungstic acid; DPPD, *n,n'*-diphenyl-*p*-phenylenediamine; RNase, ribonuclease; DNase, deoxyribonuclease; and HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

dehydrated in ethanol and propylene oxide, and embedded in Epon (42).

Pellets of secretion granule membranes in polyallomer centrifuge tubes were fixed, postfixed, and stained with magnesium uranyl acetate in the tube. At the onset of dehydration, the pellets plus adhering tube bottoms were cut into strips and subsequently embedded as units, taking care to orient the material so sectioning would include the entire thickness of the pellet. In trimming the Epon block for microtomy the residual piece of centrifuge tube was easily removable, leaving behind the embedded pellet undisturbed. Sections were placed on bar grids to further insure visualization of the entire pellet thickness. All micrographs were taken in a Siemens Elmiskop I.

Preparation of Radioactively Labeled Secretion Granules

To prepare radioactively labeled parotid secretion granules, the tissue of four parotid glands was dissected into lobules (13) and pulse labeled *in vitro* for 30 min using a mixture of fifteen ^{14}C -labeled amino acids. Pulse medium consisted of 10 ml of Krebs-Ringer bicarbonate medium (34) supplemented with unlabeled amino acids to one-half the final concentration found in Eagle's minimal essential medium and containing in addition 25 $\mu\text{Ci/ml}$ of reconstituted [^{14}C]algal protein hydrolysate. At the end of the pulse, lobules were washed with 100 ml Medium F12 (22) at 37°C and were then chase incubated in 100 ml F12 for 4 h to allow the bulk of the incorporated radioactivity to be transported to secretory granules (13). The tissue in its entirety was then fractionated to obtain secretion granules according to the protocol outlined in the Results.

Collection of Secretion Discharged from Parotid Lobules During In Vitro Incubation in the Presence of Isoproterenol

The secretion discharged by parotid lobules was obtained from incubation medium (F12) in which the tissue of two parotid glands was incubated in the presence of 1×10^{-5} M isoproterenol. Lobules were initially preincubated at 37°C for 15 min to remove loose debris and contents of damaged cells; subsequently, they were transferred to 50 ml fresh F12 containing 1×10^{-5} M isoproterenol in a siliconized 250-ml Erlenmeyer flask and incubated for 2.5 h with continuous gassing (95% O_2 , 5% CO_2). At 30-min intervals additional increments of isoproterenol (25 μl of a 0.01 M solution) each contributing 5×10^{-6} M to the secretagogue concentration were added to the incubation flask to replenish isoproterenol diminished by oxidation. Upon termination of incubation, the medium was poured from the lobules and subjected in succession to centrifugation (20,000 g_{av} , 20 min, to pellet any large particulates), lyophilization,

resuspension and desalting (over Biogel P-6 [Bio-Rad Laboratories, Richmond, Calif.] in the presence of 0.1 M triethylammonium bicarbonate), and lyophilization before solubilization for SDS-polyacrylamide gel electrophoresis.

Collection of Parotid Saliva from an In Vivo Stimulated Animal

To obtain a sample of secretion released by the parotid glands *in vivo*, the parotid ducts of a rabbit were cannulated, and saliva was collected after systemic stimulation. The rabbit was anesthetized with sodium pentobarbital, and an incision was made along the length of the jaw exposing the masseter muscle on both sides. The parotid ducts which run across the surface of this muscle were freed from the surrounding connective tissue, and small polyethylene tubes were inserted and secured by ligature. Stimulation of parotid secretion was achieved by intraperitoneal injection of isoproterenol (4×1 ml of a 20 mg/ml solution) at 15-min intervals. 1.5-ml secretion (highly concentrated in protein) was collected over 75 min. The collected saliva was lyophilized, resuspended in 0.1 M NH_4HCO_3 , desalted by gel filtration over Biogel P-2 and subsequently lyophilized to remove NH_4HCO_3 , and finally redissolved in either water or solutions directly applicable for gel electrophoresis.

Purification of Amylase by the Alcoholic Glycogen-Precipitation Procedure

Granule content (obtained by the centrifugation which resolves granule lysate into membranes and content) was concentrated in an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.) outfitted with a PM10 membrane. 1 ml of the final concentrate contained 200 U (micromoles maltose produced per minute at 30°C) amylase. The procedure of Schramm and Loyer (62) was then followed directly, making adjustments, however, to compensate for the fact that the initial amylase concentration was below the 500-U/ml level indicated in the original reference. To 2.5 ml of concentrated granule content was added dropwise 1.67 ml of cold 100% ethanol. This mixture was spun at 10,000 g_{av} for 20 min. The resulting supernate (4.1 ml) was treated with 0.20 ml 0.2 M sodium phosphate buffer, pH 8.0, 0.20 ml glycogen solution (2% wt/vol, clarified by initial centrifugation), and 0.20 ml 100% ethanol. The resulting precipitate was pelleted and washed twice by centrifugation. The final pellet was drained and dissolved for electrophoresis. The intent to perform electrophoresis in dissociating solvents rendered unnecessary glycogen digestion and limit dextrin removal steps. To check the extent of amylase removal and the effect of the procedure on other proteins of the content, the supernate of the glycogen-amylase precipitate was lyophilized and the resulting

residue was resuspended in solutions appropriate for gel electrophoresis.

Polyacrylamide Gel Electrophoresis of Granules and Their Subfractions

SDS-polyacrylamide gel electrophoresis was performed by the method of Shapiro et al. (65). Tubes 5 or 6 mm (inner diameter) containing resolving gels 10–13 cm in length and stacking gels 0.4 ml in volume were used in all instances reported here and were routinely run for approximately 12 h at a current of less than 1 mA per tube, the actual time of electrophoresis being established by monitoring the migration of the tracking dye, bromphenol blue. In initial studies resolving gels were 11% acrylamide (stock acrylamide 37.5:1, acrylamide: *N,N'*-methylene bis-acrylamide) made as specified by Maizel (45). Subsequently, the following 8–12% acrylamide gel gradient method was devised to yield optimal separation and sharpness of polypeptide bands obtained from both secretion granule membranes and content. In this procedure, seven 15-cm long, 6-mm (inner diameter) glass tubes were close packed in hexagonal arrangement using a rubber band at one end. The tube bundle was then inserted into the barrel of a 50-ml disposable (polyethyl-

ene) syringe, vertically mounted. The syringe outlet was connected to a Perpex pump (LKB Instruments, Hicksville, N.Y.) and gradient maker (Fig. 1) which contained 12% acrylamide and 12% sucrose in the back reservoir, and 8% acrylamide and 3% sucrose in the mixing chamber. The gradient was then generated by pumping in the less dense acrylamide solution first. The purpose of the sucrose gradient was to stabilize the acrylamide gradient against convection currents caused by heat evolved during polymerization. Pumping took 20 min, and the polymerization catalyst, ammonium persulfate, was reduced to 0.1 the amount recommended by Maizel (45) to insure a polymerization time of at least 40 min. The gradients thus generated were each overlaid with isobutanol (or *n*-butanol) before polymerization as suggested by Glossmann and Neville (19).

The polypeptides of all samples were subjected to disulfide reduction by heating the samples for 2 min at 100°C in the presence of 2% (vol/vol) 2-mercaptoethanol (45) or 50 mM dithiothreitol (17) before electrophoresis. After electrophoresis, tracking dye fronts were marked with India ink, and the gels were either (a) fixed in 25% isopropanol, 10% acetic acid (17) and stained simultaneously for protein with Coomassie Brilliant Blue (0.07–0.08% for 8 h) with subsequent destaining in 10%

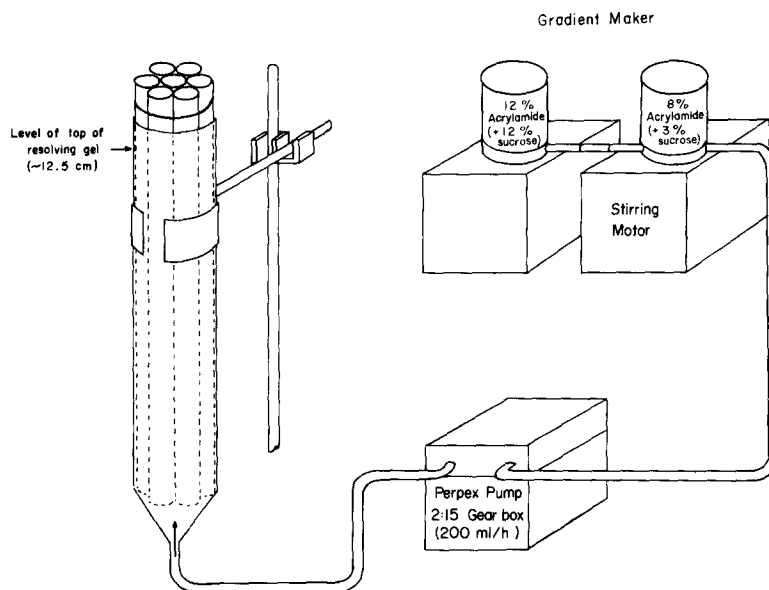


FIGURE 1 The method developed for making uniform gels with a continuous gradient of 8–12% polyacrylamide. The gradient maker chambers containing acrylamide solutions (45) are connected via a Perpex peristaltic pump (flow rate ~200 ml/h) to the 50-ml disposable syringe cartridge containing the glass tubes that ultimately contain the gels. The gradient generated (lowest density first) fills the entire syringe barrel, however, only the acrylamide solution within the tubes is overlaid with isobutanol before polymerization. 1–2 h after polymerization the base of the syringe was cut away, and the entire cylinder of polymerized acrylamide containing the gel tubes was removed; subsequently, individual tubes were dissected free of surrounding gel and secured in place in the electrophoresis chamber. Isobutanol was washed from the surface, and the resolving gels were overlaid with 0.4 ml spacer gel solution.

isopropanol, 10% acetic acid followed by 5% isopropanol, 10% acetic acid or (b) fixed only (with isopropanol-acetic acid), then reacted with periodic acid and stained with the Schiff reagent for periodic acid-Schiff (PAS)-positive carbohydrate according to the procedure of Fairbanks et al. (17).

Destained gels were photographed with a Polaroid MP3 camera and scanned in a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Great Neck, N.Y.) equipped with a linear transport device to obtain an optical density profile for polypeptides (wavelength 550 nm) and PAS-positive bands (wavelength 560 nm [17]).

Finally, gels containing radioactivity were sliced transversely into 1-mm thick sections with a gel slicer provided with multiple, equally spaced cutting wires. Each slice was dissolved in 0.5 ml of 20% H₂O₂ (36–48 h at 45°C) for scintillation counting.

Radioactivity, Chemical, and Enzymatic Assays

RADIOACTIVITY ASSAYS: Gel slices dissolved in 20% H₂O₂ were counted in a toluene-Triton X-100 (5:1 vol/vol) base scintillation fluid (58) containing 0.4% 2,5-diphenyl oxazole (PPO) and 0.05% 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene (Dimethyl POPOP). The ¹⁴C-label was counted at 75–80% efficiency in either a Nuclear-Chicago Mark I (Searle Analytic, Inc., Des Plaines, Ill.) or a Beckman LS-250 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). Comparison of radioactivity data of different samples indicated uniformity of quenching; thus the data were corrected only for background and are expressed as counts per minute.

CHEMICAL ASSAYS: RNA content of homogenates and subcellular fractions was assayed according to the procedure of Blobel and Potter (7) in which RNA is determined by spectrophotometry on alkaline extracts (0.3 N KOH) of washed PCA precipitates. RNA concentration (milligrams per milliliter) was calculated from OD₂₆₀ readings using the extinction coefficient $E_{1\text{cm}}^{1\%} = 312$ determined by Munro and Fleck (53). Since aliquots of cellular fractions were precipitated with PCA in the presence of 1 mg bovine plasma albumin, a sample of 1 mg albumin alone was processed in parallel as an assay blank. This procedure is acceptable if alkaline extraction of material other than RNA having a substantial absorbance at 260 nm is either negligible or can be quantitated (cf. Footnote † of Table II).

Estimations of total protein were performed on acid precipitates (0.5 N PCA + 0.5% (wt/vol) PTA) of subcellular fractions that had been dissolved in 1 N NaOH using either (a) the assay of Lowry et al. (41), or (b) the microbiuret assay developed from procedures presented by Chase and Williams (14) and by Gornall et al. (20). In both cases bovine plasma albumin was used as standard.

