

# SECRETION GRANULES OF THE RABBIT PAROTID

## Selective Removal of Secretory Contaminants from Granule Membranes

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### ABSTRACT

A membrane subfraction obtained from secretion granules isolated from rabbit parotid has been shown to be contaminated by residual secretory proteins to an estimated level of 25–30% of its total protein. In the present study an additional contaminant has been identified by improved mixing experiments and by comparative peptide mapping of specific polypeptides recovered from gels of membrane and content subfractions. This contaminant coelectrophoreses with (and probably comprises the bulk of) the majority component of the membrane subfraction (mol wt ~40,000). The contaminating polypeptides can be removed to a large extent by treating the membranes with low concentrations of saponin in the presence of 0.3 M Na<sub>2</sub>SO<sub>4</sub>. Although this treatment disrupts the typical bilayer structure of the granule membrane, it does not appear to cause dissociation of its phospholipids or bona fide membrane proteins.

In a previous paper (9) we have described methods for lysing (by gradual exposure to hypotonic media) secretion granules isolated from the rabbit parotid gland, and for resolving the lysate into membrane and content subfractions. The extent of contamination of the membrane subfraction by residual content (or secretory) proteins was assessed by three different procedures. First, an enzymatic assay for a major secretory protein,  $\alpha$ -amylase, was used; and the results indicated that less than 0.1% of the total activity of the original granule fraction remained with the membrane subfraction. In the other two procedures, electrophoretograms of membrane subfractions and of discharged secretion (the latter used as a physiological standard) were compared. In one variant of this approach, potential contaminants were indicated by common bands. In the other variant, granules were lysed in the presence of <sup>14</sup>C-labeled granule content, their membranes were recovered

and contaminants detected by comparing both staining and radioactivity patterns on the corresponding electrophoretograms. These semiquantitative procedures indicated an incidence of contamination by residual secretory protein much higher than that shown by the amylase assays. Each of the major secretory polypeptides contributed bands to the Coomassie blue-staining profile with a contamination level comparable to, or exceeding, that of amylase, such that the minimal estimated contamination reached 25–30% of the total protein of the membrane subfraction.

Since these secretory contaminants are present in quantities substantial enough to preclude examination of bona fide membrane components, we have sought a way to achieve their selective removal from membrane subfractions. In this report we demonstrate that low concentrations of saponin in the presence of 0.3 M Na<sub>2</sub>SO<sub>4</sub> are quite effective in approaching this goal.

## MATERIALS AND METHODS

### *Isolation of Secretion Granule Fractions and their Subsequent Subfractionation*

The isolation of rabbit parotid secretory granules was performed as previously reported (9).<sup>1</sup>

Lysis of secretion granules collected from hypertonic sucrose solutions was carried out by a stepwise reduction in tonicity in an Amicon ultrafiltration chamber (Amicon Corp., Lexington, Mass.). The granule preparations were subjected to three alternating dilutions (with 20 mM NaHCO<sub>3</sub>, 0.5 mM disodium ethylenediaminetetraacetate (EDTA)<sup>2</sup> pH 7.2) and concentrations under N<sub>2</sub> at 60 lb/in<sup>2</sup>. Clearing of the suspension at the third dilution indicated apparently complete granule lysis (9).

Separation of the membrane subfraction from granule lysates by centrifugation in a discontinuous sucrose gradient was carried out according to a two-step procedure (9). The lysates, loaded atop layers of 0.2 M, 0.6 M, and 1.5 M sucrose (each buffered with 20 mM NaHCO<sub>3</sub>, pH 7.2, and containing 0.5 mM EDTA), yielded upon centrifugation at  $1.1 \times 10^7 g_{\text{avg}}$  min a partially purified membrane subfraction concentrated at the 0.6–1.5 M sucrose interface. At this point, the membranes were treated with various agents (as specified below) to promote removal of residual secretory species. The treated membranes were layered upon 0.6 M sucrose, 20 mM NaHCO<sub>3</sub>, 0.5 mM EDTA, and pelleted through this solution at  $1.1 \times 10^7 g_{\text{avg}}$  min as in reference 9.

### *Mixing Experiments with Radioactively*

#### *Labeled Parotid Secretion*

Radioactively labeled parotid secretion was obtained from the incubation medium of isoproterenol-stimulated lobules after their *in vitro* labeling with [<sup>14</sup>C]leucine, [<sup>14</sup>C]proline, and [<sup>14</sup>C]tyrosine (10 μCi/ml of each) by procedures described previously (9). This secretion was processed by lyophilization, and desalting by gel filtration (Biogel P6, Bio-Rad Laboratories, Richmond, Calif.), and was finally stored at -20°C in 20 mM NaHCO<sub>3</sub>, 0.5 mM EDTA.

In mixing experiments performed with isolated granules, aliquots of the thawed solution were added to purified granule fractions at the last two dilution steps of the lysis protocol. A total of 12.7 mg of labeled secretory protein ( $1.4 \times 10^6$  cpm) was added to a

granule fraction with an amount of protein estimated at 15 mg. Consequently, the final postlysis concentration step in the Amicon chamber was halted at a volume of 40 ml (double the customary volume) in an effort to maintain a comparable lysate protein concentration in all experiments.

Mixing experiments were also performed to estimate the extent of adsorption of secretory protein from the fluid phase of the tissue homogenates<sup>3</sup> to the external surfaces of secretion granules. In this case, 5 mg of labeled secretory protein was added to the minced tissue before homogenization, followed by other 5 mg (total: 10 mg) immediately after homogenization. The level of radioactivity was determined for the isolated intact granules.

### *Preparation of Secretion Granules and their Subfractions Labeled Endogenously with either a Mixture of <sup>14</sup>C-Amino Acids or [<sup>3</sup>H]Leucine*

To prepare parotid secretion granules endogenously labeled with <sup>14</sup>C-amino acids, four glands were dissected into lobules (8) and pulse labeled *in vitro* for 40 min with a mixture of 15 <sup>14</sup>C-amino acids. The pulse medium consisted of 10 ml of Krebs-Ringer bicarbonate medium supplemented with unlabeled amino acids to one-half the final concentration found in Eagle's minimal essential medium, and containing in addition 25 μCi/ml of reconstituted <sup>14</sup>C-algal protein hydrolysate. At the end of the pulse, lobules were washed with 100 ml of Medium F12 (23) at 37°C and were then incubated in 100 ml of F12 for 4 h to allow the bulk of the incorporated radioactivity to be transported to the secretion granules (8). The lobules were then homogenized and fractionated to obtain a secretion granule fraction.

To label secretion granule polypeptides with [<sup>3</sup>H]leucine preparatory to their enzymatic degradation and peptide mapping, lobules dissected from four glands were incubated (after a 15-min preincubation in 50 ml of F12) at 37°C for 5 h in 50 ml of F12 containing 100 μCi/ml [<sup>3</sup>H]leucine. The lobules were then homogenized and fractionated to obtain secretion granules and their subfractions according to the procedures already outlined.

### *SDS-Polyacrylamide Gel Electrophoresis*

Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed in all cases on linear (8–12%) gradients of polyacrylamide (9). Buffers for the alkaline, discontinuous pH electrophoresis were prepared according to

<sup>1</sup> From homogenates prepared in 0.28 M sucrose containing potassium phosphate (40 mM) and EDTA (0.2 mM). The same additives were present in all sucrose solutions used in the fractionation procedure.

<sup>2</sup> *Abbreviations used in this paper:* EDTA, disodium ethylenediaminetetraacetate; F12, Nutrient Mixture F12 (a cell culture medium of defined composition [23]); PPO, 2,5-diphenyloxazole; SDS, sodium dodecyl sulfate; and TPCK, toluenesulfonylphenylalaninechloromethyl ketone (an inhibitor of chymotryptic activity).

<sup>3</sup> These proteins represent those originally present in the secretory lumina of the gland and those displaced by organelle rupture during tissue homogenization. For amylase, this soluble fraction amounts to ~40% of the total activity (9).

Maizel (28). At the end of the runs, the gels were stained in 0.08% Coomassie brilliant blue in 25% isopropanol, 10% acetic acid (16) and were destained in isopropanol, acetic acid under standardized conditions to permit the assessment, by visual comparison, of the effect of various treatments on the metachromatically staining contaminants (which, as reported, fade with extended destaining) of the granule membrane subfractions. Before electrophoresis, the polypeptide mixtures were subjected to disulfide reduction with 25–40 mM dithiothreitol (16) or 2% (vol/vol) 2-mercaptoethanol (28) for 4–5 min at 100°C. When alkylation of the samples was carried out, the mixtures of polypeptides already reduced with 25 mM dithiothreitol were treated with 80–100 mM (final concentration) iodoacetamide for at least 30 min at 22°C. The samples were then loaded directly onto gels.

Optical density scans of stained gels were obtained at 550 nm with a Beckman Acta III spectrophotometer equipped with a linear transport sample holder (Beckman Instrument Co., Irvine, Calif.).

