Biochimica et Biophysica Acta, 375 (1975) 87–105

BBA 76825

NON-PARALLEL TRANSPORT OF MEMBRANE PROTEINS AND CONTENT PROTEINS DURING ASSEMBLY OF THE SECRETORY GRANULE IN RAT PAROTID GLAND

D. WALLACH, N. KIRSHNER* and M. SCHRAMM

Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem (Israel)

(Received June 18th, 1974)

SUMMARY

The insertion of newly synthesized protein molecules into the membrane of the secretory granule of the rat parotid gland was studied by in vivo labeling with [^{3}H]-proline and [^{3}H]leucine. 2 h after the injection of the amino acid into the rat, the membrane fraction isolated from the secretory granules was found to be highly labeled with proline but only slightly labeled with leucine. The ratio of proline label in the granule membrane to that in the granule's secretory content was roughly equivalent to the ratio of total proline in the proteins of these two fractions. In contrast the ratio of leucine label in the membrane to that in the secretory content was much less than would be expected from the relative amount of leucine in both fractions.

Separation of the proteins of the granule membrane by gel electrophoresis in presence of sodium dodecylsulfate showed that a considerable amount of these proteins was unlabeled. The labeled proteins could be selectively extracted from the membrane by 0.15 M NaCl solution or by dilute buffer at pH 4.5. These extracted proteins were found to contain a high proportion of proline residues and a negligible amount of leucine residues. In the extract proline constituted 36 mole % of the total amino acids. Proline+glycine+glutamic acid constituted more than 80 mole % and leucine constituted about 1 mole % of the total amino acids.

Further analyses by gel electrophoresis in presence of sodium dodecylsulfate showed that the fractions of secretory granule membrane and secretory granule content are relatively free of contamination by proteins from other subcellular structures.

It is suggested that the proteins which will constitute the mature secretory granule are transported to the site of final assembly by two pathways. The proline-rich proteins are transported to the site of assembly in close coordination with all the exportable proteins. The other membrane proteins arrive by a different pathway. Two alternative mechanisms are suggested to explain the finding that a considerable part of the membrane proteins are not labeled.

Abbreviation: DPPD, *N,N'*-diphenyl-*p*-phenylenediamine.

* Present address: Department of Biochemistry, Duke University, Durham, N.C. 22710, U.S.A.

I. The pathway of the intracellular transport of the unlabeled membrane proteins is similar to that of the secretory proteins but the newly synthesized membrane protein molecules are diluted in a large intermediate pool — the Golgi complex.

II. The proteins that did not get labeled are derived by a process of reutilization, from membranes of granules which have previously discharged their content in the process of secretion.

INTRODUCTION

The secretory granules are membrane-bound vesicles in which secretory material is accumulated for export. The secretory material may comprise secretory proteins, peptides, or molecules of low molecular weight like the catecholamines [1–4]. The membrane of the granules was shown to play a central role in the secretion of these materials. Electronmicrographs showed that the secretory process involves fusion of the secretory granule membrane with the cell membrane and extrusion of the granule content through an opening created at the point of coalescence [2–5]. Thus, while the secretory material is carried away from the cell, the secretory granule membrane temporarily forms a continuum with the cell membrane and is subsequently reabsorbed into the cell [5]. The fate of the reabsorbed membrane is not clear. It was suggested that after the membrane is reabsorbed into the cell its constituents are reutilized in the formation of new secretory granules [6]. Another possibility suggested in a previous publication from our laboratory [7] is that after reabsorption the constituents of the secretory granule membrane are degraded and the new secretory granule membranes are constructed from constituents which are synthesized de novo. Since there is no clear ultrastructural or enzymatic marker by which this membrane can be distinguished from the other smooth membranes of the cell it is not possible to trace the granule membrane after it has been reabsorbed. For the same reason it is not possible to trace this membrane from the mature granule back to the site of its assembly. Structural studies of secretory systems failed, therefore, to give a clear cut answer to this question [2, 8, 9].

In a previous report from our laboratory [7], it was shown that following injection of a labeled amino acid mixture to the rat, radioactive proteins accumulated in the membrane of the secretory granule of the rat parotid gland. The kinetics of labeling of protein in the secretory granule membrane and in the granule content were almost identical. It was therefore concluded that the proteins of the membrane are synthesized de novo concomitantly with the secretory granule content, rather than being reutilized after secretion. The specific radioactivity, cpm/mg protein, of the membrane was identical to that of the content. This and the pattern of the labeled membrane proteins in the phenol-acetic acid gel electrophoresis method in use at that time [7], seemed to indicate that all the proteins of the membrane become labeled to the same extent.

With the development of better techniques for gel electrophoresis [10] and for isolation of secretory granules [11], we decided to study the problem further and check for differences in the extent of labeling of the various membrane proteins in order to get a better idea of the way by which the secretory granule is constructed. It is shown in the present communication that the label in the granule membrane is

localized mainly in one type of proteins which are rich in proline residues. As will be shown in a separate publication, the proline-rich proteins in the membrane fraction seem to be different from the proline-rich proteins in the granule content. The other proteins of the granule membrane are found to contain only small amounts of label. Thus our earlier conclusion [7] that all the granule membrane proteins are as highly labeled as the granule content, is incorrect. The implications of the findings in terms of the transport and assembly of the secretory granule proteins are discussed.

MATERIALS AND METHODS

Isolation of subcellular particles

Male Albino rats weighing 230±30 g, fed ad libitum were used. The rats were sacrificed by cervical dislocation and bled by heart incision. Labeled granules were isolated 2 h after injection of 50–200 μCi of the labeled amino acid to the rat. (Intravenously in 0.6 ml of saline, spec. act. 0.5–2 Ci/mmole). The parotid glands were carefully trimmed of extraneous tissue and homogenized. The secretory granules were isolated on a urograffin gradient as previously described [11]. Before applying the combined "250 × *g* sup" fraction on the 30% urograffin solution it was centrifuged once again for seven minutes at 250 × *g*. The sucrose medium which was used for the homogenization and fractionation contained 0.2 μg/ml DPPD to prevent lipid preoxidation and subsequent lysis of the secretory granules (Ben-Zvi, R., personal communication). The medium was adjusted to pH 7.5 by Na_2CO_3. Mitochondria and microsomes were isolated by differential centrifugation as described previously [7].

Isolation of the secretory granule content and membranes

The granules were resuspended with the aid of a teflon rod in the above sucrose medium (0.3 ml per one gland equivalent of granules). The suspension was dialysed for 12 h in a visking tubing against 300 vols of 10 mM Tris chloride buffer, pH 8.5 which contained also 1 μg/ml DPPD, 0.05 mM EDTA and 100 units per ml of sodium penicillin G.