ENZYMATIC ASSAYS: Amylase was assayed on samples of homogenates and subcellular fractions in 0.2% Triton X-100 containing 0.02 M sodium phosphate (pH 6.9) and 0.02 M NaCl (5). 1 U of amylase activity is defined as the amount of enzyme which liberates 1 μmol of maltose equivalents per min at 30°C.

DNase was assayed using the Kunitz hyperchromicity assay (38) as described by Price et al. (60) at pH 5.0. Highly polymerized calf thymus DNA served as substrate, and Worthington bovine DNase I (2,000 U/mg) (Worthington Biochemical Corp., Freehold, N.J.) was used as standard. 1 U of DNase is defined as the activity causing an increase in OD₂₆₀ of 1.0/min/ml (60) at 25°C.

RNase was assayed at pH 7 according to the procedure of Kalnitsky et al. (30). Yeast RNA (Sigma Fraction VI) was used as substrate, and incubation was carried out for 4 min at 37°C. 1 U of enzyme activity corresponds to the release of 1.0 OD₂₆₀ unit of acid-(25% PCA-1% uranyl acetate) soluble oligonucleotide.

Neutral lipase was assayed as prescribed by Bradshaw and Rutter (8) using as substrate β-naphthyl nonanoate in the presence of sodium taurocholate. The assay quantitates the release of β-naphthol. Purified porcine pancreatic lipase (kindly supplied by Dr. M. F. Maylie, Centre de Biochimie et de Biologie Moleculaire, C. N. R. S., Marseille, France) was used as standard.

Peroxidase activity was assayed in granule lysates by the procedure of Klebanoff (32) using *o*-dianisidine and 0.3% H₂O₂ at both pH 5 and pH 7. Horseradish peroxidase (5–10 ng/ml) served as the standard for the assay.

The presence or absence of protease activity at pH near neutrality in concentrated granule hypotonic lysates was assessed using the Azocoll assay (46).

Cytochrome oxidase was assayed in subcellular fractions using the procedure of Cooperstein and Lazarow (15) as modified by Peters et al. (59), except that reactions were run at 25°C. 0.1 ml of appropriately diluted fractions was added to 1 ml of 41 μM cytochrome *c* substrate (90% in the reduced form), and the decrease in OD₆₆₀ with time was monitored in a Gilford 2,400 spectrophotometer equipped with a linear-scale recorder set at a zero absorbance corresponding to substrate fully oxidized with potassium ferricyanide and a fullscale absorbance corresponding to fully reduced substrate (OD₆₆₀ ~0.70). Zero order kinetics were approximated by serially diluting (in twofold increments) enzyme-containing fractions until the plots of the initial decrease in optical density showed little curvature, and their slopes for consecutive dilutions changed by a factor of two, indicating half the amount of enzyme. The activity of cytochrome oxidase was converted to units of micromoles cytochrome *c* oxidized per minute using a micromolar extinction coefficient for reduced cytochrome *c* of 0.019.

N-acetyl-β-glucosaminidase was determined for all subcellular fractions by the method of Findlay et al. (18). The substrate used was *p*-nitrophenyl-*N*-acetyl-β-

glucosaminide, and the assay quantitates the release of *p*-nitrophenol which in a second step is determined (in alkaline glycine buffer) by absorbance at 400 nm (micromolar extinction coefficient for *p*-nitrophenol, 0.018). Activity units are micromoles *p*-nitrophenol liberated per minute at 37°C.

MATERIALS: Reconstituted ¹⁴C-labeled algal protein hydrolysate used in labeling secretion granules and sucrose (special RNase-free grade) used for all fractionation studies were obtained from Schwartz-Mann, Inc., Orangeburg, N.Y., and sucrose solutions were filtered through 0.45-μM Millipore filters (Millipore Corp., Bedford, Mass.). Nutrient mixture F12 used for *in vitro* incubation was obtained from Grand Island Biological Co., Grand Island, N.Y. Azocoll was purchased from Calbiochem Inc., Los Angeles, Calif. Acrylamide, *N,N'*-methylene-bisacrylamide and *N,N,N',N'*-tetramethylethylenediamine (TEMED) for gel electrophoresis were obtained from Eastman Organic Chemicals, Rochester, N.Y. Ammonium persulfate and SDS, also for gel electrophoresis, were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Isolation of a Secretion Granule Fraction from Rabbit Parotid Tissue

In procedures conventionally used for the isolation of secretion granules of approximately 1 μm in diameter, mitochondrial contamination is reduced by surface rinsing and repeated washing by resuspension and sedimentation of a granule pellet originally obtained by differential centrifugation. These operations reduce granule yield and are only partially successful in reducing contamination by mitochondria and other elements. Contamination by mitochondria in such granule fractions usually amounted to ~2% of the initial total homogenate activity (3, 21, 31, 49). Therefore, we developed a new granule isolation protocol with the intent of obtaining, through a simple procedure, an effective reduction of contamination by other cellular organelles at minimal expense to the yield of secretion granules. At all stages, a light microscope outfitted with differential interference contrast optics proved to be very useful for visually monitoring fractionation and qualitatively assessing contamination. The steps involved in this protocol (carried out entirely at 4°C and presented schematically in Fig. 2) are as follows:

1 Parotid tissue (either glands dissected free of surrounding connective tissue or incubated lobules) was divided into 0.4–0.6-g batches.

2 Each batch of tissue was thoroughly minced in a few drops of homogenization medium with a single-edge razor blade on a Teflon plate.

3 Homogenization (3 strokes at 1,300–1,600 rpm) was performed in 0.28 M sucrose, 40 mM potassium phosphate, 0.2 mM EDTA, pH 7.2, in a Brendler-type (9) tissue grinder (glass motor, serrated-tip Teflon pestle with 0.004–0.006-inch clearance and 10-ml capacity) to give a 10% (wt/vol) tissue homogenate.

4 Debris which settled by gravity was rehomogenized (3 strokes, 1,600 rpm) in 4–5 ml (precise volume depending on initial tissue weight) of homogenization medium and was combined with the supernate of the initial homogenate. The combined supernates were designated: total homogenate (5% tissue wt/vol).

5 Large debris, unbroken cells, and nuclei were pelleted from the total homogenate at 600 *g_{av}* for 8 min in conical glass centrifuge tubes in an International PR6000 centrifuge with no. 269 yoke, (Damon/IEC Div., Damon Corp., Needham Heights, Mass.). Under these conditions the resulting pellets were overlaid with a fine white layer of secretion granules.

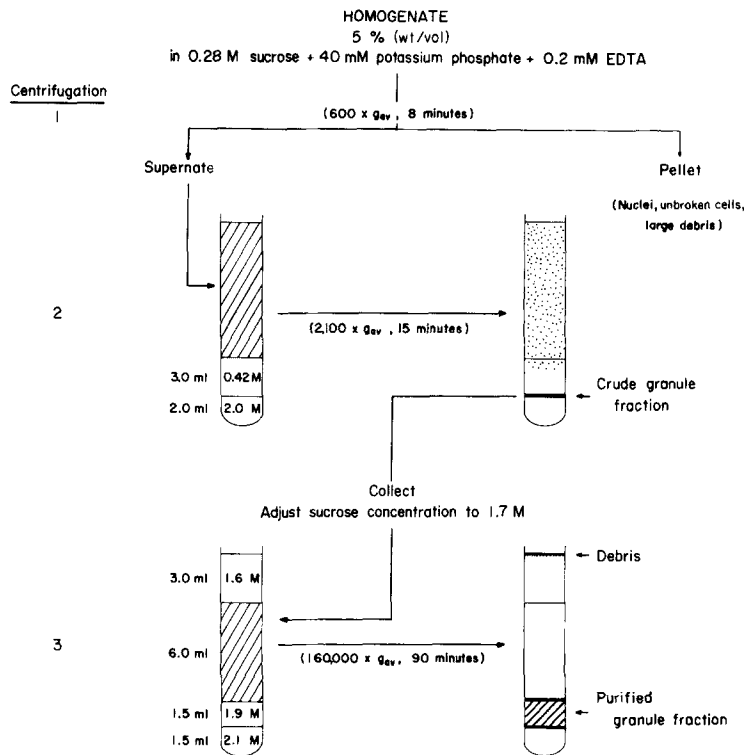
6 The resulting supernate was layered over a step gradient (in 15-ml round bottom Corex tubes) consisting of a 3-ml layer of 0.42 M sucrose, 40 mM potassium phosphate, 1 mM EDTA, pH 7.2, over a 2-ml cushion of 2.0 M sucrose, 40 mM potassium phosphate, 1 mM EDTA, pH 7.2.

7 Centrifugation carried out at 2,100 *g_{av}* for 15 min in an International PR6000 centrifuge with no. 269 yoke yielded a crude granule fraction located at the 0.42–2.0 M sucrose interface. The bulk of the mitochondria and smaller cellular organelles fail to penetrate the 0.42 M layer under these conditions.

8 The crude granule fraction was collected, and the sucrose concentration was adjusted to 1.7 M using 2.1 M sucrose, 40 mM potassium phosphate, 1 mM EDTA.

9 After thorough mixing the diluted crude granule fraction was layered centrally in a sandwich gradient having as lower layers 2.1 M and 1.9 M sucrose (1.5 ml each) and an overlay of 1.6 M sucrose (3 ml). All sucrose solutions were supplemented with phosphate and EDTA at the concentrations already indicated.

10 By centrifugation at 160,000 *g_{av}* for 90 min in an International B60 centrifuge equipped with an SB283 rotor, a purified granule fraction sedimented into the 1.9-M sucrose layer, while mem-



All sucrose solutions (except homogenization medium) contain 40 mM potassium phosphate and 1 mM EDTA

FIGURE 2 Isolation protocol for the secretion granule fraction of the rabbit parotid gland. All centrifugal forces (g_{av}) refer to the values at the center of the tubes. The tubes for centrifugations 2 and 3 were filled to capacity.

branous debris floated upwards from the load through the 1.6-M sucrose overlay.

Several features of this protocol should be emphasized. (a) Potassium phosphate buffer at pH 7.2 was present in all sucrose solutions at a final concentration of 40 mM. Granules are totally labile in unbuffered sucrose, and examination of granule stability as a function of phosphate concentration indicated that a minimum of 20 mM potassium or sodium phosphate tested over the range of pH 6.5–7.6 was required to maintain intact granules. Granule lysis could be observed in the light microscope at lower phosphate concentrations. Other salts such as potassium chloride, sodium bicarbonate, and HEPES were completely unsatisfactory as substitutes for potassium phosphate.

(b) Secretion granules readily stick to both glass and cellulose nitrate. Therefore, the glass centrifuge tubes used in steps 1 and 2 of the centrifugation schedule were siliconized, and polyallomer

centrifuge tubes were used in place of cellulose nitrate tubes for the final high-speed centrifugation.

(c) The use of high-molarity sucrose cushions as seen in centrifugations 2 and 3 as an alternative to pelleting the secretion granules at any stage was extremely helpful in reducing contamination induced by physical contact and aggregation between granules and other cellular organelles, especially mitochondria. When pelleting of granules by velocity sedimentation was attempted in early phases of these studies, mitochondria were observed to adhere strongly to granule surfaces, the intimate association being maintained throughout vigorous resuspension of granule pellets. The tenacious association could be largely overcome by avoiding pelleting of granules and other cell particulates.

(d) Finally, the inclusion of EDTA at a final concentration of 1 mM complemented the use of sucrose cushions in effectively reducing aggrega-

tion between granules and between granules and other cellular organelles. In preventing organelle aggregation the efficiency of EDTA can be readily visualized in the light microscope. It should be pointed out, however, that EDTA was used in the initial phases of fractionation at 0.2 mM final concentration. Nuclei are stabilized by divalent cations, so until nuclei are removed by the initial centrifugation, EDTA is kept at a lower concentration. This precaution was necessary since the only visual subcellular contaminant of the granule fraction was an occasional partially extracted nucleus.

Morphology of the Isolated Granule Fraction

Fixed pellets of secretion granules were studied systematically throughout their thickness and were found to be homogeneous as portrayed by Fig. 3, a representative large field, low-magnification electron micrograph.

Immediately evident in this figure are the large variety of irregular granule shapes similar to the variety observed in intact cells (13), and the absence of recognizable mitochondria and microsomes present at higher incidence in secretion granule fractions obtained by repeated differential sedimentation. As demonstrated by Fig. 4 *a*, this rabbit parotid granule fraction contains recognizable immature secretion granules (13), and the higher magnification (Fig. 4 *b*) further indicates that the limiting membranes of the granules are

well preserved, showing a reduced incidence of myelin figure formation (at the expense of membrane phospholipids) when compared to pancreatic zymogen granules (49, 50).