#### *Removal of Secretory Contaminants from Granule Membranes*

Partially purified membranes (specified above) were divided into aliquots which were diluted at least fivefold into NaHCO<sub>3</sub>-EDTA solutions (final concentrations: 20 mM and 0.5 mM, respectively) containing (except for controls) agents expected to promote the removal of contaminants. Most often the agents were 0.3 M Na<sub>2</sub>SO<sub>4</sub> plus 5–100 μg/ml saponin; but alternative treatments were attempted as indicated under Results. Treatment of each aliquot of the membrane subfraction was carried out in a total volume of 10 ml for 30–40 min with intermittent agitation. Unless specified otherwise, the temperature was 4°C. After treatment, the membranes were pelleted by centrifugation and dissolved directly in solutions appropriate for SDS-polyacrylamide gel electrophoresis. Supernates of the final centrifugation were either discarded, or concentrated for electrophoresis with a Minicon B15 dehydration chamber (Amicon Corp.), or subjected to additional centrifugation for at least  $4 \times 10^7$  g<sub>avg</sub> min to detect any slowly sedimentable material.

#### *Preparation of Pellets of Membrane Subfractions for Microscopy*

Pellets of saponin-Na<sub>2</sub>SO<sub>4</sub>-treated membrane subfractions were processed in polyallomer centrifuge tubes as in reference 9. The pellet and an underlying piece of the tube bottom were embedded as a single unit, and the tube piece was readily removed during the trimming of the specimens for microtomy.

For negative staining, pellets of membrane subfractions were resuspended in 0.1–0.2 ml of distilled water and were stained in suspension on a Formvar- and

carbon-coated 200-mesh copper grid for 5–10 s with 1% phosphotungstic acid adjusted to pH 7.

#### *Lipid Phosphorus and Protein Analyses in Untreated and Saponin-Sulfate-Treated Membrane Subfractions*

To test whether the saponin-Na<sub>2</sub>SO<sub>4</sub> treatment results in extraction of membrane phospholipids, 0.1-ml samples of membrane suspensions collected from the 0.6–1.5 M sucrose interface before saponin-Na<sub>2</sub>SO<sub>4</sub> treatment, or of membranes pelleted (after saponin-Na<sub>2</sub>SO<sub>4</sub> treatment) and then resuspended, were subjected to phospholipid extraction with chloroform-methanol according to the procedure of Folch et al. (17). The extracted phospholipids were ashed with alcoholic Mg(NO<sub>3</sub>)<sub>2</sub> and were assayed for released inorganic phosphate by the procedure of Chen et al. (11). The same procedure was applied to pellets obtained by prolonged centrifugation of supernates already cleared of the bulk of saponin-Na<sub>2</sub>SO<sub>4</sub>-treated membranes.

Protein was determined in membrane subfractions by applying a microbiuret assay to pelleted material dissolved in 1 N NaOH (10, 21). Bovine plasma albumin (20–400 μg) was used as a standard.

#### *Electrophoretic Elution of Polypeptides from Polyacrylamide Gels and Comparative Analysis of their Proteolytic Digests by Peptide Mapping*

To prepare [<sup>3</sup>H]leucine-labeled polypeptides for proteolysis, bands were carefully dissected from Coomassie blue-stained gels, minced into cubes of ~1 mm<sup>3</sup> and placed in buffer-filled glass tubes (15 cm × 6 mm, ID) restrained at the bottom by Nitex nylon screen (Tobler, Inc., Elmsford, N. Y.) and having beneath the gauze a chamber of ~300 μl bounded by a dialysis membrane. The tubes were inserted into a standard electrophoresis apparatus. The buffered mixture filling the glass tubes, the dialysis chamber, and both electrode reservoirs was 0.05 M sodium phosphate, 10 mM cysteine, and 0.1% SDS, pH 7.2. The polypeptides were eluted into the dialysis chamber above the anode by electrophoresis for 5 h at a constant current of ≥7 mA/gel. For several polypeptides excised from gels of the granule content subfraction, the efficiency of elution was ≥70% under these conditions. The eluate (containing the polypeptide, SDS, and the Coomassie blue stain) was transferred to a 1.5-ml polypropylene centrifuge tube (W. Sarstedt, Princeton, N. J.) in a total volume of ~500 μl. The Coomassie blue and SDS were removed by sequential precipitation with KCl (0.2 M final concentration), acetone + 0.1 N HCl, and acetone, according to the procedure of Bray and Brownlee (5). The final dried residue containing 60–100% of the eluted polypeptide was resuspended in 0.25 ml of 50 mM NH<sub>4</sub>HCO<sub>3</sub>, and

to it either trypsin-toluenesulfonylphenylalaninechloromethyl ketone (TPCK) or  $\alpha$ -chymotrypsin was added to a final concentration of 10–20% (wt enzyme/wt protein). The samples were incubated for 16 h at 37°C, the enzyme being replenished by the original amount at 4 h. The digests were lyophilized three times (a total of 48 h) to insure uniform desalting; the resulting residues were dissolved in water with thorough mixing and cleared by centrifugation at 16,000  $g_{\text{avg}}$  min in an Eppendorf 3200 centrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.). An aliquot of the supernate was removed for radioactivity determination to provide a guide for the duration of fluorograph exposure. The rest of the supernate was spotted as a 1-cm strip on a 20 × 20 cm cellulose MN300 plate (0.1-mm thick) (Brinkmann Instruments, Inc.). Internal markers (lysine for the electrophoretic dimension and isoleucine for the chromatographic dimension) were spotted at levels detectable with ninhydrin. Electrophoresis in the first dimension was carried out for 90 min at a constant voltage of 400 V (current 15–20 mA) in a standard pyridine-acetic acid-water buffer, pH 3.5. The plate was thoroughly dried and then chromatographed in a second dimension in *n*-butanol-acetic acid water (3:1:1 [vol/vol]) until the solvent front was approx. 2 cm from the top. The plate was then dried, sprayed with ninhydrin (color development, 5 min at 70°C), and coated with a thin layer of 2,5-diphenyloxazole (PPO) according to the procedure of Bonner and Laskey (4). The peptide maps were overlaid with a sheet of X-ray film (Kodak x-omat R) which, in the event of total radioactivity  $\leq 100,000$  dpm, was presensitized to decrease exposure times according to the procedures outlined by Laskey and Mills (26). Fluorography was carried out at  $-70^\circ\text{C}$  for periods of 2 wk to 2 mo, and the X-ray films (fluorograms) were then developed by standard techniques.

### Materials

Acrylamide and *N,N'*-methylene bisacrylamide were obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. Acrylamide was recrystallized from acetone before use. Sodium dodecyl sulfate was obtained from Alcolac, Inc. (Baltimore, Md.) and was recrystallized once from 80% ethanol before use. Saponin (derived from the bark of *Quillaja saponarica*) was obtained from Sigma Chemical Co. (St. Louis, Mo.). A stock solution 300 mg/ml in water was prepared and stored at 4°C. Trypsin-TPCK (activity: 290 U/mg) was obtained from Worthington Biochemical Corp. (Freehold, N. J.).  $\alpha$ -chymotrypsin (bovine pancreas), (activity: 1,175 U/mg and tryptic activity of 6 U/mg) was obtained from Calbiochem (San Diego, Calif.). Reconstituted  $^{14}\text{C}$ -labeled algal protein hydrolysate, L- $^{14}\text{C}$ ,U]leucine and L-[ $4,5\text{-}^3\text{H}$ ]leucine were obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). L- $^{14}\text{C}$ ,U]tyrosine and L- $^{14}\text{C}$ ,U]proline were obtained from New England Nuclear

(Boston, Mass.). All other chemicals were reagent grade.

## RESULTS

### Identification of Potential Contaminants

The two gel electrophoretograms in Fig. 1 indicate which Coomassie blue-stained bands of the membrane subfraction contain residual secretory proteins according to the experiments outlined in the introductory paragraph. Three of these bands stain metachromatically (pink) and have mobilities that correspond to mol wt 130,000, 110,000, and 95,000; they are identified as major contaminants of the membranes since they are found in parotid saliva. In the work that follows, we have exploited their metachromatic staining as a means of obtaining a rapid semiquantitative assessment of our attempts to decontaminate the membrane subfraction. Our estimate that 25–30% of the polypeptides of this subfraction represents secretory proteins is based on the assumption that the bulk of each designated band in Fig. 1 is a content contaminant. Although some contaminant bands may hide or obscure membrane polypeptides of substantial staining, we felt that even with this type of correction the level of contamination would remain high enough to require that a selective decontamination procedure be devised and tested before proceeding with the study of the average physical, chemical, and metabolic properties of the proteins of these membranes.

A second feature of note in Fig. 1 is the presence of a majority component of  $\sim 40,000$  mol wt<sup>4</sup> in the electrophoretogram of the membrane subfraction. If identified as a bona fide membrane protein, this polypeptide could be of conceivable interest to specialized functions of the granule membrane, e.g., exocytosis and content concentration and packaging. The rate of biosynthesis of the 40K component is high relative to that of other presumed granule membrane proteins; in fact, it is comparable to that of recognized secretory contaminants as demonstrated by the radioactivity profile in Fig. 1 (7). The secretion contains polypeptides of comparable mobility, and this compelled us to reinvestigate the possibility that the 40K component is a con-

<sup>4</sup> The polypeptide constituting this majority component of the membrane subfraction will henceforth be referred to as the 40K component.