The content of the dialysis tube was then carefully overlayed on twice its volume of a solution containing 10 mM Tris–Cl, pH 8.5, 30 mM sucrose and 1 μg/ml DPPD and centrifuged at 15 000 × *g* for 20 min using a T-50 Spinco Rotor. The supernatant contained the soluble content proteins of the granules. The pellet of membranes obtained from 20 to 40 glands was resuspended in a medium containing 10 mM Tris–Cl buffer, pH 8.5, 1 μg/ml DPPD and 100 units/ml penicillin (0.1 ml per one gland equivalent of membranes) and kept in ice for one hour to permit elution of absorbed soluble proteins. Then it was overlayed on the above Tris, DPPD, penicillin, sucrose medium and spun for 20 min at 100 000 × *g* in the T-50 Rotor. The supernatant was carefully sucked off and the face of the pellet carefully washed with 1 ml of the above medium. The same procedure was applied for the isolation of membranes from the mitochondria, microsomes and the "250 × *g* pellet", but in this case the precipitation of the membranes was done by centrifugation at 160 000 × *g* for 60 min.

Collection of the secretory proteins from the parotid duct

Freely fed rats were used. The cannulation of the ducts was done following the method of Robinovitch et al. [12] and Batzri et al. [13]. The amounts of the

secretogogues injected into rats of 200 g body weight were as follows: Isoproterenol 1 mg in 0.5 ml saline; phenylephrine 2.5 mg in 1 ml; pilocarpine 1.5 mg in 2 ml. The secretogogues were each injected intraperitoneally. When isoproterenol was given with phenylephrine it was given intravenously 5 min after intraperitoneous injection of the phenylephrine. In fed rats, phenylephrine did not induce any secretion while the combined introduction of phenylephrine and isoproterenol caused much higher saliva flow rate than that caused by isoproterenol alone.

Analytical methods

Protein was estimated with the Lowry reagent [14]. Amylase was determined according to Bernfeld [15]. For the estimation of the amylase in a preparation of secretory granule membranes these were incubated in 0.1 % Triton X-100 for 3 min at 30 °C before the addition of the starch solution. This was done to ensure that amylase would not remain masked in closed vesicles.

Hydrolysis of protein samples for the amino acid analysis was done in 6 M HCl at 110 °C for 22 h in an ampoule sealed under vacuum. The analysis was done with a Beckman 120 B amino acid analyser.

Gel electrophoresis

Sodium dodecylsulfate discontinuous polyacrylamide gel electrophoresis was performed according to Lammeli and Maizel [10] with the following modifications: The buffer in the sample and in the stacking gel was 0.02 M Tris phosphate, pH 6.7. The concentration of mercaptoethanol in the sample was 10 % (v/v). The electrode buffer contained 0.05 M Tris, 0.38 M glycine and 0.1 % sodium dodecyl sulfate. The final concentration of *N*, *N*, *N'*, *N'*-tetramethylenediamine was 0.05 % (v/v) in the separating gel and 0.033 % in the stacking gel. The final concentration of the ammonium persulfate was 0.05 % (w/v) in the separating gel and 0.066 % in the stacking gel. The gels were prepared in cylindrical glass tubes (12×0.6 cm) coated with Siliclad (Clay Adams). The separating gel which contained 12 % acrylamide 0.32 % *N*, *N'*-methylene bisacrylamide was prepared a day before the run. The stacking gel which contained 3 % acrylamide 0.08 % *N*, *N'*-methylene bisacrylamide was prepared just before use. The gelification mixtures were thoroughly deaerated immediately before casting the gels. The deaerated mixture and the tube rack were held in a water bath in which the temperature was kept at 5 °C. The polymerizing mixture was overlayed with isobutyl alcohol soaked with water using an Eppendorf automatic pipette and then the temperature in the bath was raised to 10 °C. After the polymerization the isobutyl alcohol was sucked off and the gel was covered with 0.375 M Tris chloride buffer, pH 8.9, containing 0.1 % sodium dodecylsulfate. The stacking gel mixture was overlayed with a solution of 0.1 % sodium dodecylsulfate. Penetration of the isobutyl alcohol into the polymerizing mixture resulted in a distortion of the bands of the analyzed proteins. Such gels could be identified by the presence of a "Schlieren pattern" in the gel matrix. The gels were checked therefore for the presence of such pattern before use. Membrane pellets and granule content, prepared as described above, were treated for electrophoresis [10] and applied to the gel without prior concentration steps. Samples of the maximal volume of 0.1 ml containing up to 200 μg protein were run. Throughout the run, the current was increased gradually at intervals of 15 min (0.5, 1, 2, and 3 mA per gel).

Fixation and staining

Staining with Coomassie Blue was done according to Fairbanks et al. [16]. Before staining the gels were fixed for 6 h in 10 % trichloroacetic acid 25 % isopropanol and then washed twice (each time for 30 min) in 10 % acetic acid, 25 % isopropanol. The membraneous proline rich protein bands were visualized by the light scattering from unstained gels that were fixed in the following way: The gel was kept for two hours in a test tube filled with 20 ml of 50 % (w/v) trichloroacetic acid, 20 % (w/v) sulfosalicylic acid solution. The solution was mixed from time to time. The bands were detected by illuminating the tube through its long axis with a light projector. The pattern was photographed perpendicularly to the light path on a black background. In order to get even illumination along the gel the tube was corked with a translucent plastic cap and placed between the slide projector and a mirror. The gels could be stained with Coomassie Blue in the presence of this trichloroacetic acid–sulfosalicylic acid solution but destaining in that solution removed the stain from the protein bands too.

Glycoproteins were stained as follows: (1) Fixation in trichloroacetic acid and washings as in the staining with Coomassie Blue. (2) 1 h in 1 % (v/v) periodic acid in 10 % acetic acid (each gel in a test tube filled with 20 ml at 4 °C). (3) Two washes, each for 15 min in 10 % acetic acid. (4) 30 min in 0.5 % (w/v) sodium arsenite in 10 % acetic. (5) Two washes each for 15 min in 10 % acetic acid. (6) 12–24 h in Schiff reagent. (7) Washing and keeping in 0.1 % (w/v) sodium metabisulfite in 0.01 M HCl. The equilibration with the various solutions was done by immersing the gels, each in a perforated plastic tube, in stirred solution with a volume of 50 ml/gel.