The Yield of Secretion Granules

An estimate of the proportion of the total secretion granule population recovered in the purified granule fraction was obtained by determining the distribution of one of the main secretory enzymes, α -amylase, taken as a granule marker. The underlying assumption is that in the intact cells the bulk of this enzyme is located within secretion granules.

Presented in Table I is a representative amylase distribution and specific activity profile for the entire fractionation scheme. The percentage distribution data are expressed in two forms: (*a*) percent relative to the fraction of origin at each step in the protocol facilitating an assessment of recovery for each step, and (*b*) percent of the total homogenate activity. 12.1% of the total amylase activity of the homogenate was recovered in the purified granule fraction in the experiment given in Table I, and the amylase specific activity of the granule fraction normalized to protein (41) was four times the homogenate specific radioactivity. The average yield of purified granules for eight different experiments was 15% of the total homogenate amylase, which compares favorably with other granule yields reported in the literature (3, 21, 49). The

TABLE I
Amylase Distribution in the Granule Isolation Scheme

Fraction	Amylase percentage distribution	Percentage of homogenate amylase	Specific activity units* mg protein (Lowry)
Homogenate	100.0†	100.0‡	75
Pellet spin 1	27.2	27.2	46
Supernate spin 1	62.6 (100.0)	62.6	94
Postgranule supernate spin 2	66.4	41.5	84
Crude granule fraction	21.9 (100.0)	13.7	200
Purified granule fraction	89.0	12.1	292
Debris and residual sucrose spin 3	7.1	1.0	n.d.
§ Mitochondria and microsomes		2.6	30
§ Postmicrosomal supernate		37.6	96

* Units: micromoles maltose produced per minute at 30°C.

‡ 100% homogenate corresponds to 14,625 amylase units.

§ Postgranule supernate spin 2 h at 160,000 g_{av} to yield mitochondria and microsomes (pellet) and postmicrosomal supernate.

|| Not determined.

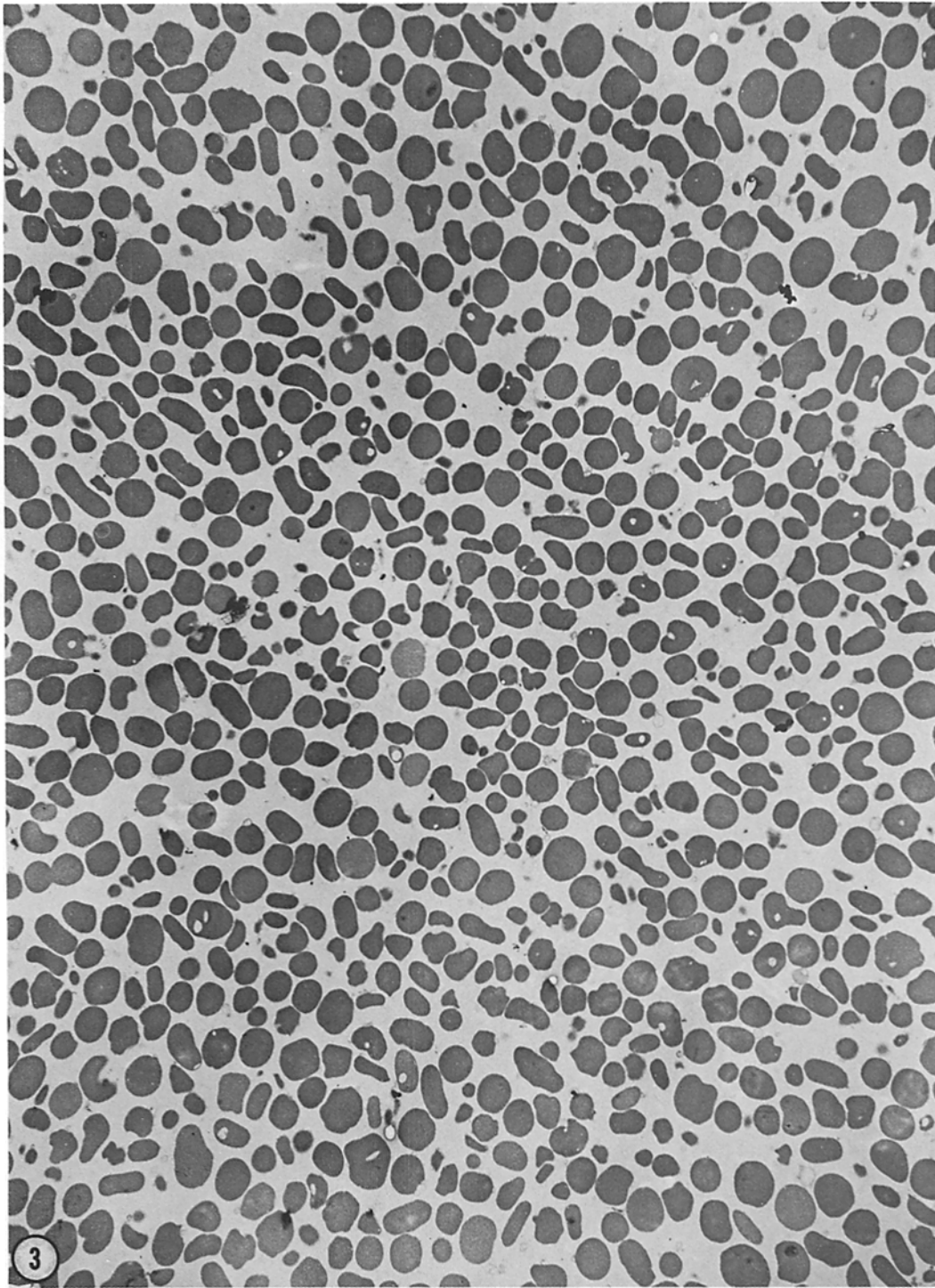


FIGURE 3 Representative electron micrograph of the purified secretion granule fraction. Samples of the granule fraction were fixed with phosphate-buffered aldehydes in suspension, then postfixated in osmium tetroxide and further processed for microscopy as pellets as described in the text. Note the variety of unusual granule shapes seen in all parts of the pellet. $\times 5,000$.

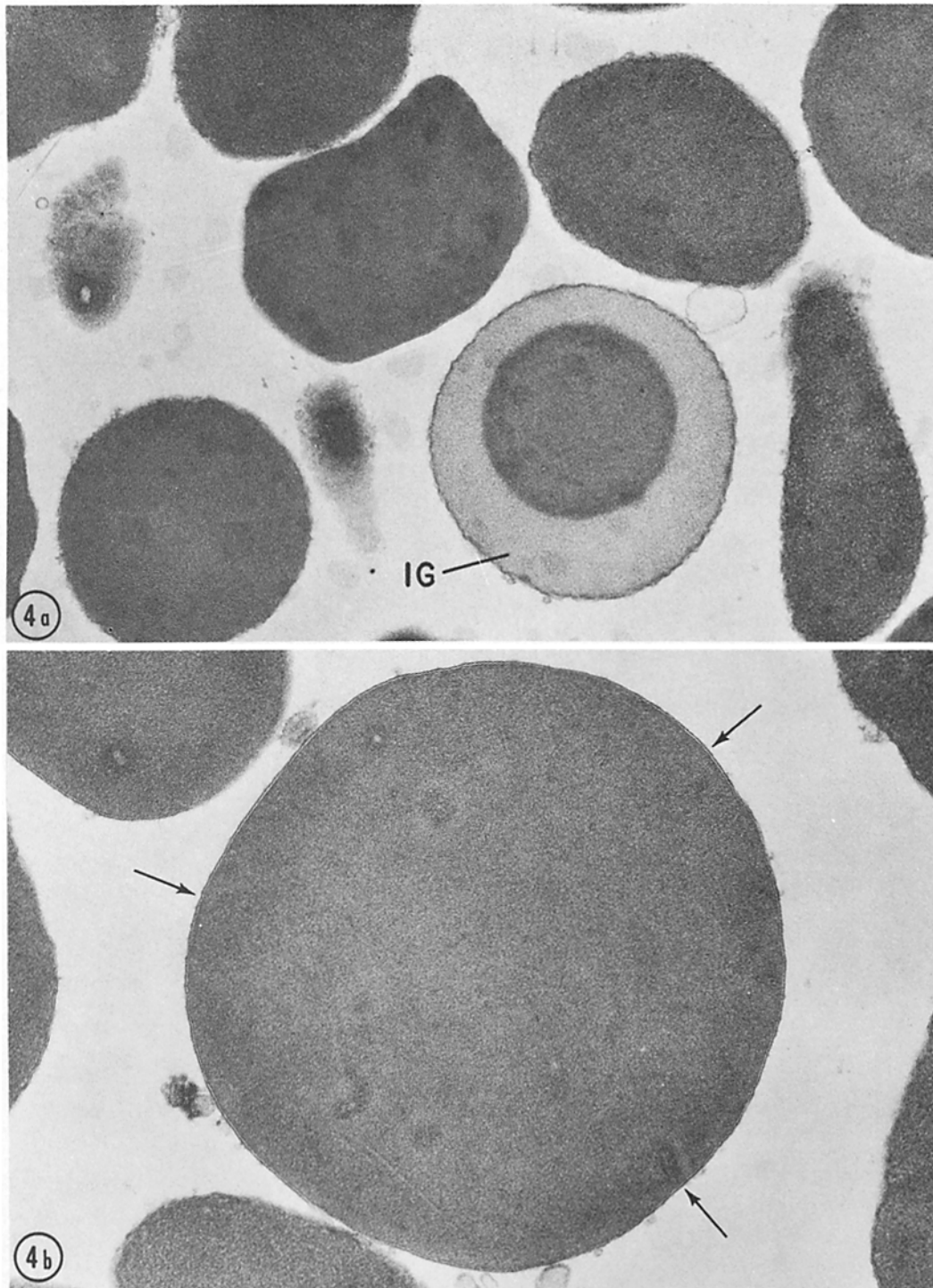


FIGURE 4 The secretion granule fraction at higher magnifications. (a) The fraction contains immature secretion granules (*IG*) recognized by their heterogeneous content (6). (b) The membranes of the granules are well preserved, the trilaminar structure being readily observed (arrows), especially in samples stained in block with magnesium uranyl acetate before dehydration. (a) $\times 36,000$; (b) $\times 56,000$.

procedure reported by Kirshner et al. (31) for rat parotid secretion granules gives a higher yield (60% of the tissue amylase activity); however, morphologically and biochemically the fraction is more contaminated than is ours. Our percentage yields of granules based on amylase activity distribution are likely to be underestimates for two reasons: (a) they are not corrected for amylase underrecovery at each step of the fractionation protocol, and (b) they are calculated on the assumption already stated, that is, amylase is localized in the tissue exclusively within secretion granules; this assumes that contributions of other compartments and of the glandular lumina to the total activity of the tissue is negligible. In any case, the yields were sufficiently high that the purified granule fraction could be considered representative of the total granule population.

Contamination of the Purified Granule Fraction by Other Cellular Organelles

Biochemical assessment of contamination of the secretion granule fraction by other subcellular

organelles was carried out using cytochrome oxidase as a marker enzyme for mitochondria, RNA as a chemical marker for rough microsomes and nuclear envelope, and *N*-acetyl- β -glucosaminidase as diagnostic for lysosomes. The distributions of marker activities for each of the steps comprising the granule isolation protocol are tabulated in Table II.

Cytochrome oxidase contamination in the granule fraction was reduced to a level of about 0.2% of the initial homogenate value, an order of magnitude better than comparable data available in the literature (3, 21, 31, 49). The final cytochrome oxidase specific activity is less than one-tenth that of the homogenate and less than one-two hundredth the specific activity expected for a purified mitochondrial fraction.

RNA, used to determine the distribution of rough microsomes, nuclei, and nuclear envelope in the fractions yielded by the protocol in Fig. 2, was reduced in the purified granule fraction to less than 0.1% of the initial homogenate value. The RNA/protein ratio of the granule fraction is at least two

TABLE II
Assessment of Contamination of the Secretion Granule Fraction by Cellular Organelles

Fraction	Cytochrome oxidase* (Mitochondria)			RNA† (Microsomes plus nuclear envelope)			<i>N</i> -acetyl- β -glucosaminidase‡ (Lysosomes)		
	Act.	%	S.A.	mg RNA	%	RNA/protein	Act.	%	S.A.
Homogenate	92.2	100.0	0.32	3.84	100.0	59.6	4.00	100.0	19.6
Pellet spin 1	9.6	10.4	0.10	1.94	50.5	43.6	1.33	33.0	18.1
Supernate spin 1	87.4	94.7	0.04	2.04	53.0	51.6	2.43	61.0	16.7
Postgranule supernate spin 2	63.2	68.5	0.38	1.64	42.7	48.0	1.78	44.5	16.3
Crude granule	6.5	7.0	0.42	0.14	3.7	60.0	0.05	1.2	5.7
Purified granule	0.25	0.27	0.025	<0.004	0.1	3.6	0.01	0.3	2.4
Debris spin 3	6.3	6.8	4.00	0.004	0.1		0.08	2.0	67.0

Act. = Activity; % = percentage distribution of activity; S.A. = Specific activity.