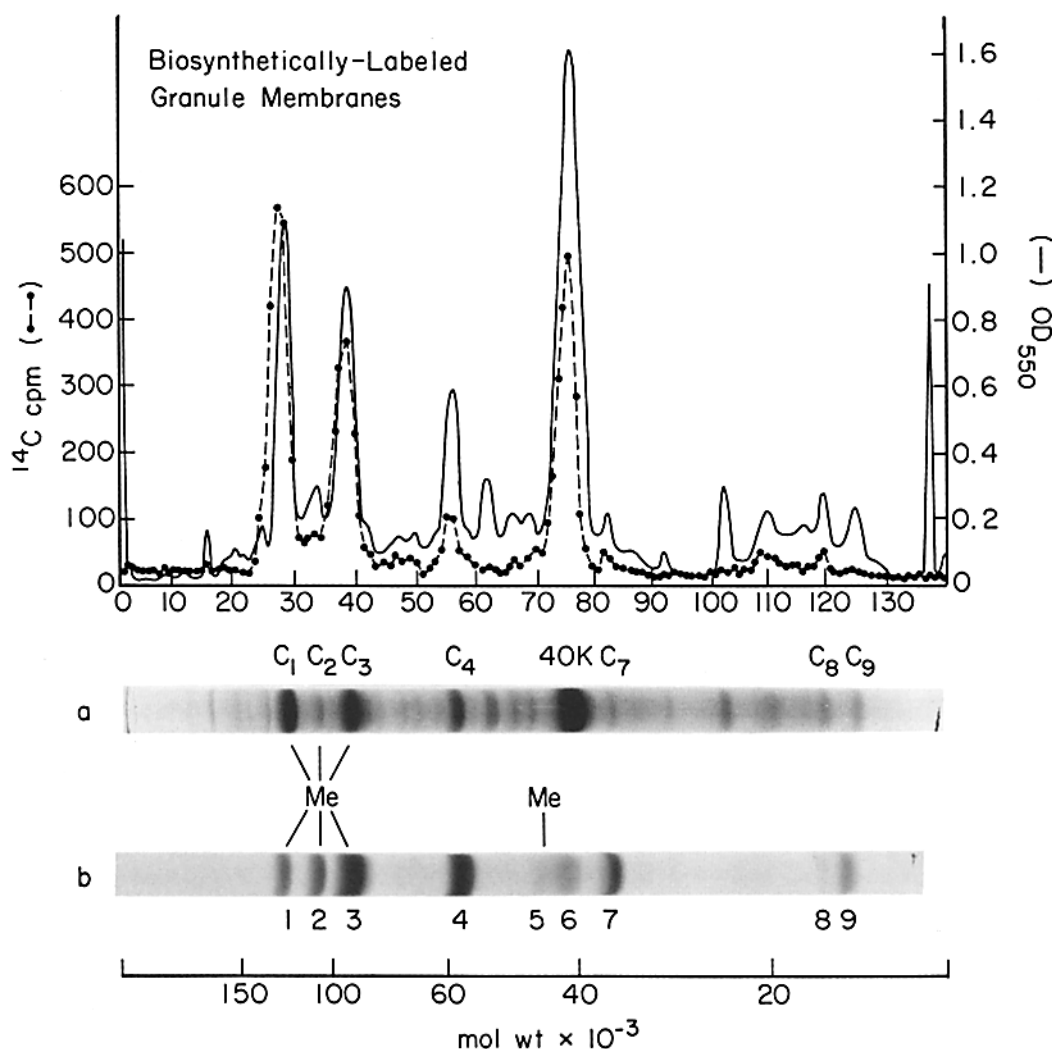


FIGURE 1 Summary of polypeptides previously recognized as secretory contaminants of the membrane subfraction of parotid secretion granules. The lower gel electrophoretogram (b) is a profile for total (unlysed) secretion granules (>98% secretory protein) which serves as a reference for content polypeptides at the concentration at which they are packaged within the granules. *Me* designates bands stained metachromatically by Coomassie blue. The upper gel electrophoretogram (a) represents the membrane subfraction prepared from secretion granules isolated from lobules labeled in vitro with  $^{14}\text{C}$ -amino acids. Stained bands already recognized as containing secretory contaminants (9) are marked C with a numerical subscript corresponding to the secretory species of identical mobility in the electrophoretogram of whole granules. 40K designates the majority component of the membrane subfraction. Incorporated radioactivity (top profile) coincides with bands  $C_1$ - $C_4$ ,  $C_7$ - $C_9$ , and 40K.

tent contaminant that could have gone unnoticed in the initial mixing experiments of our previous report.

#### *Dissociation of Secretory Contaminants from Granule Membranes*

A number of treatments were applied to the

membrane subfraction to see if they could remove secretory contaminants. In each case the efficiency and selectivity of the treatment were readily assessed by comparative SDS-gel electrophoresis against: (a) control, untreated portions of the membrane subfraction, and (b) discharged secretion. As mentioned in our previous communica-

tion, the 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, 32)), or sonication in the presence of NaBr (0.25, 0.50 M) (13, 27) did not succeed in reducing the adsorption of secretory contaminants to granule membranes. Equally unsuccessful were exposure of membrane subfractions to a 30-min extraction in 0.12 M NaCl (containing 5 mM NaHCO<sub>3</sub>, 0.5 mM EDTA, pH 7.2), at 37°C, as well as extraction in hypotonic media (20 mM sodium citrate, 0.5 mM EDTA) at acidic pH (4.5) at 0°C as well as 37°C. Similar NaCl and Na citrate treatments have been claimed by Wallach et al. (37) to be effective at 0°C for desorbing secretory contaminants from membranes of rat parotid secretion granules. We found electrophoretograms of the treated membrane subfractions to be indistinguishable from those of control preparations, the contaminants labeled in Fig. 1 all being present at visually similar levels.

Further approaches to decontamination were based on the assumption that the contaminating proteins were located primarily inside (or on the inner aspect of) the vesicles found in granule lysates. This view was supported by the results of mixing experiments in which labeled lobule secretion was added to parotid tissue before and immediately after homogenization. Since the amount of exogenously added protein was known and the amount of endogenous secretory protein present in the homogenate could be estimated (~70 mg in this experiment [~40% displaced from cellular organelles]), we calculated (from the radioactivity found in the granule fraction) that adsorption of secretory protein to the exterior (cytoplasmic) surfaces of the granules was minimal. It represents no more than 3% of the protein subsequently isolated in the membrane subfraction. Since an estimated 30% of the total protein of this subfraction consists of contaminating secretory polypeptides, only ~10% of the contamination can be on the external aspect of the membrane.

Bretz and Baggiolini (6) recently showed that treatment with 0.05% digitonin in 0.4 M Na<sub>2</sub>SO<sub>4</sub> efficiently removes myeloperoxidase (a content contaminant) from the membrane subfraction of the specific granules of polymorphonuclear leukocytes, without affecting the distribution of alkaline phosphatase (presumably a membrane protein). We, therefore, decided to apply plant glycosides to parotid granule membrane subfractions

in hopes that they could facilitate the selective removal of secretory contaminants. When granule membranes were treated with 100 µg/ml saponin in the presence of 0.3 M Na<sub>2</sub>SO<sub>4</sub>, several of the polypeptides recognized as adsorbed contaminants eluted from the membrane subfraction as seen in Fig. 2 (gel *e* showing the electrophoretogram of treated membranes, and gel *f* that of the corresponding untreated membranes). Especially striking was the nearly quantitative removal of the three metachromatically staining species (C<sub>1</sub>-C<sub>3</sub>) of estimated mol wt 130,000-95,000; their removal resulted in the unmasking of blue-staining bands which presumably represent bona fide membrane polypeptides. Also, there was a substantial decrease in contaminant bands of higher mobilities (C<sub>8</sub> and C<sub>9</sub>). Less dramatic was the effect on the contaminants in bands C<sub>4</sub> (identified as amylase) and C<sub>7</sub> (estimated mol wt, 33,000). It was further evident that the saponin-Na<sub>2</sub>SO<sub>4</sub> treatment also removed a substantial portion of the 40K component, thus emphasizing the necessity to find out whether the bulk of this band comprised secretory polypeptide(s) or represented a bona fide membrane protein solubilized by the treatment. If the 40K component were a secretory contaminant, we have to conclude that its interactions with the membranes of lysed granules are unusually strong since it is a lesser component of the secretion while it appears to be the majority component of the membrane polypeptide profile. The staining intensities of other bands in the profile of the membrane subfraction treated with 100 µg/ml saponin-0.3 M Na<sub>2</sub>SO<sub>4</sub> were visually and densitometrically similar to those of the corresponding bands in the untreated membrane preparation.

Thus three groups of polypeptides present in granule membrane subfractions can be recognized as a result of the saponin-Na<sub>2</sub>SO<sub>4</sub> treatment: (a) secretory contaminants (equivalent to those found in the physiologic secretory standard) which are readily dissociated; (b) bona fide membrane polypeptides whose distribution is unaffected by the treatment; and (c) species of uncertain physiologic identity, including the 40K component, which are partially dissociated from the membranes by the saponin-Na<sub>2</sub>SO<sub>4</sub> treatment; their similarity or dissimilarity to easily extracted, minor content proteins remains to be established by procedures more critical than those so far used.