Radioactive measurements

Liquid samples of 1 ml containing 0.5 % sodium dodecylsulfate were counted with 12 ml scintillation mixture in a Packard, liquid scintillation counter model 3320. One liter of the scintillation mixture contained: 667 ml toluene, 333 ml Triton X-100, 5.50 g 2,5-diphenyloxazole and 0.1 g 1,4-bis-[2-(4-methyl-5-phenyoxazolyl)]-benzene. Polyacrylamide gels which contained labeled proteins were sliced after partial freezing on dry ice (the slicer was prepared in the technical division of the Hadassah School of Medicine). 1 mm slices were incubated at 75 °C with 0.3 ml of 24 % hydrogen peroxide containing also 2 % w/v sodium dodecylsulfate in corked 3.5 ml disposable scintillation vials. After 24 h the vials were cooled and 3 ml of the above toluene Triton X-100 scintillation mixture was added to each vial. These vials were inserted into vials with the standard dimension and counted for 10 min. Gel pieces not containing radioactivity were solubilized by the same procedure and served as control. The counts found in the control (^{3}H, 30 cpm/vial; ^{14}C, 20 cpm/vial) were subtracted from all experimental samples.

Materials

The sources of chemicals for the preparation and staining of the gels were: Acrylamide, *N*, *N'*-methylenebisacrylamide, *N*, *N*, *N'*, *N'*-tetramethylethylenediamine and ammonium persulfate, Eastman. Sodium dodecylsulfate and bromo phenol blue Matheson Coleman and Bell. Coomassie Brilliant Blue R-250, Schwartz-Mann. Basic fuschin, Allied Chemical. Hydrogen peroxide, sulfosalicylic acid, Merck. Acrylamide

was recrystallized from chloroform [17]. Sodium dodecylsulfate was recrystallized from ethanol. The isopropanol and the acetic acid that were used for the periodic acid Schiff stain were of analytical grade. Urograffin was from Schering AG, Berlin. The sources of labeled amino acids were: L amino acid ^{14}C mixture (NEC-445) New England Nuclear, L-[4-^{3}H]leucine (TNCN 24) Nuclear Research Centre, Negev, L-[G-^{3}H]proline (TRA 83) The Radiochemical Centre, Amersham.

RESULTS

In vivo labeling of the proteins of the secretory granule membrane by [^{3}H]proline and [^{3}H]leucine

2 h after injection of [^{3}H]proline or [^{3}H]leucine into the rat the secretory granules of the parotid gland are found to be heavily labeled. The recovery of proline or leucine label in the secretory granule fraction is about equal to the recovery of secretory proteins in that fraction (50 % of the total in the gland).

Since the granule membrane represents less than 5 % of the total proteins of the granule, the absolute amount of label in the membrane is obviously low. Yet, as shown in Table I, the specific radioactivity of the proline labeled membrane is even

TABLE I

LABELING OF THE SECRETORY GRANULE MEMBRANE AND CONTENT PROTEINS BY ^{3}H PROLINE AND ^{3}H LEUCINE

The granules were labeled by injection of 50 μCi [^{3}H]proline of [^{3}H]leucine to each rat 2 h before the isolation of the granules. The amount of secretory granule content proteins which remains in the membrane preparation was estimated by amylase determination to be 5 % of the total protein in the membrane preparation. According to the known specific radioactivity of the content proteins, this contamination forms 4 % of the radioactivity in the proline-labeled membrane and 25 % of the label in the leucine-labeled membrane. The specific radioactivity in the latter was corrected accordingly from the obtained value of 620 cpm/mg protein to 500 cpm/mg protein*. The amino acid analysis was carried out on the proline-labeled preparation. The complete analysis is given in Table II.

	Proline		Leucine	
	Relative amount (moles per 100 moles amino acid	Spec. act. (cpm/mg protein)	Relative amount (moles per 100 moles amino acid)	Spec. act. (cpm/mg protein)
Membrane	16**	3300	6	500
Contents	11	2300	9	3000
Ratio $\frac{\text{membrane}}{\text{contents}}$	1.5	1.4	0.7	0.17

* This correction is based on the assumption that the quantitative ratio between the amylase and the other secretory proteins in the membrane preparation is identical to that which exists in the secretory granule content. The pattern of the membrane proteins on acrylamide gel (Figs 1 and 2) does not show any gross violation of this assumption.

** Much higher values for the relative amount of proline and the extent of labeling of the membrane were achieved with batches of membranes which were washed only once, briefly. Since such membranes contained significantly higher amounts of secretory granule content proteins they were not used here.

higher than that of the secretory granule content proteins. The ratio of labeling in the membrane to that in the content is about equal to the ratio of the proline content in the two fractions.

In contrast, the labeling of the membrane by leucine is very low and the ratio of the specific radioactivities of the leucine labeled membrane and content is significantly lower than the ratio of the leucine content of the two fractions. This discrepancy was studied further by analyzing the individual membrane proteins separated with the aid of disc gel electrophoresis.

The pattern of the secretory granule proteins in sodium dodecylsulfate gel electrophoresis

The patterns of the proteins of the secretory granule content and the secretory granule membrane in gels stained by Coomassie Blue and peroidic acid Schiff stain are given in Fig. 1.

The secretory granule content is composed of five major bands C_3, C_5, C_6, C_7, C_8 (C_7 can sometimes be shown to be composed of two closely located glycoprotein bands) and several minor bands. Two of the major bands ($C_{6,7}$) and several

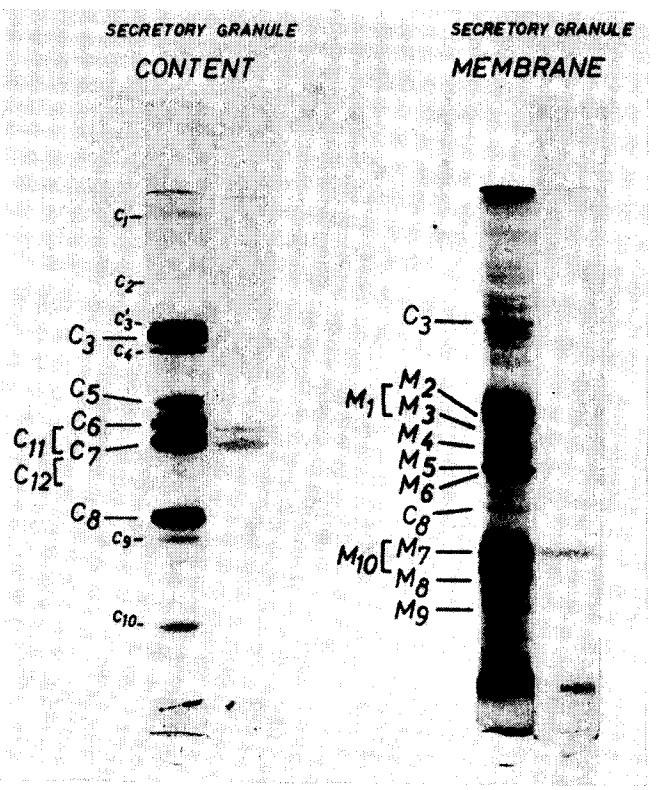

Fig. 1. Sodium dodecylsulfate gel electrophoresis of the secretory granule content and secretory granule membrane proteins. The gels were stained with Coomassie Blue (left of each pair) or periodic acid Schiff stain (right of each pair). On each gel 50 μg of protein were applied. The brackets show the localization of the diffuse purple proline-rich bands (C_{11}, C_{12}, M_1 and M_{10}).