* Cytochrome oxidase: activity expressed as micromoles cytochrome *c* oxidized \times min⁻¹. Specific activity: activity \times milligrams protein⁻¹. The values above represent one of four determinations of cytochrome oxidase distribution. For the four different experiments the cytochrome oxidase activity of the purified granule fraction represented 0.17-0.30% of the total homogenate activity.

† RNA: content expressed as mg RNA. Normalization: milligram RNA \times gram protein⁻¹. We chose the assay of Blobel and Potter (7) for RNA determination rather than the orcinol determination because we anticipated interference by glycoproteins in the latter assay. However, the RNA content we have recorded for the purified granule fraction represents an upper estimate since protein no longer precipitable by PCA after KOH extraction represents a major contribution to the observed OD₂₆₀ for this fraction. By subjecting radioactively labeled granule protein to the extraction and subsequent reprecipitation, we determined that as much as 75% (.003 mg) of the presumed RNA could be protein. For other cellular fractions (with the exception of Debris spin 3); however, nonprecipitable protein contributes negligibly to the quantities of RNA determined.

‡ *N*-acetyl- β -glucosaminidase: activity expressed as micromoles β -naphthol released \times min⁻¹. Specific activity: activity \times grams protein⁻¹. The above data represent one of two lysosomal distribution experiments. In both experiments 0.03% of the homogenate *N*-acetyl- β -glucosaminidase activity was recovered in the purified granule fraction.

times lower than that of the purest pancreatic zymogen granule fractions available (21, 25, 49) and tenfold lower than that of the parotid secretion granule fraction of Amsterdam et al. (3). A typical pancreatic rough microsomal fraction (28) has an RNA specific activity more than twofold that of the homogenate, corresponding to approximately 40 times the specific activity of the purified fraction of rabbit parotid secretion granules.

N-acetyl- β -glucosaminidase activity is effectively segregated from the secretion granules by both discontinuous gradient centrifugations. It was reduced to 0.3% of the initial homogenate value in the purified granule fraction and had a final specific activity one-eighth the initial homogenate value and less than one-hundredth the specific activity expected for a purified rat liver lysosomal fraction (39).

In each of these assays at least one-fourth of the entire purified granule preparation was processed as a single sample and, with the exception of cytochrome oxidase, levels of activity detected were at or near the limit of sensitivity of the assay.

In summary, the fraction of rabbit parotid secretion granules obtained by this new protocol exhibits satisfactory morphological purity and at the biochemical level is quite thoroughly freed of subcellular contaminants at acceptable expense to the yield of granules.

Other Enzymatic Activities Associated with Granules

Additional characterization of the secretion granule fraction was limited to assaying for a number of potential secretory enzymes. All assays were carried out on hypotonic lysates of the fraction.

Protease activity at neutral pH was not detectable using an assay based on dye released from Azocoll (46) which should detect less than 1 μ g/ml trypsin equivalents. Neutral lipase assayed using as substrate β -naphthyl nonanoate in the presence of sodium taurocholate (8) gave a positive reaction barely above the colorimetric level of the blank only when a large fraction (one-fourth to one-third the quantity of a single experiment) of the total granule protein was assayed as a single sample. These assays confirmed the initial assumption that the parotid might represent a promising exocrine system in contrast to the guinea pig exocrine pancreas (49, 50) for the study of undegraded intracellular membranes.

Peroxidase, a secretory enzyme extensively studied in rat parotid, (23) was not detected at either pH 5 or pH 7 in rabbit parotid, confirming observations by Thomson and Morell (67) as well as making it unnecessary to include in fractionation media the antiperoxidatic agent DPPD (24) which has been used by Amsterdam et al. (3) as preventive for possible peroxidation of membrane lipid.

The nucleases, DNase and RNase, however, were both found to be present in lysates in easily detectable amounts, increasing to three the number of known enzymatic activities in the rabbit parotid secretion. DNase had a specific activity in the granule lysate of 19 U/mg protein which can be compared to a value of \sim 2,000 U/mg for Worthington purified bovine pancreatic DNase I. RNase has a lysate specific activity of about 70 U/mg protein, whereas purified porcine pancreatic ribonuclease A has a specific activity of 3,000 U/mg. If it is assumed that the specific activities of these purified nuclease standards are identical to the specific activities of the respective purified parotid nucleases, then it can be calculated that DNase would account for about 1% and RNase about 2% of the total exportable protein packaged in parotid secretion granules. However, these estimates should be regarded with some caution on two accounts: (a) protein in pancreatic standards and lysates was determined by different methods (gravimetric and Lowry [41] assays, respectively), and (b) recent work by Lundblad et al. (43) demonstrated that antibodies raised against pancreatic RNase and parotid DNase (both of bovine origin) failed to inhibit the enzymatic activities of the respective parotid and pancreatic counterparts where controls—pancreatic RNase reacted with antipancreatic RNase and parotid DNase reacted with antiparotid DNase—showed strong inhibition of enzymatic activity.

The search for activities associated with the secretion granule content was not carried past this point since primary interest was focused on the resolution of lysed granules into their limiting membranes and packaged content.

Lysis of Secretion Granules and Subsequent Separation of Granule Membranes and Secretory Content

To reduce the high tonicity of the solution in which the purified granule fraction is initially suspended (1.9 M sucrose + 40 mM potassium

phosphate + 1 mM EDTA) and thereby induce hypotonic lysis, we devised a procedure based on stepwise dilution and pressure dialysis in an Amicon ultrafiltration cell. In this manner it was possible (a) to avoid the long time periods required for standard dialysis against media low in salt concentration as were used by Amsterdam et al. (3) for lysis of rat parotid secretion granules, and (b) to prevent fragmentation of membranes resulting from too abrupt a change in tonicity which occurs when granules are pelleted from high molarity sucrose and resuspended directly in lysis medium. The lysis procedure comprises the following steps:

1 Isolated granules along with 3 vol of lysis medium (20 mM NaHCO₃, 0.5 mM EDTA, pH 7.2) were placed in an Amicon ultrafiltration chamber outfitted with a PM10 sieving membrane (which retains molecules of 10,000 mol wt or greater). At this point sucrose and phosphate concentrations were approximately 0.5 M and 10 mM, respectively.

2 The diluted granules were concentrated to one-half the initial volume under pressure (N₂ at 60 lb/in²).

3 Lysis medium was added up to the original volume, thus reducing approximate sucrose and phosphate concentrations to 0.25 M and 5 mM, respectively. At this stage partial clearing of the suspension was observed.

4 The rediluted granules were then concentrated to one-third the original volume under pressure.

5 Lysis medium was then added up to the original volume, thus reducing the approximate sucrose and phosphate concentrations to 0.08 M and 1.6 mM, respectively. At this point the suspension completely cleared indicating apparently complete granule lysis.

6 The lysate was concentrated to 15 ml and removed from the Amicon chamber. The chamber was washed with an additional 5 ml lysis medium, the wash being combined with the concentrated lysate.

In the case of the guinea pig pancreatic zymogen granules, the procedure for resolving granule lysates into membranes and content took into account the unusually low density of the granule membranes. These membranes, which had accumulated free fatty acid secondary to the action of endogenous pancreatic lipase on tissue triglycerides and membrane phospholipids (50), banded at a 0.7–1.0 M sucrose interface, while residual

mitochondria pelleted through the 1.0 M sucrose during centrifugation. Since rabbit parotid secretion granules contain little or no lipase activity, the membranes of lysed granules would not be expected to undergo any significant shift to lower density. We resolved the final concentrated hypotonic lysate into membranes and packaged content using the two-step centrifugation scheme shown in Fig. 5. The granule lysate was layered over a discontinuous sucrose gradient containing steps of 0.2 M, 0.6 M, and 1.5 M sucrose each supplemented with 20 mM NaHCO₃, 0.5 mM EDTA, pH 7.2. It was intended that the slightly hypotonic 0.2-M layer would provide a wash of the ghost fragments before any resealing of ghosts anticipated to take place in the 0.6-M layer. Centrifugation using an International B60 ultracentrifuge with an SB 283 rotor was carried out for 60 min at 160,000 *g*_{av} to separate membranes (at the 0.6–1.5-M sucrose interface) from granule content (remaining in the original load). The granule content was collected (including some of the 0.2-M layer beneath) and used according to the requirements of the experiment, either directly for enzyme assays, or concentrated for amylase precipitation, or processed for use in polyacrylamide gel electrophoresis (lyophilization, resuspension in 0.1 M

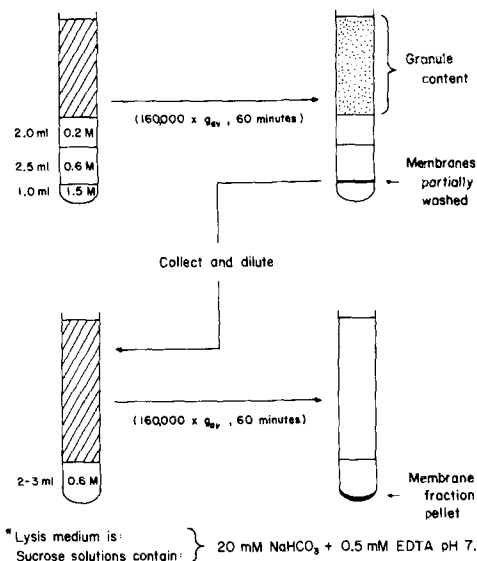


FIGURE 5 The separation of membranes and content from secretion granule lysates. Centrifugal forces (*g*_{av}) refer to the values at the center of the tubes, all tubes being filled to capacity. Sucrose solutions contain 20 mM NaHCO₃, 0.5 mM EDTA, pH 7.2.

NH_4HCO_3 , desalted over a column of Biogel P-2, lyophilization and resuspension in water). The granule membranes were harvested and diluted fivefold with lysis medium. The diluted membranes were then layered over a 0.6-M sucrose cushion (containing 20 mM NaHCO_3 , 0.5 mM EDTA, pH 7.2) and pelleted at 160,000 g_{av} for 60 min. The resultant pellets were resuspended for chemical or enzymatic assay, fixed for morphological observation, or dissolved in SDS for polyacrylamide gel electrophoresis.

Assessment of the Degree of Separation of Content and Membranes

We carried out an enzymatic assessment of the degree of separation of granule membranes and secretory content achieved by the above protocol by determining the amount of residual amylase activity present in the purified granule membranes relative to that present in either the granule content or unlysed granules. A representative amylase distribution is presented in Table III. Consistently, membranes were freed of amylase to a level of 0.03–0.07% of the initial total granule complement suggesting, on the basis of a single major secretory protein, a very satisfactory separation.

Morphology of the Membrane Fraction

Fig. 6, a low-magnification electron micrograph of the membrane fraction, shows a homogeneous

population of unusual oblong structures that appear to be collapsed or folded membrane vesicles. Throughout the thickness of the pellet, cut to expose the entire cross section of sedimented material, the appearance did not differ at all (only the spacing of vesicular elements differed) from that seen in Fig. 6, a field taken from the middle of the pellet. Higher magnification (as seen in the *inset* of Fig. 6) revealed that the membranes were indeed quite clean, and generally organized in closed vesicles as indicated by the absence of free ends. At many points the membranes of these vesicles showed typical bilayer structure, and at certain points along the inner aspect a fine fibrillar material (marked *f*) is evident.

Contamination of the Membrane Fraction by Other Cellular Organelles

Attempts to assess the degree of contamination of the membrane fraction using marker assays for other cellular organelles were, for the most part, unsuccessful since the use of half the entire membrane fraction per assay failed to give values above the assay background. Cytochrome oxidase, the mitochondrial marker, was, however, detectable somewhat above assay background in amounts representing 16–20% of the total activity associated with the granule fraction, while its specific activity in membrane fractions was five times that of the secretion granule fraction, indicating that mitochondria contaminating the granule fraction tended to cosediment with granule membranes after granule lysis. Despite this cosedimentation, it can be approximated that, at most, 5% of the protein of the granule membrane fraction can represent mitochondrial contamination.²

SDS-Polyacrylamide Gel Electrophoresis of Granule Membranes, Granule Content, and Secretion Collected by Cannulation of Parotid Ducts

Representative Coomassie brilliant blue-stained 11% polyacrylamide gels of granule membranes,

² By morphometric measurement mitochondria constitute 5% of the rabbit parotid acinar cell volume (69); hence, in obtaining a pure mitochondrial fraction, at least a 20-fold enrichment (relative to the homogenate) of cytochrome oxidase activity is expected if it is assumed that the protein concentration of the mitochondrion is the same as that of the entire cell. We have based our approximation on this consideration.