We stress that the decontamination of the membranes took place only when Na<sub>2</sub>SO<sub>4</sub> was present

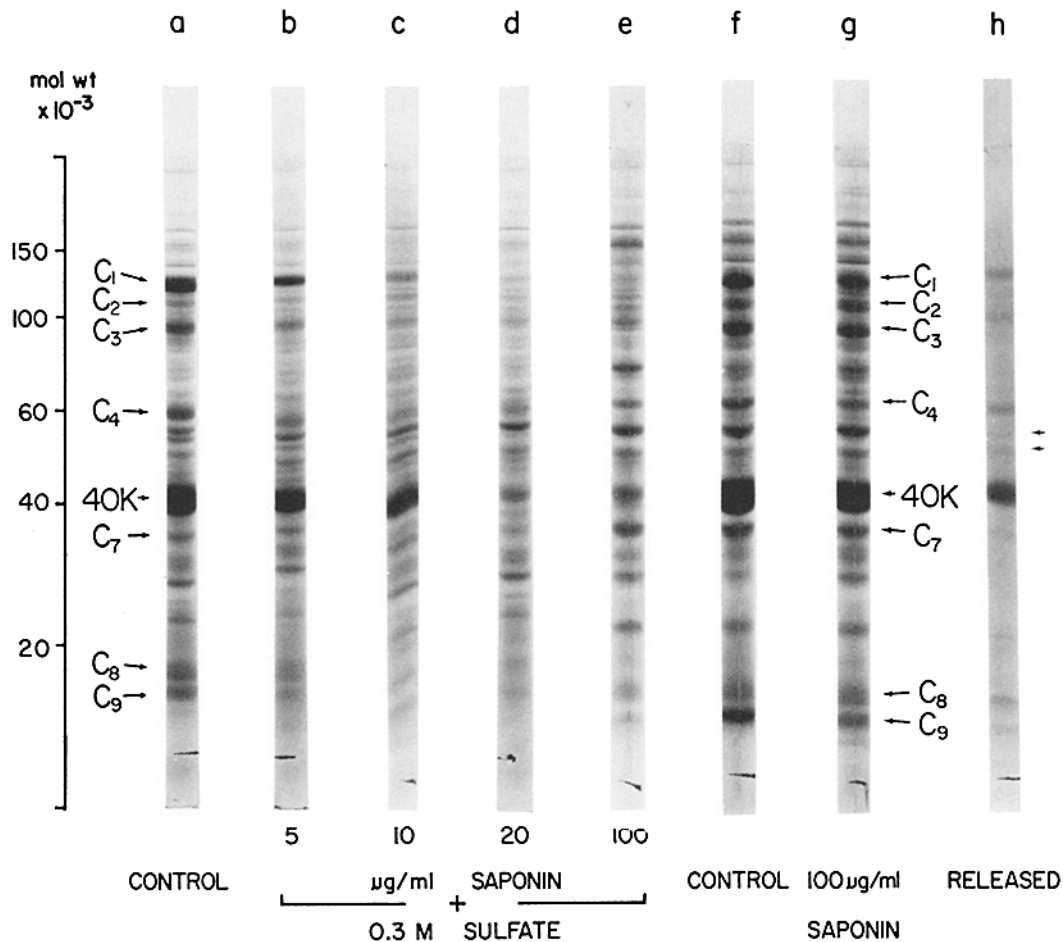


FIGURE 2 Gel electrophoretograms demonstrating the selective decontamination of a granule membrane subfraction by saponin in the presence of 0.3 M  $\text{Na}_2\text{SO}_4$ . The bands which contain secretory contaminants are marked  $C_1$ - $C_4$ ,  $C_7$ - $C_9$  on the electrophoretogram of untreated membranes (gel *a*). Gels *b*-*e* present the effects of increasing saponin concentrations (5, 10, 20, and 100  $\mu\text{g}/\text{ml}$  saponin, respectively) in the presence of 0.3 M  $\text{Na}_2\text{SO}_4$ . The progressive loss of all contaminants (especially  $C_1$ - $C_3$ ,  $C_8$ ,  $C_9$ ) and of 40K with increasing saponin concentration is evident. On gel *e* (for which the protein load was  $\sim 35\%$  higher than for gels *a*-*d*) the blue-staining species, originally obscured by the metachromatically staining contaminants, ( $C_1$ - $C_3$ ), are clearly seen. An electrophoretogram for untreated membranes at the same load as gel *e* is shown in gel *f*. The latter gel also provides a comparison for an electrophoretogram of membranes treated only with 100  $\mu\text{g}/\text{ml}$  saponin (gel *g*), which shows that saponin alone has little effect on the level of contamination. Gel *h* is an electrophoretogram of polypeptide aggregates recovered by centrifugation at  $5 \times 10^7 g_{\text{avg}}$  min from the supernate of a membrane fraction treated with 20  $\mu\text{g}/\text{ml}$  saponin-0.3 M  $\text{Na}_2\text{SO}_4$ . Trace amounts of presumed bona fide membrane polypeptides (correlating with the low incidence of membranous vesicles observed in these pellets) are identified by arrows.

in addition to the saponin, since the gel profile of an equal amount of membranes treated solely with 100  $\mu\text{g}/\text{ml}$  saponin (gel *g* of Fig. 2) was practically indistinguishable from its control. Conversely, treatment with  $\text{Na}_2\text{SO}_4$  alone did not reduce the level of contaminant species. The latter finding supports the assumption previously men-

tioned that the contaminating proteins are located within the vesicles and represents either incomplete extraction or adsorption on the inner aspect of the granule membrane.

Further examination of Fig. 2, especially the profiles for control preparations (gels *a* and *f*) and for preparations treated with 20 and 100  $\mu\text{g}/$

ml saponin (gels *d* and *e*), indicates that there are a few notable differences in the Coomassie blue-staining patterns for the presumed bona fide membrane polypeptides. Aside from minor mobility differences arising from slight non-uniformity of the polyacrylamide concentration gradients, the presence of a prominent band consisting of polypeptide of apparent mol wt 70,000 in gels *e* and *f*, found in low levels at best in gels *a* and *d*, is unexplained. Bands of mobility corresponding to a mol wt of 50,000–55,000 (slightly below band  $C_4$ ) are variably present as a singlet (gels *d*–*f*) or as a doublet (gel *a*). The intensity of the lower band of the doublet apparently is directly related to the stability of polypeptide sulfhydryls in the reduced form.

Examination of sectioned samples in the electron microscope indicated that treatment of membrane subfractions with 100  $\mu\text{g/ml}$  saponin-0.3 M  $\text{Na}_2\text{SO}_4$  caused wholesale disorganization of the membrane bilayer structure (Fig. 3). Only occasional small segments of recognizable unit membrane were evident; the bulk of the subfraction had the appearance of a tortuous, amorphous network possibly interconnecting the bilayer segments. Despite the extensive damage done to the bilayer structure, the membranes were still sedimentable at  $1.1 \times 10^7 g_{\text{avg}} \text{ min}$ .

In hopes of better preserving the structural integrity of the granule membrane while simultaneously achieving the same level of selective removal of secretory contaminants from the membrane subfraction, we titrated the saponin concentration downward, maintaining  $\text{Na}_2\text{SO}_4$  at a level of 0.3 M (since membranes treated with this amount of  $\text{Na}_2\text{SO}_4$  alone were indistinguishable in appearance from the untreated membranes presented in Fig. 3*e*). At each saponin concentration the membrane structure was examined, and levels of residual secretory contaminants were assessed in parallel by SDS-polyacrylamide gel electrophoresis. When saponin was tested at 20, 10, and 5  $\mu\text{g/ml}$  (Fig. 2), the lowest level was effective to the extent that staining intensities of secretory contaminants, especially those of metachromatically staining species ( $C_1$ – $C_3$ ), were reduced to ~50% of those of control preparations. Also, roughly half of the 40K component remained with the membrane subfraction. Saponin at 10  $\mu\text{g/ml}$  increased considerably the dissociation especially of the metachromatically staining species although most of the secretory contaminants were still visually detectable. 20  $\mu\text{g/ml}$

saponin in 0.3 M  $\text{Na}_2\text{SO}_4$  was very nearly as effective as the highest saponin level (100  $\mu\text{g/ml}$ ) tried (cf. gels *d* and *e* in Fig. 2).

As can be seen in Fig. 3, the decrease in saponin concentration resulted in less perturbation of the membrane bilayer structure. At 20  $\mu\text{g/ml}$  saponin (Fig. 3*c*) the extent of disorganization was considerably decreased relative to that seen at 100  $\mu\text{g/ml}$  with a concomitant increase in frequency and length of bilayer segments. At 10  $\mu\text{g/ml}$  saponin (Fig. 3*b*), lengthy arrays of bilayer are connected by more contained and less contorted regions of disorganization, the latter having the appearance of patches of laminar felt.<sup>5</sup> The appearance of membranes subjected to saponin at 5  $\mu\text{g/ml}$  (Fig. 3*a*) was not very different from that of controls (Fig. 3*e*). At this lowest saponin concentration tested, the membranes appeared as closed vesicles, although they were less collapsed than the vesicles of untreated membranes.