minor bands (like C_1, c_2, c_3', c_4) are stained by periodic acid Schiff stain and therefore seem to be glycoproteins. A study of the properties of these different content proteins will be published separately.

The pattern of the membrane proteins of the secretory granule is more complex. In contrast with the phenol acetic acid method by which only one major band and two minor bands could be related to the secretory granule membrane [7] the sodium dodecylsulfate gels exhibit at least four major bands ($M_{2,3,5,7}$) several secondary bands and minor bands. The number of detectable minor bands increases with the amount of protein applied to the gel. When stained with the less sensitive stain amido black, most of them are not detectable. Of the major bands only M_7 is stained faintly by the periodic acid Schiff stain. Several additional high molecular weight fine bands (of which one is positively stained by periodic acid Schiff stain) were separated on gels which contain less acrylamide (10 %). In two regions of the gel (M_1 and M_{10}) there are diffuse faint bands which stain lightly with Coomassie Blue in a reddish colour. After fixation in 50 % trichloroacetic acid 20 % sulfosalicylic acid solution these membrane proteins are specifically visualized as sharp light scattering bands. As shown in Fig. 2, each of these bands turns out by this method to be composed of

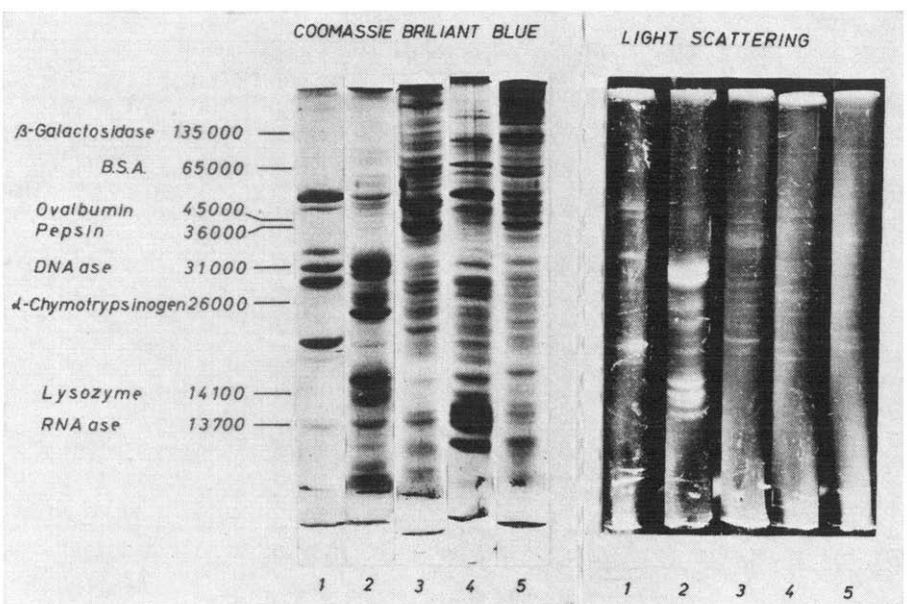

Fig. 2. Sodium dodecylsulfate gel electrophoresis of the secretory granule content and various membrane fractions from parotid gland. (1) Secretory granule content. (2) Secretory granule membrane. (3) Mitochondrial membrane. (4) Microsomal membrane. (5) Membranes isolated from the "250 × *g* pellet". The relative mobility in this electrophoresis system of several known proteins is given for an approximation of the molecular weight of the different protein bands. 20 μg of secretory granule content proteins were applied on the gel which was stained with Coomassie Blue. On all the other gels, 50 μg protein of the various samples were applied. Light scattering by the protein bands was visualized after fixation with 50 % trichloroacetic acid, 20 % sulfosalicylic acid as described under Methods and Materials. Because of low contrast of the photograph of the periodic acid Schiff stain the bands were retouched with a soft pencil.

several distinct bands. It will be shown in this paper that the proteins in these bands are rich in proline residues.

The purity of the granule membrane and the granule content preparation

In Fig. 2 the protein pattern of the secretory granule membrane is compared to that of the secretory granule content and to membranes prepared from the mitochondria, microsomes and the "250 × *g* pellet" prepared from the parotid gland. The proteins of the secretory granule membrane definitely differ by their relative mobility in the gel from the secretory granule content proteins. The minor contamination by the principal content bands (C_3 and C_8) in this pattern is variable (compare Figs. 1, 2, 4, 6). The glycoproteins C_6 and C_7 do not appear at all in the pattern of periodic acid Schiff stain of the membranes. By estimating the amylase activity in the membrane preparation the amount of content proteins is found never to exceed 10 % of the proteins of the preparation.

Two other contaminating minor bands in the pattern of the secretory granule membrane are those which run a little ahead of the contaminating C_3. These bands seem to correlate with the major bands of the mitochondrial membrane. It might be that the multitude of very fine bands which form the background colour on the upper part of the gel correlate to the other mitochondrial bands. A very small amount of contaminating mitochondria was indeed reported to be present in this granule preparation [11]. It is, however, obvious from Fig. 2 that even the major mitochondrial bands do not contribute significantly to the pattern of the secretory granule membrane proteins. No sign of contamination by microsomal membrane proteins can be seen on the gel of the secretory granule membrane proteins, the only common bands being the remnants of the secretory proteins which can be identified in the microsomal membrane too. (This is in contrast to membranes of granules which were isolated by differential centrifugation [7]. These membranes always show some amount of the major bands of the microsomes.) The "250 × *g* pellet" fraction which contains cell membranes and nuclei shows a multitude of high molecular weight protein and glycoprotein bands which do not appear in the secretory granule membrane at all. The proline rich membrane bands M_1 and M_{10} can be easily overlooked in the Coomassie Blue staining pattern because they are rapidly destained. Therefore, the presence of these proteins was checked in the different membranes and in the secretory granule content by the light scattering from gels after fixation in trichloroacetic acid, sulfosalicylic acid solution. As shown in Fig. 2 the proteins which form the bands M_1 and M_{10} are unique to the secretory granule membrane.