TABLE III
Removal of Amylase from Secretion Granule Membranes

Fraction	Units amylase activity	Percentage distribution
Secretion granules	1,670	100.0
Granule content	1,608	96.3*
Granule membranes	1.1	0.06

* The recovery of granule amylase activity after concentration of the lysate in an Amicon chamber varied from 66–100% in eight experiments. To consistently recover over 90% of the amylase activity at this step, it is necessary to siliconize the walls of the chamber and to use a sieving membrane that has been exposed to granule protein previously: (more than 10% of the total granule amylase activity has been found to stick to a sieving membrane that has not been conditioned with protein before use).

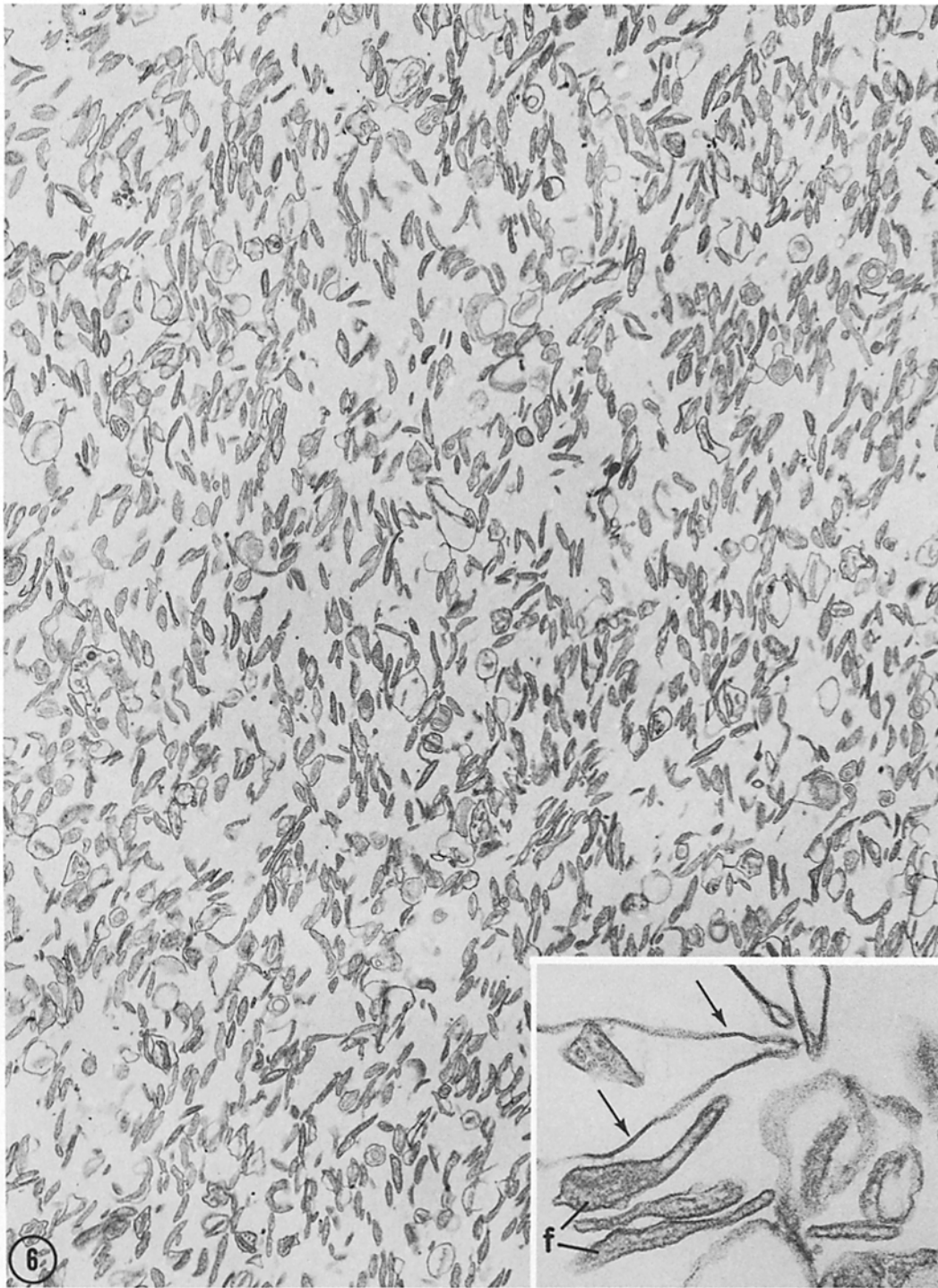


FIGURE 6 Electron micrograph of the granule membrane subfraction taken from the center of the pellet, but representing in composition all levels of the pellet. Evidently the membranes do not have the appearance of spherical granule ghosts. Instead, they are, for the most part, collapsed and infolded vesicles. The *inset* shows a portion of the pellet at higher magnification. The trilaminar structure of normally cut membranes is indicated by arrows. At many points along the inner aspect a fine fibrillar material (*f*) can be seen which appears to form a quasicontinuous layer in regions of folding. $\times 15,000$; *inset*, $\times 62,000$.

granule content, and material collected by cannulation of parotid ducts after *in situ* stimulation are presented in Fig. 7 accompanied by a logarithmic molecular weight scale based on the mobilities of β -galactosidase, albumin, ovalbumin, chymotrypsinogen, and cytochrome *c*. The staining patterns for granule content and duct collection are quite similar and show a surprising number of bands representing exportable proteins, considering the paucity of identified enzymatic activities. Although there was considerable variability in the amounts of various secretory components from experiment to experiment, three bands in the content are distinguished as major components having mol wt of approximately 58,000, 33,000, 12,000.⁹ The pattern observed for granule membrane, however, is considerably more complex with 20–30 Coomassie brilliant blue-staining bands being readily visible, the majority having mol wt greater than 30,000. Of particular interest is a single band of estimated mol wt 40,000 which stands out as a major component. Another interesting property of two low mobility bands evident in gels of the secretion obtained from cannulated parotid ducts (designated *Me* in Fig. 7 and a pair of high molecular weight species present in gels of granule membranes (also designated *Me*) is that they stain pink with Coomassie brilliant blue. This metachromatic staining is unstable, fading with time, but is reestablished by restaining (compare Fig. 7 and Fig. 9) indicating qualitatively that loss of visibility of these components is not a result of protein loss from the gel to the isopropanol-acetic acid fixative. The granule content has only a single metachromatic band corresponding to the higher mobility metachromatic species seen in the secretion collected from the duct, but it faded before photographing this gel. PAS-positive bands are indicated by an arrow alongside the corresponding Coomassie brilliant blue-stained band. Note that the major content species of estimated mol wt 58,000 and 12,000 are PAS-negative and thus fill the additional role of internal controls in this assay.

⁹ The molecular weights given should clearly be considered first approximations since electrophoretic mobility in SDS-polyacrylamide gels is not strictly a log-linear function of molecular weight and is known to depend on the polysaccharide content of the protein (19, 63) and on the sample volume loaded.

Identification of Amylase on Gels

The enzymatic identity of only one of the bands present in the granule content, i.e., amylase, has been established. Amylase was purified as described in the Materials and Methods by the procedure of Schramm and Loyer (62). The purified enzyme electrophoreses in SDS-polyacrylamide gels as a single band, and its mobility corresponded to that of the content band of ~58,000 mol wt. The fraction of the granule content left soluble after ethanol precipitation of the amylase-glycogen complex was devoid of the 58,000-mol wt component.

Comprehensive Identification of Residual Content Polypeptides Contaminating Secretion Granule Membranes

A necessary prerequisite to investigations of the physical and chemical properties of granule membranes, the rates of synthesis of the polypeptides of content and membranes of secretion granules, and the relative turnover of the polypeptide components of these two granule subfractions is a rigorous assessment of the degree of contamination of granule membranes by residual secretory proteins in a more comprehensive manner than by enzymatic assay for one or two representative species. Since we had identified only three enzymatic activities associated with the secretion granule fraction (one major [amylase] and two minor [DNase and RNase]), and since the SDS gel electropherograms reveal at least seven polypeptide components in the granule content, an exhaustive examination of granule membranes for contaminant enzymatic activities was considered useless. Instead, we chose an alternative approach which aimed at the identification of all granule content proteins which contaminated the granule membrane fraction, the identification being carried out by comparative SDS-polyacrylamide gel electrophoresis.

To obtain disk gels of satisfactory uniformity and optimal porosity for the entire molecular weight range represented in both membranes and content, resolving gels with an 8–12% polyacrylamide continuous gradient were made in groups in a single container as described in detail in the Materials and Methods section. As a result, bands throughout the molecular weight range were sharp,

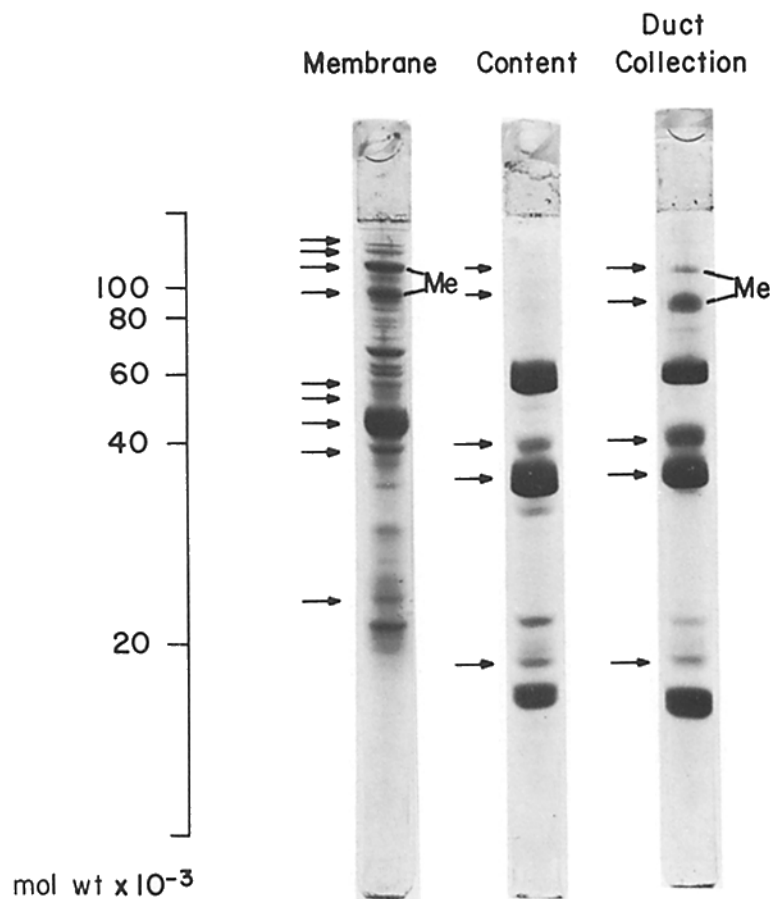


FIGURE 7 11% polyacrylamide gel profiles of granule membrane, content, and secretory protein collected by cannulation of parotid ducts. Samples for electrophoresis were dissolved in SDS and reduced as described in the text. Sample loads were 125 μg , 100 μg , and 140 μg protein (41) for membrane, content, and duct collection, respectively. Loads for gels reacted with the PAS technique were 150–200 μg protein (*Me*) denotes bands that stain metachromatically with Coomassie brilliant blue. Arrow indicates periodic acid-Schiff positive band.

and mobilities of identical proteins were quite comparable from gel to gel.