Micrographs of negatively stained preparations of membranes untreated (Fig. 3*f*) or treated with saponin at 5 and 20  $\mu\text{g/ml}$  in 0.3 M  $\text{Na}_2\text{SO}_4$  (Fig. 3*g*, *h*) indicate that the disorganization seen in thin sections is correlated with the presence of presumed saponin-induced pits in the membrane similar in appearance to those described by Bangham and Horne (3), Glauert et al. (19), and Seeman (33).<sup>6</sup>

At 10  $\mu\text{g/ml}$  saponin, a level at which contaminant removal is extensive (but not complete) and membrane disorganization is still limited, we tried a number of variations on the 10  $\mu\text{g/ml}$  saponin-0.3 M  $\text{Na}_2\text{SO}_4$  treatment in hopes of achieving a more extensive removal of contaminants while retaining a reasonable degree of membrane integrity. Removal of secretory contaminants was not enhanced by either increasing the  $\text{Na}_2\text{SO}_4$  concentration to 0.5 M, or replacing it by 0.5 M NaCl, or adding 2 mM EDTA, 0.5 mM EGTA (to the saponin- $\text{Na}_2\text{SO}_4$  solution), or adding  $\text{CaCl}_2$  to a net concentration of 0.1 mM over residual EDTA. Finally, we supplemented the saponin-

<sup>5</sup> The lengths of the membrane arrays at this level of saponin were substantially increased over those of untreated membranes, which implied that the treatment caused some degree of reorganization (by fusion?) of the original vesicles.

<sup>6</sup> Unpublished observations (J. D. Castle and W. L. Hubbell) indicate that parotid secretion granule membranes contain cholesterol at a level more than sufficient to promote formation of saponin-cholesterol complexes (20).



Na<sub>2</sub>SO<sub>4</sub> solutions with a mixture of the electrolytes found in Krebs-Ringer-bicarbonate medium in an attempt to mimic the ionic composition of primary saliva at the level of the acinar cells (29, 30, 36), on the assumption that, during the physiologic exocytosis, complete dissociation of granule content from granule membranes obtains in a similar medium. Again, a decontamination more extensive than that obtained with saponin-Na<sub>2</sub>SO<sub>4</sub> alone was not achieved.

Efforts to avoid the use of saponin by sonicating (15 s, three times on ice) a suspension of granule membranes in the presence of 0.3 M Na<sub>2</sub>SO<sub>4</sub> were successful only to the extent that secretory contaminants were reduced by 10–20% relative to the levels for untreated membranes. Ferritin was used as a probe for vesicles opening during sonication; it was apparent from the electron microscope examination of such preparations that the efficiency of sonication in creating ferritin-patent openings (and thus accessibility to the inner aspect of the membrane) was low.

Thus, in using saponin in 0.3 M Na<sub>2</sub>SO<sub>4</sub> to dissociate residual secretory proteins selectively from the membrane subfraction, we are faced with the situation that effective removal of the contaminants cannot be obtained without extensive membrane disorganization which is probably needed either to gain access to intravesicular adsorbed proteins and/or to detach such proteins from a tight association with the membrane.

When the supernate of the pelleted saponin-Na<sub>2</sub>SO<sub>4</sub>-treated membranes was diluted twofold with 20 mM NaHCO<sub>3</sub>, 0.5 mM EDTA and spun at  $5 \times 10^7$  g<sub>avg</sub> min, a small, well-packed white pellet was obtained. The electrophoretogram of this (solubilized) pellet (gel *h* of Fig. 2) indicated that it was composed primarily of secretory contaminants plus the 40K component removed from granule membranes. The removed contaminants

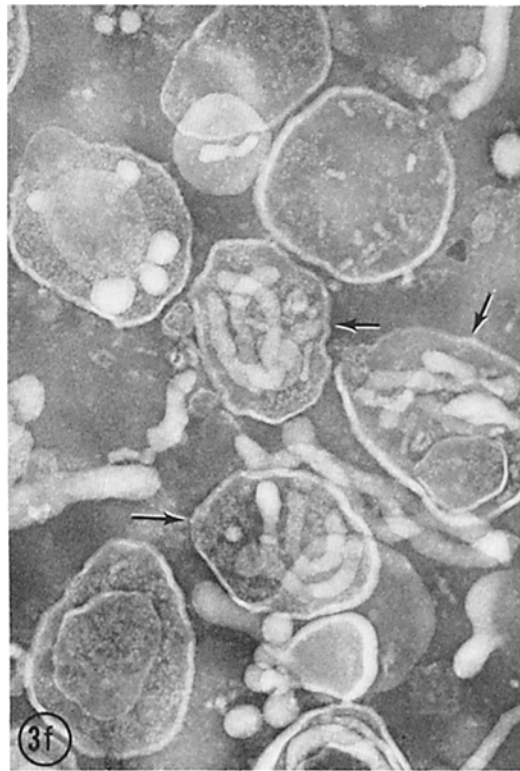
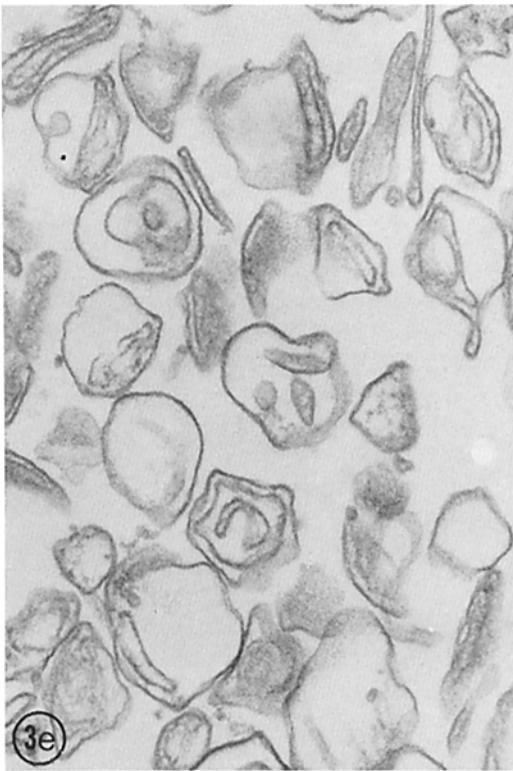
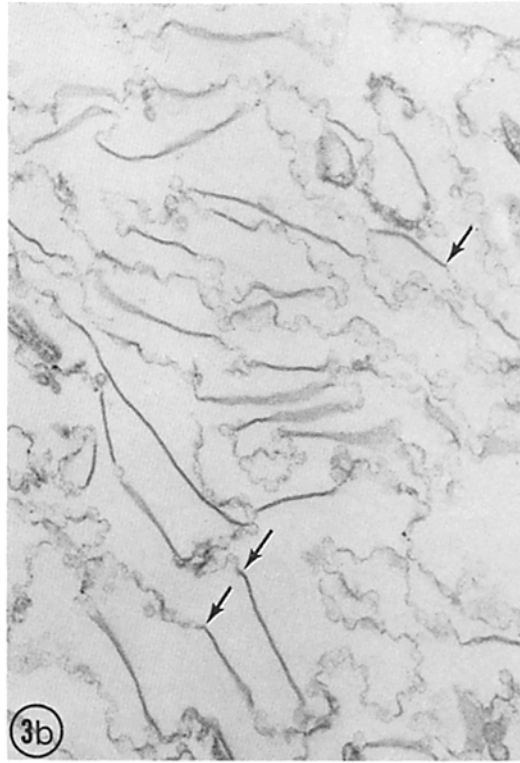
must be extensively aggregated since sedimentation under the above conditions requires a sedimentation coefficient of at least 50S corresponding to a mol wt  $>2.2 \times 10^6$ . Approx. 50% of the polypeptides freed by saponin-Na<sub>2</sub>SO<sub>4</sub> treatment were recovered in the  $5 \times 10^7$  g min pellet (estimated from the absorbance of Coomassie blue-stained gel bands); the other half was not sedimentable under our experimental conditions.