Fig. 3 shows a comparison of the secretory granule content proteins with the proteins that are actually secreted and appear in the gland duct after in vivo stimulation by various secretogogues. All the three patterns of secretory material are identical to that of the secretory granule content proteins. This is in spite of the fact that the different pharmacological agents used have divergent effects on the gland cell [13]. The preparation of secretory granule content seems therefore not to contain soluble proteins of contaminating subcellular particles or proteins of the secretory granule which would not be secreted.

The pattern of the labeling in the secretory granule proteins

Staining of acrylamide gels on which proline-labeled proteins of secretory

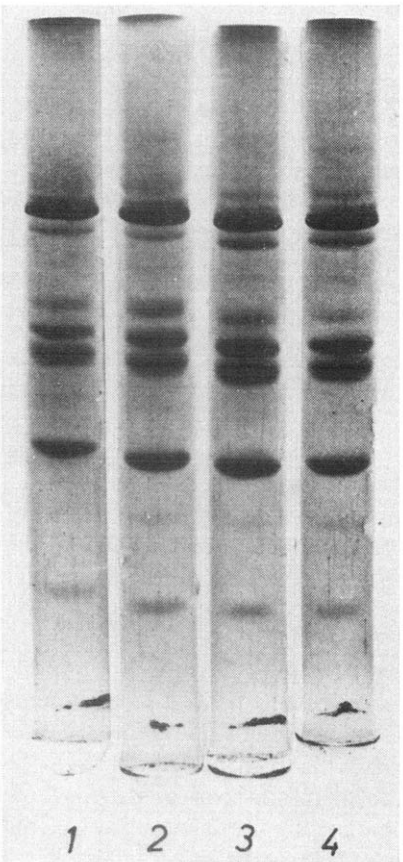

Fig. 3. Sodium dodecylsulfate gel electrophoresis of secretory granule content proteins and the salivary proteins of the parotid gland. The gels are stained with Coomassie Blue. (1) Secretory granule content proteins. (2) Saliva secreted by induction with isoproterenol. (3) Saliva secreted by induction with isoproterenol after injection of phenylephrine. (4) Saliva secreted by induction with pilocarpine. 100 μg of protein were applied to each gel.

granule membrane have been applied, caused a nearly complete extraction of the label. This is in contrast to leucine-labeled proteins of the granule content which were fully recovered from the gel after staining. Unstained gels were therefore analyzed and the radioactivity pattern was compared to the staining pattern of another gel on which the same material has been applied (Fig. 4). In this way a complete recovery of tritium label was achieved.

Fig. 4A shows the pattern of labeling of the secretory granule content proteins by proline in comparison to labeling by an amino acid mixture. All the major bands are found to be heavily labeled by both ways. In two regions (around C_6 and C_7 and in particular C_5) the proteins are found to be selectively more labeled with proline than with the amino acid mixture. In a study that will be published separately we have shown that the protein C_5 and the proteins C_{11} and C_{12} that overlap with C_6 and C_7 are highly enriched with proline residues.

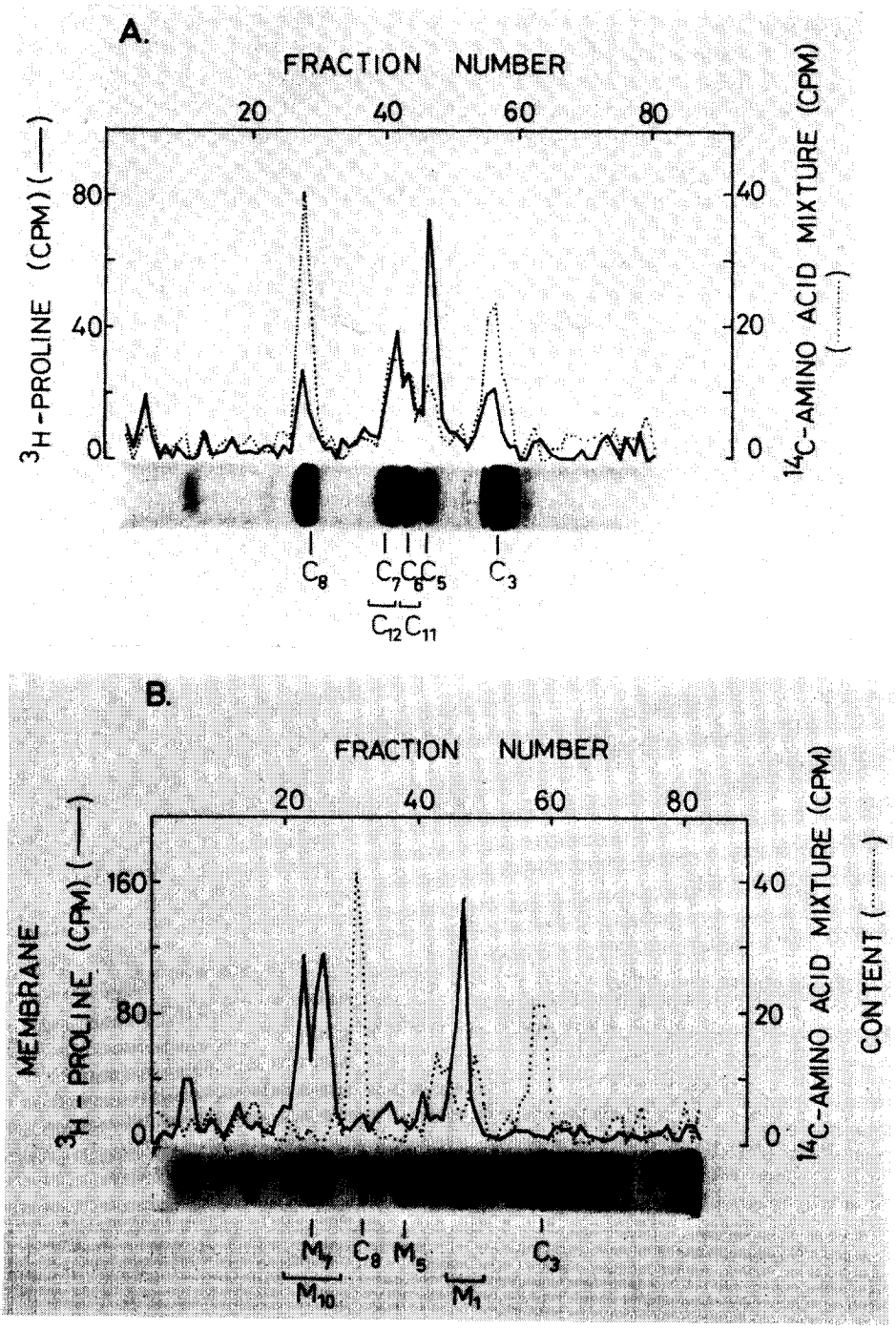

Fig. 4. The labeled proteins in the secretory granule content (A) and in secretory granule membrane (B). The location of [^{3}H]proline-labeled proteins of the membrane and of the content on unstained gels is compared to the staining pattern with Coomassie Blue. Secretory granule content proteins