Complete assessment of content contaminants was achieved in two complementary types of experiments. I To carry out the first type, radioactively labeled granule content was prepared by pulse labeling lobules with [^{14}C]amino acids, chase incubating the tissue until the incorporated label had reached mature secretion granules, isolating, and finally lysing the granules to obtain the labeled soluble extract.⁴ This radioactive extract was then

⁴ The term extract rather than content will be used in the rest of the paper for reasons that will soon become evident; the extraction procedure employed does not fully remove the content.

used for two mixing experiments: (a) an isolated granule fraction was lysed in the presence of ~ 0.1 μCi of the radioactive extract, and the membranes were subsequently isolated and processed for gel electrophoresis; (b) in the second type of experiment, isolated granule membranes solubilized in SDS were mixed just before gel electrophoresis with radioactive extract (an amount corresponding to one-fifth the radioactivity loaded on gels of extract alone). These two types of experiments should establish which bands in the gel electropherogram of a usual membrane fraction represent content contaminants. Such bands should be easily identifiable by (a) their comigration with radioactivity, (b) their increased absorbancy at 550 nm, and (c) their identical mobility to bands present in

the gel electrophoretic pattern of the extract. The results of the two mixing experiments are presented in Fig. 8 accompanied by the radioactivity and optical density (Coomassie brilliant blue) profiles given by a gel electropherogram of the radioactive extract used in these experiments. Also included as a control is the staining pattern of the gel of an unlabeled, untreated granule membrane fraction. A comparison of the four composites in this figure reveals that content contaminants not removed by the extraction procedure can be easily identified in control membrane preparations since, as expected, they have the same mobility as radioactivity peaks and amplified optical density peaks in the mixing experiments.

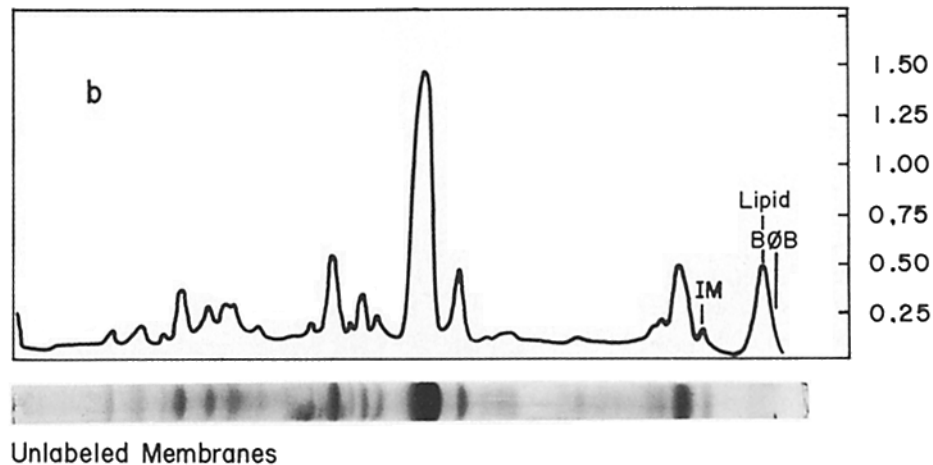
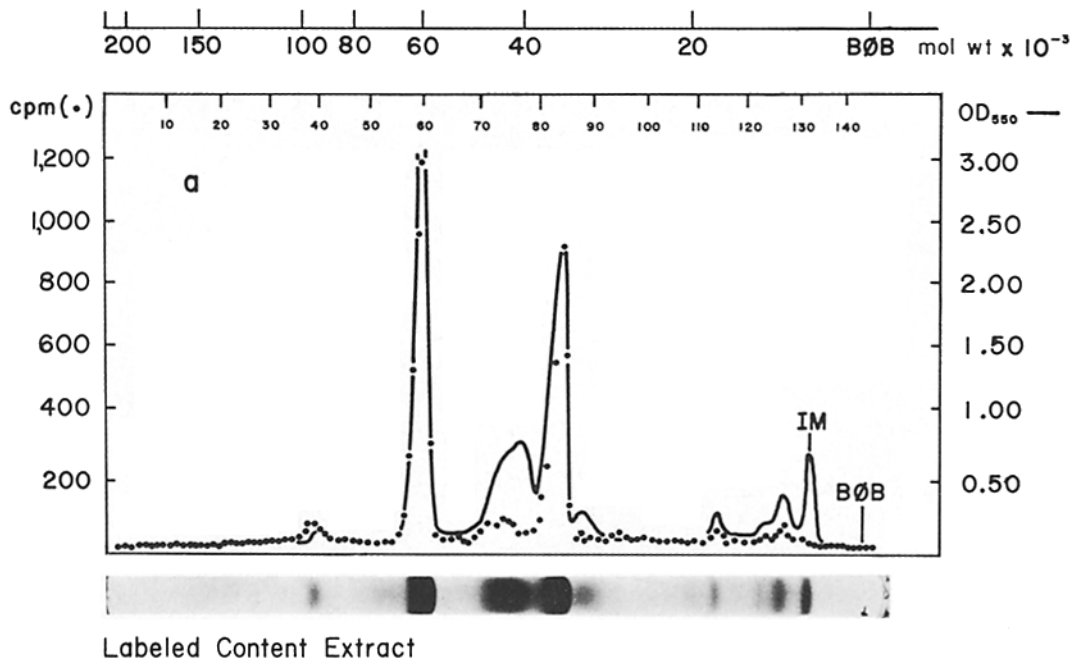
Amylase, for example, recognized at mol wt 58,000 in the electropherogram of the extract (panel *a* of Fig. 8) was present as a substantial band in the gel profile of untreated membranes (panel *b* of Fig. 8) even though enzymatic assay showed that 99.93–99.97% of the total granule complement had been removed postlysis. The gel pattern of isolated membranes mixed with radioactive granule extract just before electrophoresis (panel *c* of Fig. 8) demonstrates very clearly that content amylase coelectrophoreses with the band recognized as containing amylase contaminant in the gel profile of untreated (control) membranes. In general, a rather large number of the proteins of the granule extract appear to comigrate with bands present in the membrane gel profile and can be recognized as contaminants of the membrane based on the criteria already mentioned. Specifically, contaminants are easily identified at estimated mol wt 95,000, 58,000 (amylase), 33,000, and 12,000. These bands have been consistently observed in all our granule membrane gel profiles to date; however, there are quantitative variations in the amounts of each residual secretory species. For example, the two different membrane preparations used in the experiments in Fig. 8—unlabeled membranes and membranes isolated from a granule fraction lysed in the presence of labeled extract—show this variability. Residual amylase, is much more prevalent in the unlabeled (control) membrane preparation than it is in membranes lysed in the presence of radioactive extract, while the reverse is true for 33,000 mol wt contaminant species.

Further, as can be seen in the stained electrophoretic profile of membranes mixed with labeled extract just before electrophoresis in Fig. 8, the metachromatic, pink-staining band present in

granule membranes at mol wt 95,000 coelectrophoreses with radioactivity associated with the metachromatic band present in the granule extract suggesting possible identity of the two species.

Finally, an estimate of the magnitude of adsorption of radioactive extract to the granule membranes which were exposed to the label during granule lysis was provided by the observation that 1% of the radioactivity initially added to the lysis chamber was present in the final membrane preparation as determined from summation of the gel radioactivity profile.

II In the second study a comprehensive assessment of contamination of membranes by secretory content was carried out by comparing gel electropherograms of granule membranes, granule extract, unlysed granules, saliva collected from cannulated ducts (see Materials and Methods), and secretory protein discharged into the incubation medium from isoproterenol-stimulated lobules. The last two samples provided references for the identification of all granule protein destined for secretion. In choosing these references we realize that we have administered an analogue of a physiologic secretagogue at nonphysiologic concentrations, but we assume that what we collect as secretion represents the mixture of proteins released under physiologic conditions. Hence, we will refer to such samples as physiologic standards. Furthermore, the relative amounts of various secretory proteins present in the collected secretion and in unlysed granules allowed direct and independent assessment of the thoroughness of the nonphysiologic means (20 mM NaHCO₃, 0.5 mM EDTA, pH 7.2) of extracting granule content proteins and thereby separating them from granule membranes. The comparison of gel electropherograms of granule membranes and extracted content with those of unlysed granules and the two standards—protein released into ducts (duct collection) or into incubation media (lobule secretion)—is presented in Fig. 9. Since protein in the membrane fraction represents at most 3% of the total granule protein, nearly the entire population of granule membranes of a single isolation was subjected to electrophoresis on a single gel. On the other hand since content represents the bulk of granule-associated protein, very small fractions (less than 1%, ~90 μ g (Lowry et al. (41)) of (*a*) the total secretion (duct collection or processed incubation medium), (*b*) the total granule population of a single isolation, and (*c*) the total soluble extract of a single lysis experiment were subjected



to electrophoresis on the appropriately designated gels. From this study, it is evident that all bands which stain metachromatically with Coomassie brilliant blue and not just the band of higher mobility (identified in the mixing experiments) belong to the secretory content and, in fact, should be considered major components of the secretory mixture. In stained profiles of gels of the physiologic standards and especially in those of unlysed granules, these bands represent a significant fraction of the staining intensity of the entire profile

indicating that in recovering secretory content postlysis, a considerable amount of these high molecular weight, metachromatically staining species were lost by tenacious adsorption to the granule membranes and probably (because of the quantities involved) by adsorption to the walls of the Amicon lysis chamber.

Combination of sonication with extraction by NaCl solutions at low (50 mM) as well as high (500 mM) concentrations (used to free membranes of other systems of loosely adsorbed proteins [1, 61])

MIXING EXPERIMENTS

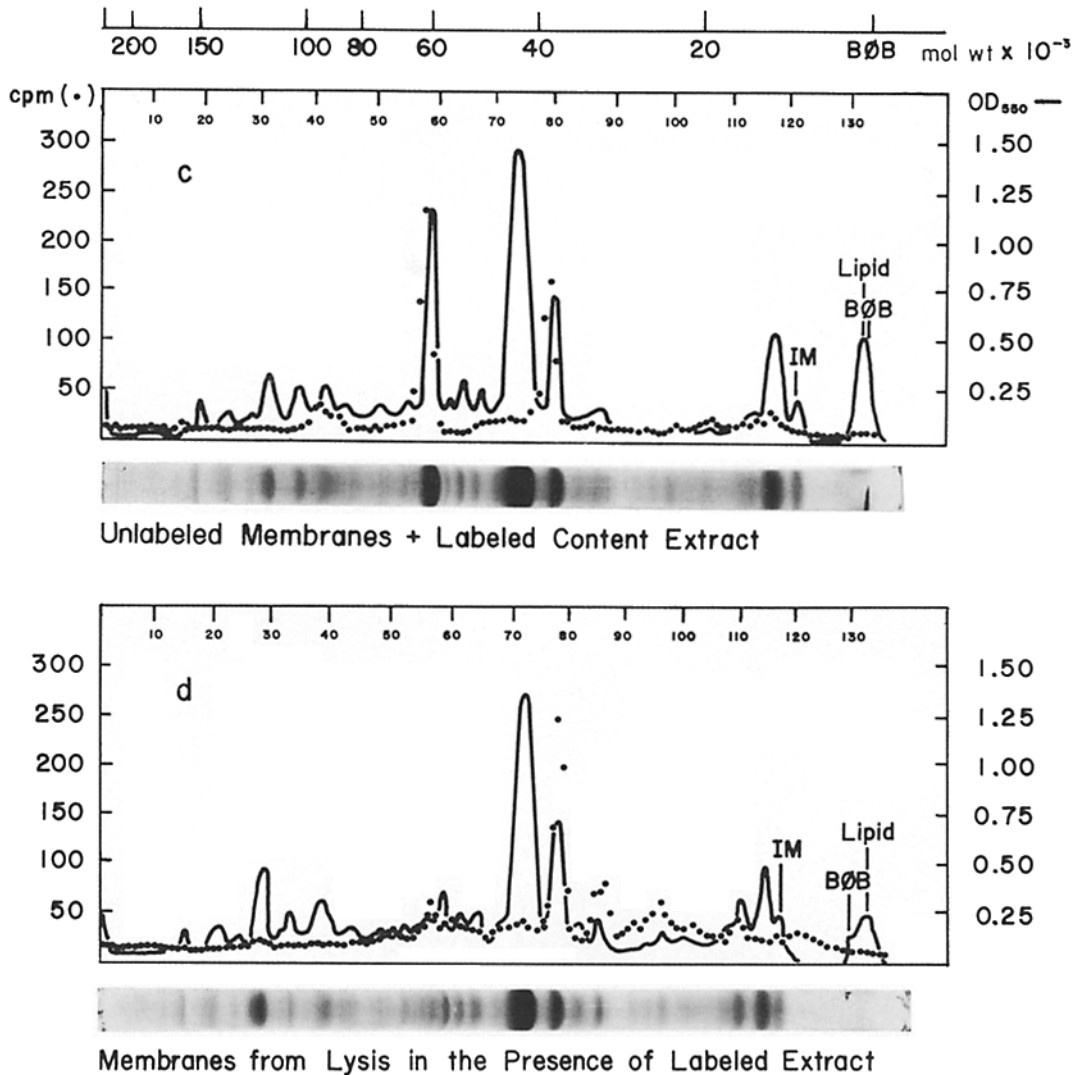


FIGURE 8 Mixing experiments used to identify bands in electropherograms of granule membrane subfractions that represent residual content contaminants. Panels *a*, *c*, and *d* of the figure are composites of gel photographs, OD_{550} scans of the staining pattern (—), and radioactivity profiles (·) obtained by slicing and counting the gels. Panel (*b*) is a composite which has no radioactivity profile. Gels are polyacrylamide continuous (8–12%) gradients. (*a*) [^{14}C]granule extract which was used for both mixing experiments that are presented in panels *c* and *d*. (*b*) Unlabeled (control) gel profile of the membrane subfraction. By comparison of this profile with that of the two mixing experiments (panels *c* and *d*), it is possible to identify which bands composing a usual membrane subfraction represent or comigrate with residual content contaminants. (*c*) Mixing experiment in which labeled extract (one-fifth the amount of panel *a*) was added to SDS-solubilized granule membranes (the identical membrane preparation viewed in panel *b*) just before electrophoresis. Contaminants can be recognized as comigrating with radioactivity and having an amplified staining intensity in comparison to bands present in the profile of panel *b*. (*d*) Mixing experiment in which 0.1 μCi [^{14}C]granule extract was added to secretion granules in the Amicon chamber before lysis. The electrophoretic pattern of the membrane subfraction separated from the ensuing lysate is presented in this panel. Most bands which comigrate with radioactivity can be recognized as containing residual content contaminant. A few additional bands between $\sim 20,000$ – $30,000$ mol wt, however, appear labeled. The labeling of this region of the gel has not been reproducible in subsequent mixing experiments. IM = Internal marker: myoglobin. BøB = Bromphenol blue.