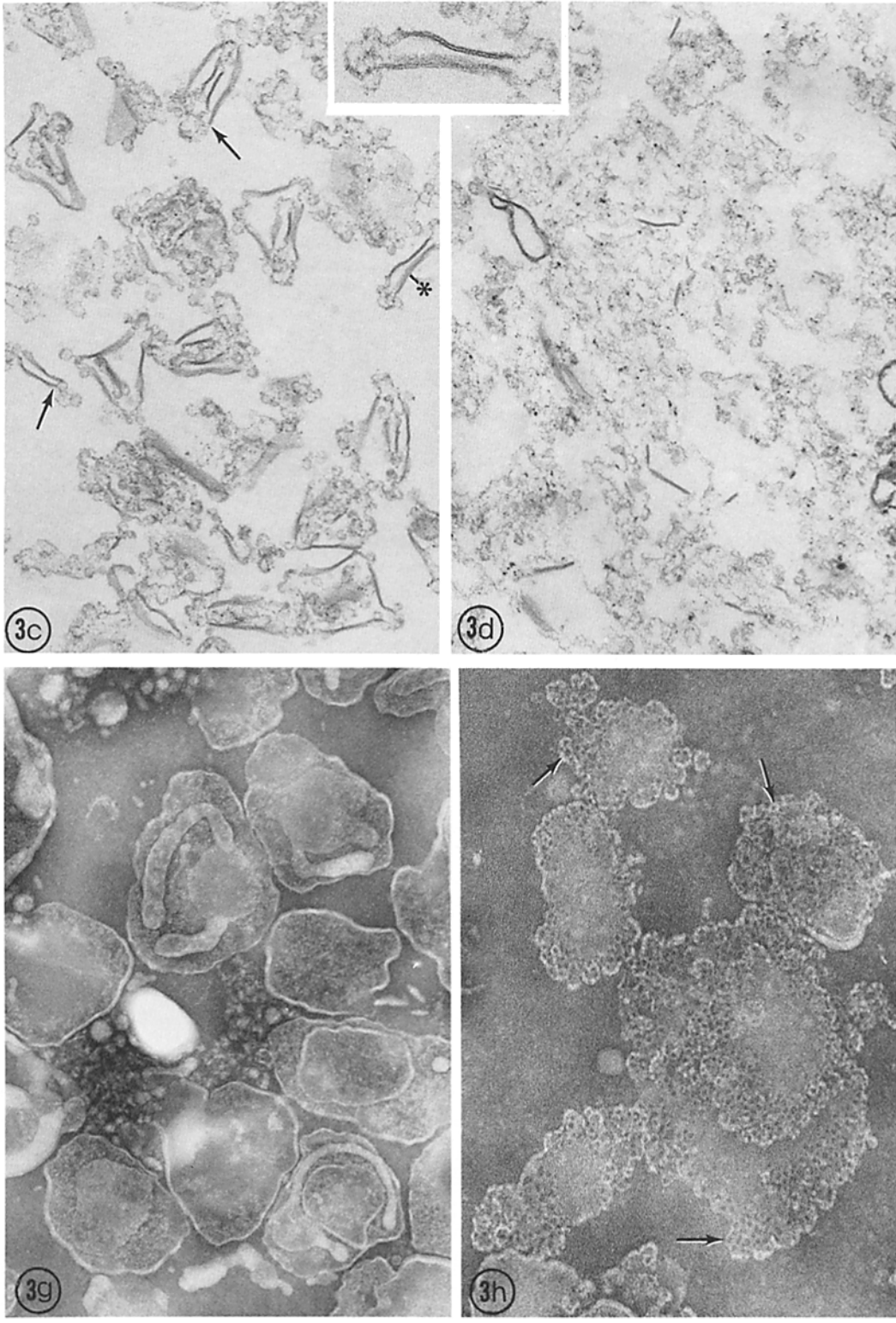
When examined in the electron microscope, the bulk of the pellet was found to consist of an amorphous matrix containing a few scattered vesicles of small diameter (100–150 nm). These vesicles were probably formed during the membrane reorganization by saponin-Na<sub>2</sub>SO<sub>4</sub> treatment. SDS-gel electrophoretograms showed that presumed bona fide membrane polypeptides were present at very low concentration in this pellet.

The distribution of membrane phospholipid was examined before and after treatment with 20 μg/ml saponin-0.3 M Na<sub>2</sub>SO<sub>4</sub>. In an experiment in which we recovered 93% of the lipid phosphate originally associated with the membrane subfraction, 80% of the recovered amount remained associated with the saponin-Na<sub>2</sub>SO<sub>4</sub>-treated membrane. The remainder was located in the pelleted fraction of removed aggregates and, in all likelihood, was associated with the small vesicles found in this subfraction. We concluded from the distribution and extent of recovery of the phospholipid, and from the detection of small bilayer vesicles in the small pellet mentioned above, that the bulk of the membrane phospholipid retained its bilayer organization and was not solubilized by the saponin-Na<sub>2</sub>SO<sub>4</sub> treatment. Furthermore, since the retention of phospholipid by the treated membranes (at least 80% of the total) and the retention of the 40K component in this same fraction (by densitometry, <20% of the Coomassie blue stain in the electrophoretogram) were

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FIGURE 3 The effect of saponin-Na<sub>2</sub>SO<sub>4</sub> treatment on the structure of secretion granule membranes. Fig. 3*a-d* show thin-sectioned preparations of the titration series of 5, 10, 20, and 100 μg/ml saponin (respectively)-0.3 M Na<sub>2</sub>SO<sub>4</sub>. Panel 3*e* shows an untreated membrane subfraction. The progressive disruption and reorganization of the membranes is evident. The arrows in Fig. 3*b* and *c* mark points of continuity between persisting bilayer structures and patches of "unraveled" membrane. A profile (asterisk) in Fig. 3*c* is shown in the *inset* at a magnification high enough to demonstrate clearly the bilayer structure. (*a-e*)  $\times 50,000$ ; *inset*,  $\times 100,000$ . Fig. 3*f-h* show negatively stained preparations of untreated membranes and membranes treated with 5 μg/ml and 20 μg/ml saponin-0.3 M Na<sub>2</sub>SO<sub>4</sub>, respectively. Vesicles resembling mitochondria in the untreated preparations (arrows) are in fact distorted granule membranes. Apparent surface discontinuities, 60–70 Å in diameter (arrows), are evident only upon treatment with 20 μg/ml saponin-0.3 M Na<sub>2</sub>SO<sub>4</sub>. (*f* and *g*)  $\times 68,000$ ; (*h*)  $\times 105,000$ .





not parallel, the 40K component is not likely to be predominantly associated with the small vesicles pelleting with the contaminant aggregates. This assumption was supported by the observation that the 40K polypeptide was already noticeably removed from membrane fractions treated with the lowest concentration of saponin used (5  $\mu\text{g}/\text{ml}$ ) in  $\text{Na}_2\text{SO}_4$  (gel *b* of Fig. 2), its removal apparently preceding the onset of gross structural alteration of the membrane (Fig. 3*a* and *g*).

The identification of aggregates of secretory contaminants, which sediment far more readily than the molecular weights of their individual polypeptides would predict, serves as a valuable lesson in harvesting membrane subfractions after various treatments. Had prolonged centrifugation been used to pellet the membranes, the contaminant aggregates desorbed by saponin- $\text{Na}_2\text{SO}_4$  also would have pelleted to recontaminate the fraction.

#### *Mixing Experiments: Adsorption of Components of Labeled Lobule Secretion to Granule Membranes and their Removal by Saponin- $\text{Na}_2\text{SO}_4$*

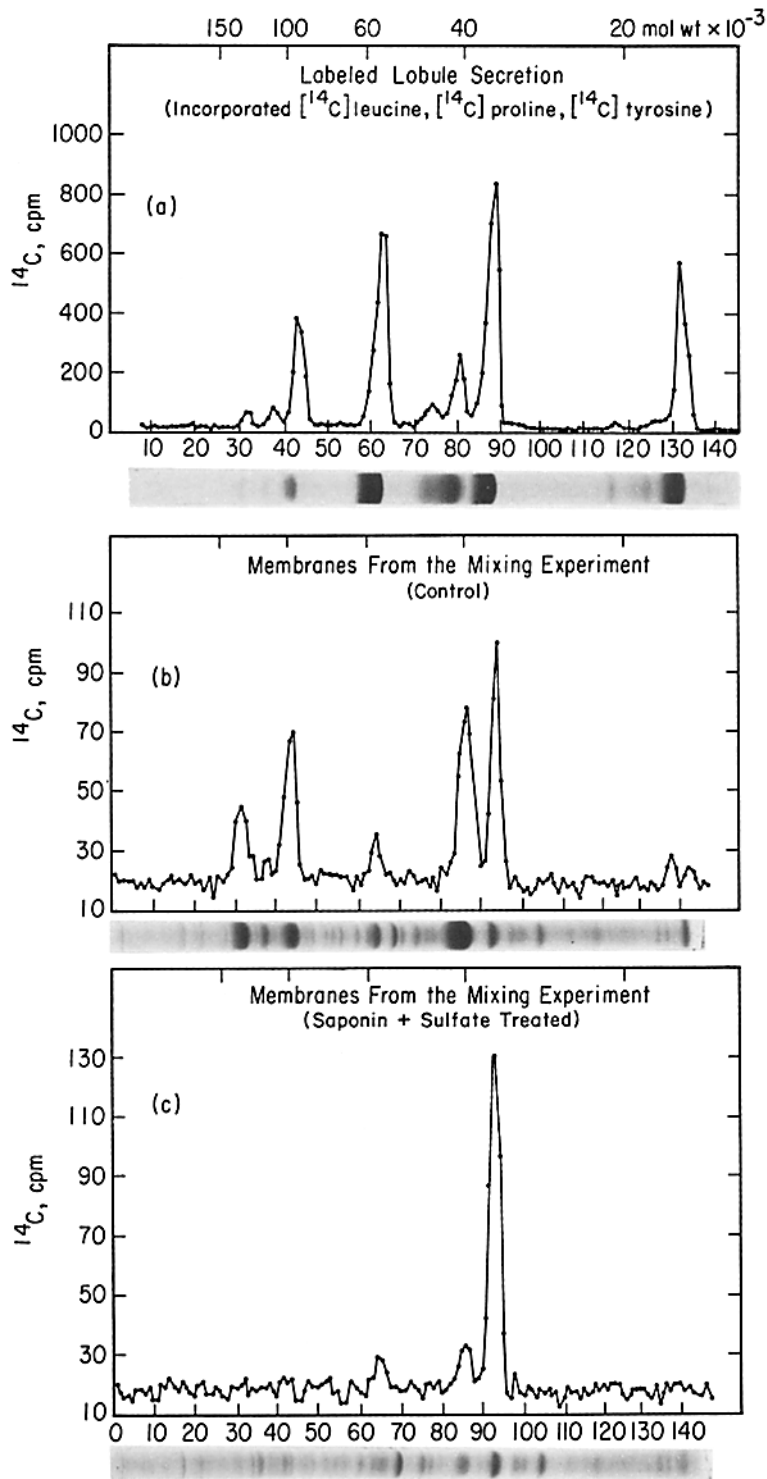
[ $^{14}\text{C}$ ]leucine, [ $^{14}\text{C}$ ]proline, and [ $^{14}\text{C}$ ]tyrosine-labeled parotid secretion<sup>7</sup> (a gel staining and radioactivity profile of which is presented in Fig. 4*a*) was mixed with granule fractions during lysis as specified under Methods. Granule membranes obtained from the lysate were purified after dilution and treatment with media not containing (control) or containing 20  $\mu\text{g}/\text{ml}$  saponin-0.3 M  $\text{Na}_2\text{SO}_4$ . The staining and radioactivity profiles of the electrophoretograms of these preparations are presented in Fig. 4*b* and *c*. Compari-

<sup>7</sup> As discussed in our previous communication (9), radioactive secretion of lobules is the standard of choice for mixing experiments, since it can be collected and processed easily and since it is a more representative sample of the spectrum of secretory polypeptides than the extracted content. However, comparison of Fig. 4*a* with profile *b* of Fig. 1 illustrates that the relative concentrations of secretory polypeptides present in the standard differ from those found in intact secretion granules. Apparently, nonparallel loss of secretory protein during collection and processing of the radioactive standard cannot be avoided.