The proline label in the membrane (Fig. 4B) is found to be almost exclusively located in two regions: M_1 and M_{10}. In the stained gels these regions are faintly stained in a reddish colour which is not clearly distinguished in the photograph. In order to check whether the band M_1 identifies with any of the proteins of the secretory granule content, ^{14}C-labeled secretory granule content proteins were run admixed with the [^3H]proline-labeled membrane proteins. By comparing the pattern of the ^{14}C label and the ^3H label it can be seen that M_1 does not coincide with the closely located C_5 and C_6 bands.

Further analyses were done in order to show that no other membraneous protein is labeled to a similar extent by any amino acid. When large amounts of proteins of membrane from which M_1 and M_{10} proteins were extracted (see below) are applied on the gel, no labeled bands other than the remnants of M_1 and M_{10} proteins are revealed. No membraneous band other than M_1 and M_{10} is found to be labeled when membranes which were labeled with uniformly labeled amino acid mixture are applied on the gel. When leucine-labeled membranes are applied to the gel no membraneous band can be shown to be labeled at all. The only peaks of label are those of the minute bands of contaminating secretory granule content proteins.

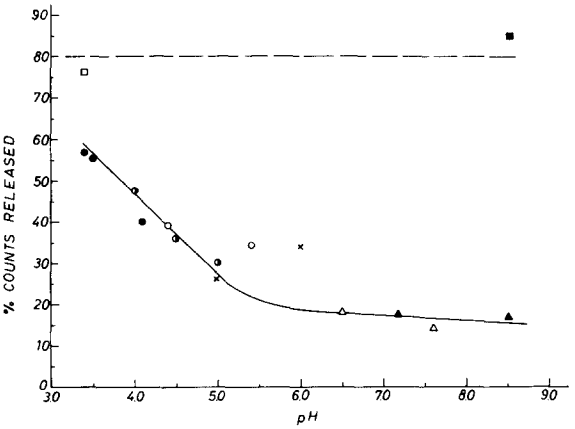

Fig. 5. Extraction of the proline-labeled proteins from the membrane. Labeled membranes were isolated 2 h after injection of 100 μCi [^3H]proline to each rat. The washed membranes (7000 cpm/mg) were divided into equal samples of 50 μg proteins by resuspension in the Tris chloride buffer, pH 8.5 (0.15 mg protein per ml), and sedimented in polycarbonate tubes. The pellet was resuspended in 10 mM buffer or in 10 mM buffer plus 150 mM KCl. After 1 h at 0 °C the membranes were sedimented. The amount of radioactivity which was released in that hour was assayed by counting the supernatant and the precipitated membranes. Before counting the pellet was solubilized with 0.1 ml of 2 % (w/v) sodium dodecylsulfate. ●, sodium citrate; ◑, sodium acetate; ○, sodium succinate; ×, sodium maleate; △, imidazole chloride; ▲, Tris chloride; □, sodium citrate plus 150 mM KCl. ■, Tris chloride plus 150 mM KCl.

which were labeled by ^{14}C-labeled amino acid (· · ·) were run admixed with the [^3H]proline-labeled proteins (—) in the unstained gels, both in A and B. Note the poor staining in the regions of the proline-labeled membrane proteins. The granules were labeled in vivo by injection of either 200 μCi [^3H]proline or 100 μCi ^{14}C-labeled amino acid mixture to each rat. 50 μg of proteins were applied to the gels that were stained. 60 μg of ^{14}C-labeled secretory granule content proteins and either 40 μg of [^3H]proline-labeled proteins or 70 μg of [^3H]proline-labeled membrane proteins were applied for the analysis of the radioactivity pattern.

Extraction of the proline labeled proteins from the membrane

Resuspension of labeled membranes and incubation for an hour in dilute buffer cause release of part of the label to the medium. About 15 % of the label are released in the Tris–Cl buffer, pH 8.5 which is used in the preparation of the membranes. (This accounts for the variability in the proline content in different batches of membrane preparations.)