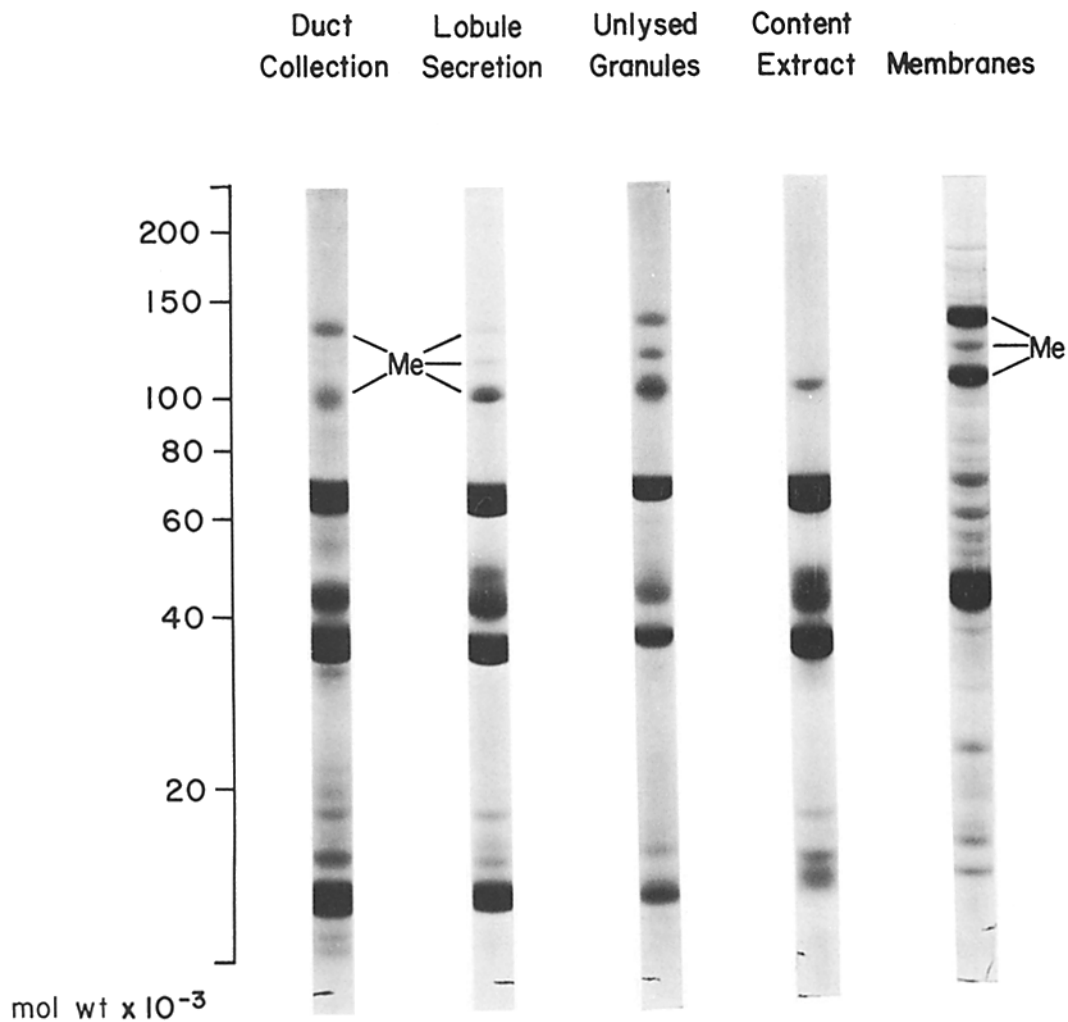


FIGURE 9 The physiological reference system for comprehensive identification of all secretory proteins of the acinar cells of the rabbit parotid gland. The electropherograms of the physiologic standards—secretory protein collected from cannulated ducts and from stimulated lobules—and that of unlysed granules indicate the entire spectrum of proteins destined for export. Evidently, the metachromatically staining species (designated *Me*) present in the membrane profile between 95,000–130,000 mol wt are all proteins destined for export; however, they remain tenaciously adsorbed to membranes after the non-physiologic process of granule extraction. Note in the electropherogram of duct collection that one metachromatic species is missing. A faint band was present but faded before the gel was sufficiently destained to be photographed; however, the bulk of this species as well as a notable proportion of all metachromatically staining polypeptides of the lobule secretion may have been lost in processing these samples for gel electrophoresis.

or sonication in the presence of the mild chaotropic agent sodium bromide (0.25–0.50 M) (16, 44) did not succeed in further reducing adsorption of these proteins to granule membranes.

In summary, a physiologic reference system has

been used which uniquely defines on high resolution gel electropherograms which proteins are truly destined for release from granules. Not only did it establish the entire spectrum of secretory proteins, but additionally it provided a reliable

base of reference for assessing the efficiency of the procedure used for the extraction of the content of the granules. At present the assessment is semi-quantitative; it can be made quantitative by amino acid analysis (as a sensitive measure of composition and total protein) of content versus membrane contaminant gel bands. Furthermore, it complemented the mixing experiments performed with labeled granule extract to provide critical means for the identification of the content components which contaminated the granule membranes, especially where correlations between polypeptides visible as stained bands on gels and enzymatic activities have not been established. The gels in Fig. 10 summarize pictorially the bands identified as content contaminants of the granule membrane fraction. The arrows indicate bands representing or containing secretory contaminants in the membrane fraction; the remaining bands are considered for the present to be truly membrane-associated polypeptides. The companion gel of unlysed granules represents less than 1% of the total granule fraction of a single isolation.

DISCUSSION

Since no data were available concerning the density of secretion granules of the rabbit parotid as well as their permeability to the suspension media, we determined the parameters of the granule isolation procedure empirically. The technique is based essentially on differential velocity sedimentation with three modifications: (a) a cushion of high molarity sucrose (through which granules will not penetrate) was loaded in the bottom of tubes for centrifugation designed to sediment secretion granules. In this manner close contact and aggregation between granules and other cellular organelles as well as damage to granules incurred by resuspension of tightly packed pellets were largely avoided; (b) an intervening layer of sucrose was introduced above the cushion in the second centrifugation to minimize contamination of the sedimented fraction by particulates smaller than granules; and (c) a final purification step which makes use of high density sucrose media served to remove residual membranous debris by flotation and to wash simultaneously the sedimenting granules. We monitored the results of the procedure by morphology (for homogeneity) and by enzymatic assay (for recovery and extent of contamination). We

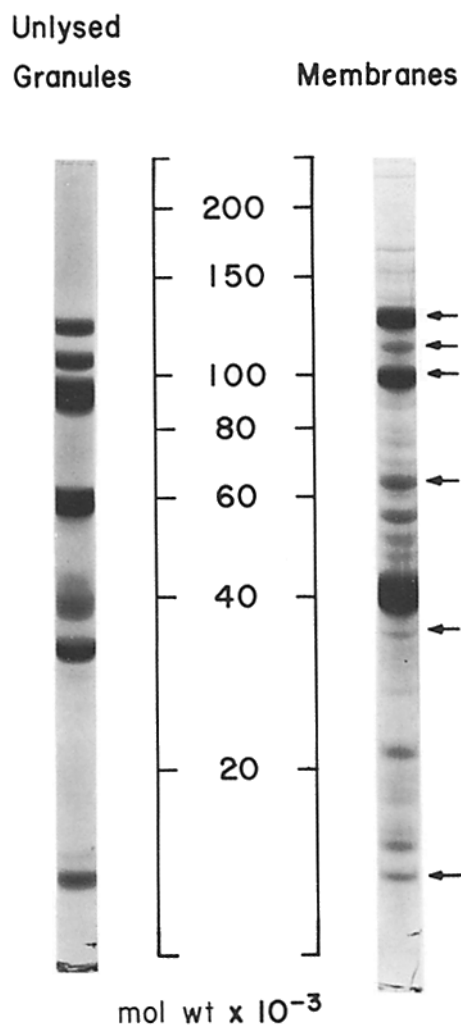


FIGURE 10 The Coomassie brilliant blue-stained species present in gel electropherograms of the membrane subfraction which readily identified as contaminants representing residual granule content by comparison to the polypeptide spectrum of unlysed granules. Arrow indicates granule content contaminant.

made no attempt to attain full recovery, but instead considered a fraction representing 15% of the total tissue-associated amylase sufficient to warrant its use in subsequent experiments. The high purity of the granules can be ascribed in part to their large size and high density ($\sim 1.26 \text{ g/cm}^3$).

The procedure we developed for lysing the secretion granules of the rabbit parotid exploited granule lability in hypotonic media rather than slightly alkaline pH (7.5–8.0), known to be very

effective in the lysis of pancreatic zymogen granules (21, 25, 49), since in phosphate buffer the granules remained relatively stable when pH was adjusted upwards to mild alkalinity. The use of an Amicon ultrafiltration cell proved advantageous since tonicity could be reduced much more rapidly than by standard dialysis, yet the decrease in sucrose concentration is gradual and can be controlled. Under such conditions the granule membranes are recovered as large, apparently intact vesicles; if granules are transferred directly from the high-molarity sucrose in which they are isolated into very hypotonic lysis medium, wholesale fragmentation of the granule membrane occurs.

The granule membrane subfraction obtained by our procedure is unusual in that it is not a population of thoroughly extracted spherical ghosts, but rather it appears to be a homogeneous population of collapsed and infolded vesicles. The fine, fibrillar material seen protruding from the inner aspects forms a quasicontinuous layer in regions where the vesicles are flattened, suggesting that the special form of the granule ghosts is due to the interaction of the fibrillar material lining the apposed faces of the granule membrane. This material may possibly represent the bulk of the secretory protein identified in gels as adsorbed contaminants of the purified granule membrane subfraction.

Polyacrylamide gel electrophoresis of secretion granule extract and the released secretion collected by cannulation of parotid ducts has revealed that the spectrum of polypeptides destined for export in the rabbit parotid is considerably more complex than generally assumed in the literature or suggested by the somewhat limited enzymatic characterization carried out in this study. Several major bands spanning the mol wt range of 12,000–130,000 are visualized by staining with Coomassie brilliant blue; however, the number does not approach the 20–40 bands visualized by Meyer and Lamberts (52) for human parotid saliva subjected to electrophoresis in gels not containing detergents. Amylase has been identified and used throughout the present studies as an enzyme characteristic of secretion granules, and it has been localized to the region of estimated mol wt 55,000–60,000 in SDS-polyacrylamide gels.⁵ The nucleases, DNase and RNase, al-

⁵ A similar means of identifying parotid amylase in SDS-polyacrylamide gels has been reported recently (40).

though easily assayable in granule lysates, appear to represent only minor components (hence minor gel bands of the electrophoretic pattern) of the parotid secretion based on calculations which assume that their specific activities are equal to those of crystallized pancreatic standards. Thus at least six major content species resolved on the basis of molecular weight remain unidentified in terms of activity and function. They might represent digestive enzymes (or zymogens) for different substrates like the kallikrein described in human parotid saliva (4) or proteins with or without enzymatic activity acting as antibacterial agents. Lysozyme might be present as it is in other secretions, especially since peroxidase is absent (32). Additionally, we should test for the presence of the polypeptides rich in glutamic acid, proline, and glycine found in human parotid saliva by Bennick and Connell (4) and Oppenheim et al. (56).