A second point we wish to emphasize is that labeling with amino acids that are found at either substantial or consistent frequencies in the proteins of interest (8) can increase the sensitivity of contaminant detection in the analyzed membrane gels.

son of the three profiles leads to the following observations: (*a*) The secretory species of mol wt 130,000, 110,000, and 95,000 (the three metachromatically staining species) and 40,000 are not the major radioactive components of the parotid secretion; yet, relative to the species of mol wt  $\sim 58,000$  (amylase) and 12,000, they show preferential adsorption to the membranes of lysed granules. Taken in concert with the demonstrated effectiveness of the saponin- $\text{Na}_2\text{SO}_4$  treatment, the finding suggests that the 40K component represents, in large part, strongly adsorbed secretory protein which under physiologic circumstances is destined for secretion. (*b*) Two secretory polypeptides, amylase and the 33,000 mol wt species, of both endogenous and exogenous (labeled) origins, are less effectively removed than the other contaminants by treating the granule membranes with saponin- $\text{Na}_2\text{SO}_4$ . In fact, the treatment appears to enhance slightly the association of the 33,000 mol wt component to the membranes. (*c*) The two preceding points are part of the more general observation that the components of the mixed radioactive secretion associate in an apparently equivalent fashion to granule membranes during granule lysis as do the endogenous secretory proteins. Since the association of *both* endogenous and exogenously added polypeptides is reversible only by the vigorous conditions of saponin- $\text{Na}_2\text{SO}_4$  treatment and since the ensuing removal of both types of contaminants is incomplete (Fig. 4*c*), we do not believe that with the evidence at hand it is possible to classify the endogenous contaminants as "perimembrane" proteins chemically distinct from secretory polypeptides (37).

If complete equivalence of exogenous and endogenous secretory protein with regard to its capacity to associate with membranes during granule lysis is assumed, it is possible to calculate approximate levels of contamination of the membrane subfractions from the results of the mixing experiments. With experimentally determined values for the quantities of granule protein and labeled secretion mixed to obtain the data in Fig. 4*b* (refer to Methods) and the average specific radioactivity ( $1.1 \times 10^5$  cpm/mg protein) of the lobule secretion (Fig. 4*a*), we estimate that 40% of the protein of the control membrane preparation (Fig. 4*b*) and 18% of the protein of the membrane preparation cleaned by the saponin- $\text{Na}_2\text{SO}_4$  treatment (Fig. 4*c*) represents secretory contaminants. For the latter preparation, 14 out



**FIGURE 4** Mixing Experiments. (a) Gel electrophoretogram and radioactivity profile of secretion sample discharged by lobules labeled *in vitro* with [ $^{14}\text{C}$ ]leucine, [ $^{14}\text{C}$ ]proline, and [ $^{14}\text{C}$ ]tyrosine. This preparation was used in subsequent mixing experiments. Gel load, 90  $\mu\text{g}$  protein. (b) Gel electrophoretogram and radioactivity profile of a membrane subfraction isolated from secretion granules mixed with labeled secretion (panel a) before lysis. Radioactivity associated with the contaminant bands is due entirely to the exogenously added secretory protein. Gel load,  $\sim 75 \mu\text{g}$  protein. (c) Gel electrophoretogram and radioactivity profile of an aliquot of (b) after treatment with 20  $\mu\text{g}/\text{ml}$  saponin-0.3 M  $\text{Na}_2\text{SO}_4$  to test the removal of all contaminants, endogenous as well as exogenous; the latter were provided by the labeled secretory proteins added before lysis. Gel load,  $\sim 55 \mu\text{g}$  protein.

of the 18% contaminating protein can be ascribed to the 33,000 mol wt polypeptide. Thus, we have made substantial progress toward obtaining an electrophoretogram consisting solely of bona fide membrane polypeptides of parotid secretion granules; the only major contaminant left is the 33,000 mol wt component.<sup>8</sup>

*Comparative Peptide Mapping of the 40K Component and the Secretory Content Polypeptide Having the Same Electrophoretic Mobility*

To evaluate the extent of similarity or dissimilarity between the polypeptide of the 40K component which appears to be synthesized at a rapid rate during short-term labeling and the secretory content polypeptide which has a similar mobility in SDS gels, parotid lobules were incubated for 5 h (sufficient to allow substantial labeling of mature secretion granules [8]) in a medium containing 100  $\mu$ Ci/ml [<sup>3</sup>H]leucine. From these lobules, secretion granules were isolated; membrane and content subfractions were prepared and subjected to SDS-polyacrylamide gel electrophoresis to obtain the usual electrophoretograms (Fig. 1). The Coomassie blue-stained bands of interest were dissected from gels of each of the subfractions, and eluted electrophoretically. The eluted polypeptides were processed into a medium suitable for proteolytic digestion as described in Methods. After digestion with TPCK-trypsin (or  $\alpha$ -chymotrypsin) the peptide mixtures were subjected to two-dimensional mapping on cellulose thin layers. Fluorograms of the resulting maps were prepared to locate the peptides containing [<sup>3</sup>H]leucine. Fig. 5 gives the results obtained with tryptic digests and shows clearly that the labeled peptide maps are very similar for the two polypeptides (40K and 6 - in Fig. 1) of equal mobility excised from the gels of membrane (Fig. 5a) and content (Fig. 5b) subfractions, respectively. The agreement of the fluorograms is extensive but not perfect. There is a peptide present in the 40K component (marked

by a long arrow in Fig. 5a) which is not found in the fluorogram of the content-derived species, and there are additional labeled peptides of high electrophoretic mobility and low chromatographic mobility (marked by short arrows in Fig. 5b) in the corresponding content band. Most of these discrepancies are explained by the identification of the same peptides in the fluorogram (Fig. 5c) of a second content polypeptide (designated 5 in Fig. 1) only partially resolved from the 40,000 mol wt content component (designated 6 in Fig. 1) at the position of excision from the original gel. The coincidence of the fluorograms of the chymotryptic maps (not illustrated) is slightly less extensive than for the tryptic maps, but most of the major labeled peptides are common to the 40K component and its corresponding content polypeptide. In general, the fluorography of the peptide maps reveals a 90-95% coincidence of endogenous radioactivity for the 40K component and its secretory counterpart. We assume that the remaining discrepancy represents variation in the extent of proteolysis since the ratio protease:substrate was not exactly the same for all samples. This assumption is based on observed differences in relative intensities of spots of identical mobility in the two fluorograms (Fig. 5a and b) and on variability observed in duplicate fluorograms of content-derived species.

The results obtained lead us to the conclusion that the two 40,000 mol wt polypeptides found in the membrane and content subfractions are highly similar, if not identical. Hence, the 40K component cannot be considered as a unique, bona fide membrane polypeptide, characterized by its high rate of synthesis (cf. references 7 and 9). It has a counterpart of similar composition and metabolism among the secretory proteins and it appears to be in part, or in toto, a secretory contaminant of the membranes.

#### DISCUSSION

In principle, during the hypotonic lysis of parotid secretion granules, adsorption of residual secretory polypeptides can occur on both the inner and outer aspects of the granule membranes (eventually isolated as a membrane subfraction). The results of the mixing experiments in which radioactive secretory protein was added to the homogenization medium and the homogenate indicate that relocation onto the outer membrane surface is minimal. Therefore, the contamination problem resides primarily with the inner aspect of the

<sup>8</sup> The unusual behavior of the 33,000 mol wt species (contaminant C<sub>2</sub>) may indicate that the nature of its binding to components associated with the inner aspect of the granule membrane is different from that of other secretory polypeptides. Characterization of these interactions as well as evaluation of their consequences to the secretory packaging process remain the subjects of future investigation.