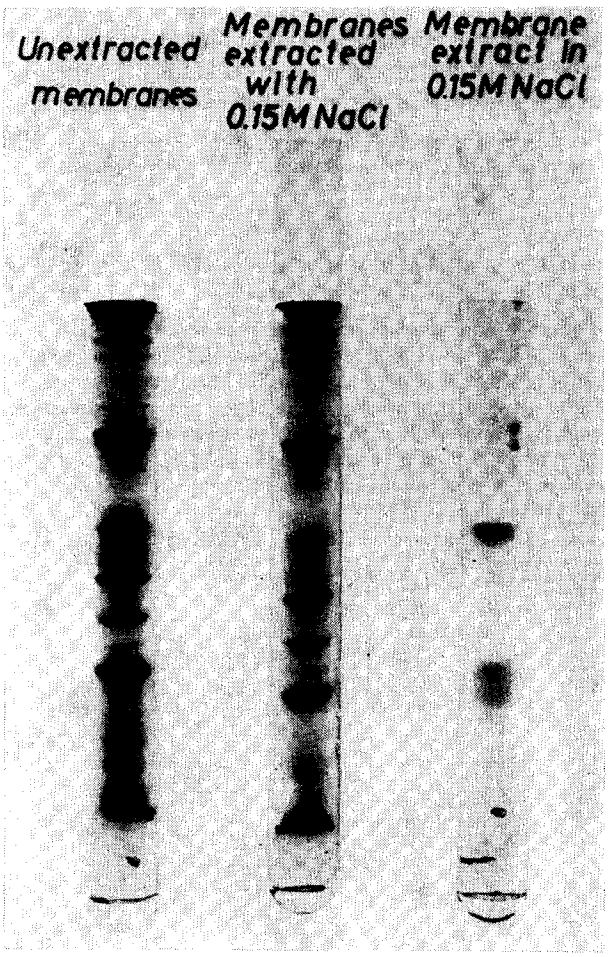

Fig. 6. Sodium dodecylsulfate gel electrophoresis of the proline-rich proteins which were extracted from the membrane. The gels were stained by Coomassie Blue. Washed membranes from six rats were divided into two equal parts and washed again with 10 mM Tris–Cl buffer pH 8.5. One of the samples was washed twice with 0.15 M NaCl solution containing also 10 mM Tris–Cl buffer, pH 8.5, 0.2 μg/ml DPPD and 100 units/ml penicillin. The suspension of the membrane in the NaCl solution was incubated in ice–the first time for 60 min and then for 30 min. 50 μg of protein of the NaCl-treated and untreated membranes and 20 μl of the first NaCl extract were applied to a gel. The pattern of the proteins which were released from the membranes in 10 mM sodium citrate buffer, pH 4.5, was identical to that of the 0.15 M NaCl extract (though the washed membranes still show some amount of the M_1 and M_{10} proteins). Traces of the glycoprotein M_7 appeared in the periodic acid Schiff stain staining pattern of the second NaCl wash.

As shown in Fig. 5, the release of the label is more prominent at low pH. Inclusion of 0.15 M KCl or NaCl in the extraction medium causes an almost complete release of the label independent of the pH of the buffer*.

Fig. 6 shows the pattern of the extracted proteins on the acrylamide gel. The extract is composed exclusively of the proteins of bands M_1 and M_{10}, except for two very faint bands in the region of C_3. By periodic acid Schiff stain it was found to be free of the glycoprotein M_7. Longer extractions with salt solution cause the release of M_7 from the membrane too. As shown in Fig. 2, the proteins of bands M_1 and M_{10} can be visualized by light scattering after fixation in 50 % trichloroacetic acid, 20 % sulfosalicylic acid. When the pattern of the extracted proteins is studied by the light scattering of the bands after such a fixation, the pattern of the extracted material is found to be identical to that of the whole untreated membrane while the 0.15 M NaCl treated membranes do not show any light scattering band at all. This visualization

TABLE II

AMINO ACID COMPOSITION OF THE MEMBRANE PROTEINS AND OF THE SECRETORY GRANULE CONTENT PROTEINS

The washed membranes were divided into two samples and washed twice more with 10 mM Tris-Cl buffer, pH 8.5. One of the samples was treated twice with 0.15 M NaCl solution as described in the legend to Fig. 6. Amino acid composition of the washed and unwashed membranes, of the first NaCl extract and of the counted proteins is given. The efficiency of the Lowry method in determination of the different proteins is estimated by comparing the results of the Lowry assays which were performed before hydrolysis with the weight of the amino acids in the hydrolyzate. The latter was estimated by multiplying the amount of the identified amino acids by their molecular weight.

Amino acid	mmoles per 100 moles of amino acids			Content proteins
	Untreated	Extracted with 0.15 M NaCl	0.15 M NaCl extract	
Proline	17.3	8.8	35.6	11.5
Glycine	15.9	14.3	22.8	10.1
Glutamic acid	14.0	8.7	22.2	9.6
Aspartic acid	7.2	7.9	2.4	13.2
Arginine	3.6	4.8	5.8	2.9
Lysine	4.2	8.2	2.7	3.4
Histidine	1.9	2.5	0.6	1.9
Threonine	3.9	4.6	1.1	4.7
Serine	5.6	6.6	2.3	6.8
Alanine	5.6	6.9	1.1	5.2
Valine	4.6	5.7	1.1	7.3
Isoleucine	3.3	4.1	0.6	4.1
Leucine	6.3	7.5	1.3	10.0
Cysteine	0.6	0.9	0	0.9
Methionine	1.2	1.5	0.3	1.0
Tyrosine	2.4	2.7	0.3	3.3
Phenylalanine	2.8	3.8	0.4	3.7
Lowry proteins (mg)	1.0	0.8	2.0	0.8

* An abstract reporting extraction of the membrane proline rich proteins by a similar procedure has recently appeared [18].

procedure, therefore, seems to be specific for the proteins that are extracted from the membrane with 0.15 M NaCl.

The amino acid composition of the 0.15 M NaCl extract (the M_1 and M_{10} proteins) is given in Table II. The amount of proline in these membrane proteins is very high. The amount of most of the amino acids, except glycine glutamic acid and proline, is very low. By comparing the proline content of the membranes, the NaCl treated membranes and the NaCl extract, the proline which is found in the M_1 and M_{10} proteins is calculated to comprise more than 60 % of the total proline in the untreated membranes. On the other hand, the leucine in the M_1 and M_{10} proteins is calculated to comprise only 4 % of the total leucine of the untreated membrane. Thus the difference between the extent of labeling of the membrane by leucine and by proline means that of the membraneous proteins only the proline-rich (leucine poor) ones become labeled. The underestimation of the latter by the Lowry method (Table II) results in higher values for the specific radioactivity (label to "Lowry protein" ratio) of the total membrane. This might give the impression that all the membrane proteins are labeled [7].

DISCUSSION

The proteins of the membrane represent less than 5 % of the total proteins of the secretory granule. In contrast the proteins which remain in the membrane fraction prepared in a similar way from mitochondria or microsomes represent about 50 % of the proteins of the particles [7]. For that reason incomplete removal of the secretory granule content proteins or slight contamination of the granule preparation with other subcellular particles results in high contamination of the membrane preparation with foreign proteins. The pattern of the proteins of the secretory granule membrane was compared in this work to that of the secretory granule content and of other membranes from the parotid gland cell. The secretory granule membrane was shown by that way not to be largely contaminated by the secretory granule content proteins or by mitochondrial or microsomal membranes and probably not by plasma membrane or nuclear membrane.

Since the pattern of the secretory granule content is found to be identical to that of the proteins secreted by the gland it also seems unlikely that granules with soluble content other than the secretory granules, like lysosomes or peroxisomes are found in the granule preparation. The membrane of the secretory granule is found to contain a smaller number of proteins than the mitochondrial and microsomal membranes. This is perhaps expected from the limited amount of functions that this membrane is supposed to serve. Yet this pattern of secretory granule membrane proteins is more complex than that shown previously in our laboratory by the phenol–acetic acid method [7]. Similar differences between the behavior in the two gel systems was reported for the membrane proteins of the secretory granules from guinea pig pancreas [19]. A severe drawback of the phenol–acetic acid method is that the phenol, which serves for the solubilization of the proteins of this membrane cannot be included in the ingredients of the acrylamide gel. For that reason some of the applied proteins precipitate at their entry into the gel. It seems that the two bands which appeared in the phenol–acetic acid acrylamide gels [7] correlate with the bands M_1 and M_{10} of the sodium dodecylsulfate gels. These proteins are quite soluble in the acetic acid

which is included in the phenol–acetic acid gels and therefore they penetrated into these gels while all the other membrane proteins precipitated in the heavy band which is found at the start of the phenol–acetic acid gel. If that is the case, since M_1 and M_{10} are the only proteins which become labeled, it is evident why the radioactivity pattern on the phenol–acetic acid gel was found to be identical to the staining pattern [7]. Another point which gave the wrong impression that all the proteins of the membrane are labeled, was the identity of the specific radioactivities of the membrane and the content proteins. As shown in this paper, the proline-rich proteins of the membrane are underestimated by the Lowry determination, possibly because of the low content of tyrosine and tryptophan. These proline-rich proteins comprise a large part of the proteins in the membrane preparation. The measure of specific radioactivity (label to "Lowry protein" ratio) used in the earlier communication from our laboratory was therefore misleading.