SDS-polyacrylamide gel electrophoresis of granule membrane subfractions has shown that the polypeptides of this subfraction, presumably membrane polypeptides, can be resolved into at least 20 bands (neglecting content contaminants) of distinct molecular weight. What is most striking about this electrophoretic pattern is that a single band, ~40,000 mol wt, predominates. Other polypeptide bands recognized as membrane components are primarily greater than 40,000 mol wt.

Comparison of the gel electrophoretic pattern obtained for the membranes of the secretion granules in other systems (e.g. zymogen granules of the exocrine pancreas [44, 48] and chromaffin granules of the adrenal medulla [70]) seems to indicate that membranes specialized for storage of exportable products have a relatively simple composition, that is, are resolved into a limited number of Coomassie brilliant blue-stainable bands and contain a few polypeptide species which constitute a large fraction of the total protein as estimated from stain distribution in the entire profile. This apparently common feature remains to be tested by determining whether these major components in granule membranes prepared from the parotid or other sources are or are not bona fide membrane proteins. Such a situation should be contrasted with that found for other intracellular membranes for which the number of resolvable bands is considerably higher and in which majority components have seldom been detected (19, 36, 48).

Available evidence suggests that secretion gran-

ule membranes are functionally specialized to recognize cellular membrane on the apical front of the cell and to interact with it in exocytosis. If there exist no other functions performed by this membrane, then the presence of a few majority components becomes understandable. The majority components so far recorded in the membranes of secretion granules are, however, of different molecular weights: 40,000 for parotid; 74,000–85,000 for pancreas (44).

Several of the polypeptide bands in the gel electropherogram of the granule membrane fraction reacted positively with the PAS procedure suggesting the presence of bound polysaccharide. At least three of the reactive species can be recognized as content bands contaminating the membrane gel pattern (at 130,000, 95,000, and 33,000 mol wt) by correlating electropherograms in gradient and 11% nongradient gels. The rest presumably represents true membrane glycoproteins. It should be understood that the comprehensive identification of the carbohydrate-containing species requires more stringent criteria than PAS reactivity. In the future it would be of interest to determine by appropriate means (surface-labeling techniques) if the glycoproteins of the granule membrane are asymmetrically distributed as is the case for cell membranes in other systems (10, 12, 27, 64, 66).

As indicated in the Results, a comprehensive determination of the extent of contamination of granule membranes by residual secretory species is best carried out by establishing a physiologic standard which should be qualitatively and quantitatively an accurate index of all content species released from the granules. For these experiments the standards consisted of parotid secretion collected in two ways: (a) by cannulation of parotid ducts and (b) from incubation media after isoproterenol stimulation of parotid lobules. Unlysed secretion granules can be used as an additional convenient reference since they are expected to contain the polypeptides of the granule membrane in negligible or undetectable amounts and since they constitute a control for any alteration of the polypeptide composition the secretion standards may incur during desalting and lyophilization before electrophoresis. The reference systems were defined in terms of bands resolved by gel electrophoresis since in the absence of known functions for all the secretory proteins of the parotid, activity assays could not be considered comprehensive enough as checks for contamination. With this

approach we immediately recognized the metachromatically staining bands of low mobility which were very prominent in the electrophoretic pattern for granule membranes as being absorbed proteins which under physiologic conditions are released from secretion granules and, in fact, represent a substantial fraction (as roughly estimated from relative staining intensities) of the secretory protein packaged within the granules.

The mixing experiments using radioactive granule extract were useful for sorting out from a complex electrophoretic profile, in an exact manner, which polypeptides of the membrane subfraction comigrate with material recognized as residual contaminating content. In retrospect, the use of radioactive secretion collected from *in vitro* incubated lobules, in place of radioactive granule extract, would have been advantageous for these mixing experiments since in this way the added mixture would have included all content proteins, especially all metachromatically staining species, which are lost from the extract by adsorption to granule membranes and presumably to the walls of the Amicon ultrafiltration chamber during the concentration of the granule lysate. Moreover, the gel electropherograms of the two physiologic standards show very similar staining patterns (although one of the metachromatically staining species [$\sim 110,000$ mol wt] is absent from the gel pattern depicting saliva collected from the cannulae). Apparently, the secretion of acinar cells undergoes little if any modification in protein composition by cells lining the major ducts of the gland. Considering the missing metachromatically staining species described above along with the general poor recovery of all metachromatic species in content extracts of granule and with the decreased quantities of these same three polypeptides in the electropherogram of lobule secretion relative to the levels observed in the pattern for unlysed secretion granules, it appears as if these polypeptides are selectively lost during the process used to prepare the released proteins for gel electrophoresis.

In summary, although the paucity of assayable enzymes in the parotid secretion of the rabbit necessitates devising alternative means for achieving comprehensive assessment of residual content contaminants of granule membranes, the use of comparative electropherograms for recognizing contaminants as stained gel bands is advantageous since (a) it allows identification of contaminants, endowed as well as not endowed, with enzymatic

activity, and (b) it defines the level below which enzymatic activities of the residual content must be reduced to qualify as negligible contaminants. In the study at hand, the amylase that remains in the membrane subfraction after granule lysis and membrane isolation (0.03–0.07% of the total granule amylase) is associated with one of the prominent bands in the stained profile of the membranes.

The adsorption of soluble proteins as a source of significant contamination of cellular fractions and membrane subfractions has generally received little attention and has seldom been examined with the intent of testing what is actually the amount of adsorbed species relative to the bulk of the protein. The process of tissue homogenization displaces the organelles from their physiologic environment and, as suggested in Table I, results in considerable organelle disruption with subsequent mixing and relocation of once-segregated proteins. Hence, possibilities are created for the adsorption of these proteins to, and their copurification with, particulates in the homogenate. Furthermore, at the level of disrupting and subfractionating a cellular organelle, especially of the type specialized for participation in the packaging and export of secretory protein, the amount of nonmembrane or packaged protein is in great excess relative to the membrane protein. Even in the absence of specific adsorption of soluble proteins to the membrane, the tendency for disrupted membranes to form closed vesicles and thus trap soluble proteins in their cavities can lead to the appearance of contaminants of the membrane fraction at easily detectable levels. The expression of this contamination as a percentage of the total activity associated with the fraction, as shown in our study, is misleading; in the case of secretion granules where membrane protein can be estimated to constitute 2–3% of the total protein of the fraction, the presence of a total of 0.1–0.2% of residual content species in the membrane subfraction can be readily calculated to account for 5–10% of the total protein of the latter subfraction (even more, if the membranes have not been quantitatively recovered).

Recently, studies performed in three granular systems other than the rabbit parotid—the guinea pig exocrine pancreas, the rat liver, and the bovine adrenal medulla—have given indication that removal of polypeptides known not to be membrane proteins to limiting levels of detectability has not been attained (47, 37, 26, 68, 70). In these studies

the contaminating proteins are again secretory proteins identified by enzymatic activity, by comparative gel electrophoresis with soluble extracts of the fractions under investigation, or by a characteristic high rate of incorporation of radioactively tagged amino acid during pulse labeling. The membrane ghosts prepared by mild alkaline extraction of pancreatic zymogen granules of the guinea pig were found by enzymatic assays to contain low levels (0.1–0.2% of the total granule component) of a number of residual enzymes and zymogens. However, gel electropherograms of granule membrane subfractions prepared from [³H]leucine pulse-labeled and chase-incubated slices showed that nearly all radioactivity could be identified as residual contamination by content polypeptides based on comparison with gels of the alkaline granule extract (47). Furthermore, studies in which radioactively labeled secretion (discharged by pancreatic lobules stimulated *in vitro*) was included in the media used for homogenization of unlabeled tissue (in effect, as a marker for the substantial quantities of secretory proteins freed from cellular compartments by the shearing forces of homogenization) indicated that adsorption of soluble proteins to the membranes of cellular fractions is extensive, not easily extracted, and, in fact, poorly exchangeable with nonradioactive secretory protein (A. Tartakoff, private communication).

In the case of rough microsomes isolated from rat liver, treatment with low concentrations of sodium deoxycholate, shown to create discontinuities in the microsomal membranes (35), promotes only partial extraction of short term-labeled (30-min postpulse) polypeptides identified as protein destined for export from the liver (37). Thus both these studies suggest that adsorption to either the cytoplasmic or cisternal surfaces of membrane-bounded compartments as a consequence of cell or cellular compartment disruption in nonphysiologic media is both substantial and difficult to overcome by a variety of extractants.

Finally, contrary to the general assumption that membrane polypeptides should be different from those traditionally identified as content polypeptides, it has been suggested that the membranes of the chromaffin granules of the bovine adrenal medulla actually contain as part of their structure a protein which simultaneously exists in soluble form packaged within the granule—dopamine β -hydroxylase (68, 70). Hörtnagl et al. (26) have

concluded, based on electrophoretic mobility, comparative amino acid analysis and immunological cross-reaction, that the soluble dopamine β -hydroxylase is the same protein as that termed chromomembrin A, one of the major "membrane-associated" polypeptides. After our demonstration of the tenacious sticking to parotid secretion granule membranes of selected secretory proteins, in particular the metachromatically staining species, it is tempting to suggest that the situation with dopamine β -hydroxylase is similar, especially since means other than repeated hypotonic washing were not attempted to test the nature of the association of the residual enzyme with the membrane. However, caution must be exercised in concluding that insoluble dopamine β -hydroxylase is an adsorption artifact because chromaffin granule membranes as isolated by Winkler et al. (70) have a protein to lipid weight ratio of 0.45 mg protein/mg phospholipid, which is already lower than that of most biological membranes (0.7–4.0 mg:mg)⁶ (33). Thus the further removal of dopamine β -hydroxylase (chromomembrin A), which is one of the major polypeptides of the chromaffin granule membrane subfraction would decrease the protein to lipid weight ratio to levels not encountered thus far for biological membranes. Whether such reservations affect any of the polypeptides found in the membrane subfraction of secretion granules of the rabbit parotid remains to be determined by further investigation.

In summary, although the purity of the secretion granule fraction we have obtained from the rabbit parotid makes this fraction very attractive for the study of the biosynthetic rates of components of the granule membrane relative to those of the secretory content and for the examination of components possibly involved in exocytosis, the resolution of the granules into membrane and content subfractions is incomplete as assessed at the level of SDS-polyacrylamide gel electrophoresis. In general, our work and the recent work of others indicates that unambiguous ascription of a protein as belonging to the membrane subfraction of a cellular organelle is considerably complicated by adsorption artifacts. For the purposes of this

⁶ The ratio does not appear to be artificially lowered as a result of endogenous lipolytic activity as is the case in the pancreas (cf. Meldolesi et al. [49]) even though these membranes contain large amounts of lysolecithin relative to other membranes of the cell (6).

identification we have found it valuable to establish physiologic reference standards against which membrane subfractions can be tested for potential contaminant proteins and to perform mixing experiments—membranes with a radioactively labeled standard—to assess in sensitive fashion the extent of contamination and the precise electrophoretic mobility of contaminating polypeptides relative to the presumed true membrane polypeptides. As demonstrated by the selective loss of granule content species after parotid secretion granule lysis (consequently the necessity to refer to the isolated content as a soluble extract), the best choice of reference is one which is independent of the organelle subfractionation procedure and is subject to minimal compositional alteration.

Until the adsorption problem is overcome, physical chemical parameters of membranous subfractions such as density and protein to lipid ratio have little real meaning, and data from the investigation of average properties of the membrane must often be subjected to substantial, even prohibitive, corrections resulting from the contributions of the adsorbed species.

The authors are grateful to Louise E. Castle for her technical assistance and to Frank R. Hubbard for preparation of line drawings used in the illustrations.

The research was supported in part by Public Health Service Research Grant AM 17389 from the National Institute of Arthritis and Metabolic Diseases. J. David Castle is presently a postdoctoral fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

This work constitutes a part of a doctoral thesis submitted by J. David Castle to the faculty of The Rockefeller University, New York, N.Y., in December 1973.

Received for publication 19 July 1974, and in revised form 30 September 1974.

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