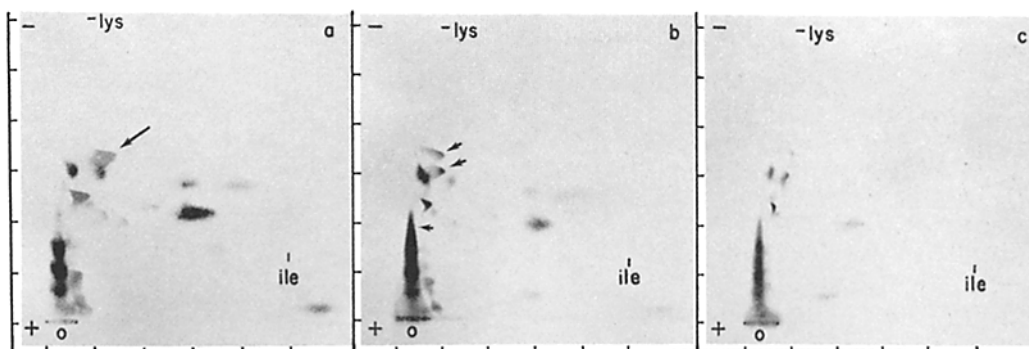


FIGURE 5 Peptide map fluorograms of tryptic digests of [ $^3\text{H}$ ]leucine-labeled polypeptides eluted from gels of membrane and content subfractions. The sample was applied to each map at the origin, a 1-cm strip in the lower left corner (marked *o*). The direction of the electrophoretic separation (first dimension) is marked by the electrodes +, - and by the position of the internal marker, lysine (*lys*). The direction of the chromatographic separation (second dimension) is indicated by the internal marker, isoleucine (*ile*), which has a mobility 0.7-0.8 that of the solvent front. (a) Fluorogram obtained for the 40K component harvested from gels of the membrane subfraction. Load: 200,000 dpm. (b) Fluorogram of the content polypeptide (designated band 6 in Fig. 1) of mobility corresponding to that of the 40K component. Load: 100,000 dpm. (c) Fluorogram of a second content polypeptide (designated band 5 in Fig. 1) only partially resolved in the original SDS-gel from that presented in Fig. 5*b*. Load: 30,000 dpm. Short arrows in Fig. 5*b* mark peptides for which corresponding peptides are not observed in the 40K component (a). A single peptide indicated by a long arrow in Fig. 5*a* is not found in the other two fluorograms.

membrane and is likely to represent either incomplete extraction of the content or adsorption secondary to granule lysis. The second alternative, which is based on the assumption that during lysis the membrane is temporarily permeable to polypeptides in both directions, is strongly supported by the results of the mixing experiments initiated with intact granules.

The membranes of the lysed granules apparently reseal as closed vesicles with residual secretory polypeptides preferentially or exclusively interacting with their inner aspect such that successful decontamination requires that sustained access be established to this compartment. The contaminants dissociated by an appropriate aqueous medium can then pass freely to the external medium.

A number of surface-active agents have been used successfully to lyse biologic as well as synthetic phospholipid membranes by creating (and in most cases stabilizing) discontinuities in the membrane bilayer structure. Lysolecithin and deoxycholate are among the many agents used for such purposes (25, 33); but the concentration of deoxycholate required to achieve partial extraction of cisternal (content) polypeptides from microsomal fractions too closely approaches that at which actual solubilization of the membrane begins to occur (25). A number of compounds of

different chemical structure, e.g., vitamin A alcohol and aldehyde (2, 18), polyene antibiotics (filipin and nystatin) (24, 33, 35), and certain glycosides (digitonin and the saponins) (3, 15, 19, 33-35), apparently create membrane discontinuities secondary to their interaction with membrane cholesterol. From this group, we chose to investigate the glycosides. The activities of a number of mitochondrial membrane enzymes are stable in digitonin (12, 14, 22) and, as already mentioned, Bretz and Baggiolini (6) have reported a selective decontamination of the membranes of leukocytic specific granules by using low concentrations of digitonin (50  $\mu\text{g}/\text{ml}$ ) in the presence of 0.4 M  $\text{Na}_2\text{SO}_4$ . In that case, the efficiency of the procedure was evaluated for a single content protein (peroxidase, removed) and a single membrane protein (alkaline phosphatase, retained).

For the decontamination of the membrane subfraction obtained from parotid secretion granules, we chose to use saponin as opposed to digitonin for two reasons. First, although the saponin preparation is less pure than is digitonin, it is much more soluble in water than the latter. Second, there is evidence that saponin, at low concentrations, induces highly restricted membrane damage, probably less extensive than that

caused by digitonin. Seeman et al. (34) have carefully demonstrated that structural discontinuities in erythrocyte membranes created by 30  $\mu\text{g}/\text{ml}$  saponin are pits no larger than 50–60  $\text{\AA}$ , diameter. A recent direct comparison of the effects of digitonin and saponin showed that the latter alters to a lesser degree the structural integrity of erythrocyte membranes (I. Ohtsuki, J. D. Jamieson, and G. E. Palade, personal communication).

The electrophoretogram and radioactivity profile of Fig. 4*b* reveal the entire spectrum of secretory contaminants of a representative membrane subfraction. The selective removal of these polypeptides is achieved in large part by treatment with saponin- $\text{Na}_2\text{SO}_4$ , a procedure which most probably assures sustained access to (and egress from) the internal compartment of the secretion granule ghosts. In the presence of 0.3 M  $\text{Na}_2\text{SO}_4$ , 20  $\mu\text{g}/\text{ml}$  saponin represents the minimum concentration for which maximal decontamination is achieved. Membrane preparations subjected to this treatment show, upon negative staining, frequent apparent discontinuities not found at the lowest saponin concentration, thus suggesting that sustained access to the internal compartment may entail a stabilized reorganization of the usual membrane structure.

The 40K component, the nature of which was still uncertain at the beginning of this study, was also extensively but not completely removed by the saponin- $\text{Na}_2\text{SO}_4$  treatment; it was still detectable as a distinct band in the electrophoretogram of the residue of the membrane subfraction. For this reason, we felt obliged to obtain additional information concerning the relationship of this band (especially of its rapidly synthesized fraction) to the secretory proteins. To this intent, the analysis was taken one step further to the level of comparative mapping of labeled peptides generated by enzymatic digestion of the corresponding polypeptides after their extraction from gel bands. The results of the mapping studies indicate quite clearly that the rapidly synthesized polypeptide of the 40K component is (within the qualification previously mentioned) a secretory protein, rather than a unique membrane species. This finding does not preclude the existence of a bona fide membrane polypeptide hidden under the contaminant and having only limited (or negligible) endogenous labeling. We assume, however, that such a polypeptide, if present, would represent at most a small fraction of the 40K component for

two reasons. First, Fig. 4*c* shows that at least a part of the residual 40K component must be incompletely removed endogenous secretory contaminant, since the radioactive, exogenously added polypeptide of the same mobility is incompletely removed by saponin- $\text{Na}_2\text{SO}_4$ . Second, previous estimates of the specific radioactivity (cpm/mg protein) of the 40K component (obtained from gels comparable to that of the endogenously labeled membranes of Fig. 1) are quite similar to values obtained for individual granule content species from the same experiment (7).

Thus, in total, approx. 40% of the protein of the untreated granule membranes represents residual secretory contamination. Accordingly, estimates of the relative amount of presumed bona fide membrane protein must be revised downward to  $\sim 1.5\%$  of the total granule protein, from the  $\sim 3\%$  previously mentioned (7, 9). Calculated on the basis of this corrected figure, protein contributed by other cellular membranes (primarily mitochondrial) could now represent as much as 10–15% of the total protein of the granule membrane subfraction, instead of the 5% of the earlier estimate (9).

As a result of this investigation which focuses on the selective decontamination of secretion granule membranes, we wish to emphasize several points which concern the nature of membrane-secretory polypeptide interaction. First, as indicated by our mixing experiments, the association of contaminants is definitely asymmetric, i.e., apparently restricted to the inner aspect of the granule membrane. Furthermore, the interaction is of such strength that complete decontamination is not achieved even with a major reorganization of the membrane structure. This asymmetric and tenacious association, as well as the content-content interactions inferred from the sedimentation of removed contaminants as aggregates, may have functional implications in connection with the formation and stabilization of the secretion granules, achieved during the concentration, packaging, and storage of the secretory products. Second, we are obliged to distinguish our conditions of granule lysis (with the ensuing outflow of secretory contents) from the physiologic discharge attained through exocytosis. For the latter process, the removal of content species is presumed to be complete. Finally, we should point out that the strength of the interaction may render difficult (or uncertain) in some cases the identification of “peripheral” membrane proteins distinct from se-



cretory species; special criteria (such as select differences in amino acid sequences) may be required for their identification.

In this study we have used comparative peptide mapping to advantage to equate (bearing in mind our qualifications) a lesser polypeptide collected from the granule content to that part (probably the bulk) of the 40K component which is synthesized (and labeled) during a 5-h in vitro incubation of parotid tissue. We conclude that granule membranes do not possess, as we assumed in the past (7, 9), a bona fide majority component having a high rate of biosynthesis comparable to that of secretory proteins. Further, at the level of resolution afforded by the membrane electrophoretogram of Fig. 1, none of the bona fide membrane polypeptides of parotid secretion granules shows a rate of biosynthesis approaching that of the packaged content. In this respect the acinar cells of the rabbit parotid are analogous to those of the guinea pig pancreas (31).

By its nature, the technique of comparative fluorography of peptide maps cannot detect heterogeneities in composition of stained gel bands when the multiple components of a given band are labeled at very different rates. Such heterogeneities will be identified, however, when it will be possible to apply enzymatic digestion and peptide mapping to amounts of polypeptides sufficient for visualizing the entire spectrum of their derived peptides by reaction with fluorescent compounds (fluorescamine or dansyl chloride) or ninhydrin. We assume that the techniques of comparative peptide mapping will prove useful in the future, not only for the analysis and identification of adsorbed membrane contaminants, but also for evaluating possible biogenetic relationships among polypeptides isolated from the membranes of distinct cellular organs or compartments.

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