The apparent discrepancy between the high radioactivity of the granule membrane when proline is used and the low radioactivity when labeled leucine is used is resolved through the analyses by gel electrophoresis. It was found that of the different membrane proteins only the proteins which form the bands M_1 and M_{10} become labeled to an extent similar to that of the secretory proteins. Because of the high proline content and very low amount of leucine in these proteins the membrane is found to be heavily labeled with proline and only slightly labeled with leucine. In studies on the granule content we have found a series of proteins, each relatively rich in one of the following amino acids: proline, leucine, half cystein (to be published). It is obvious from these findings and from those discussed above, that amino acid incorporation experiments, using a single labeled amino acid, can be quite misleading if the composition of the proteins under study is not known.

The fact that the proteins which form the bands M_1 and M_{10} are readily released from the membrane preparation by mild treatments like elevation of the ionic strength or lowering the pH, raise the question of whether these proteins are really membrane proteins, or may be remnants of the soluble content of the granule. Proline-rich proteins, which are underestimated by the Lowry method, whose amino acid composition and peculiar staining properties on acrylamide gel are very similar to those of M_1 and M_{10} proteins, were reported to be present in parotid saliva [20, 21]. These secretory proteins are probably derived from the content of the secretory granules. In a study that will be published separately we show that the secretory granule content indeed includes some proline rich proteins (C_5, C_{11} and C_{12}), but that these differ in their mobility in sodium dodecylsulfate gels from the proteins which form the bands M_1 and M_{10}. Yet this segregation to membrane-bound and soluble, proline-rich proteins might not reflect the true situation in the intact secretory granule. Studies on isolated secretory granules of the pancreas have shown that washing of the lysed secretory granules under certain conditions effectively solubilize part of the proteins of the content of the granule while other proteins, which are without doubt secretory proteins (trypsinogen and lipase) remain insoluble [22]. On the other hand, the fact that the proteins which form the bands M_1 and M_{10} are readily detached from the membrane by mild treatments does not necessarily imply that they are not membrane components. Several membrane proteins, which were named 'peripheral membrane proteins' [23] are similarly found to be detached from the membrane by treatments which interfere with electrostatic bonds [24]. Such

peripheral proteins of the secretory granule membrane might be detached from the membrane in the secretion process after the exposure of the inner face of the membrane to the high ionic strength of the saliva which fills the lumen of the gland [25]. It is not impossible therefore that these proteins might be found as minor components in the parotid saliva.

If the proline-rich proteins are indeed part of the secretory granule membrane before secretion, one might expect that the secretory granules would lyse during treatments which cause the detachment of these proteins from the membrane. This indeed seems to be the case. The pH-dependence curve of the stability of the isolated secretory granules is very similar to the pH-dependence curve of the interaction between the proline-rich proteins and the membrane. In contrast with the secretory granules of the pancreas [22] the secretory granules of the parotid are quite stable in alkaline and neutral pH, but release their content at pH lower than 5.5 [26, 27].

Another similarity is the effect of isotonic salt solutions. As shown here isotonic solution of KCl releases the proline-rich proteins when it reaches the interior of the granule. (Membranes of granules which were incubated without prior lysis, in 0.15 M KCl solution still contain the proline-rich proteins.) Similarly, introduction of isotonic KCl into the interior of the granules by treating the granule with sulfhydryl blocking reagents results in lysis of the granule [28]. It therefore seems possible that interaction between the proline-rich proteins and the membrane does take place in the intact granule too and is important for the stability of the granule structure.

In contrast with the membraneous proline-rich proteins, the 'integral' [23] proteins of the secretory granule membrane are not found to be labeled to any extent comparable to that of the secretory granule content. A similar situation was reported with the membrane proteins of the secretory granules of the guinea pig pancreas [29] and with the chromaffin granules from the bovine adrenal medulla [30]. This difference between the secretory content and the membrane of the granule implies that the integral proteins of the membrane which envelop the secretory proteins were already present in the cell before the incorporation of the labeled amino acid into new proteins. Autoradiographic studies had shown that the labeled secretory proteins are concentrated in new secretory granules rather than being introduced randomly into mature old granules [31, 32]. The proteins of the granule membrane have therefore to be ready in the cell in some intermediate pool awaiting arrival of the newly synthesized exportable proteins. From what is known on the secretory cell it is most probable that this pool is in the Golgi complex. The fact that the proteins of the granule membranes are older than the proteins in the granule content can thus be explained by one of the following hypotheses.

I. The proteins in the pool are derived mostly from membranes of previously discharged granules by a mechanism of reutilization.

II. The only source of the membrane proteins in the pool is de novo synthesis but since the amount of proteins in this pool is much larger than the amount which gets labeled after a single injection of labeled amino acid, the labeled proteins undergo extensive dilution with unlabeled proteins and therefore only a small part of the label reaches the membrane of the new secretory granules at the time of the experiment.

III. Unlike the synthesis of the secretory proteins whose rate is only slightly changed throughout the secretory cycle of the gland [33] the synthesis and accumulation of the secretory granule membrane protein occurs only in a specific period

in this cycle (for instance, after the induction of secretion) and might therefore get labeled only in this period.

In order to be able to distinguish between these alternatives we should be able to track the secretory granule membrane proteins back into the intermediate pool and to follow them forth in the process of exocytosis and after it. As shown in this paper, the membrane of the secretory granule is composed of relatively few proteins which seem to be unique to this membrane. The presence of proteins which are specific to the secretory granule membrane was reported for the secretory granules of the pancreas too [19, 34]. It seems possible therefore that these proteins might form a tool by which the secretory granule membrane might be traced in the different transformations that it passes through in the cell.

ACKNOWLEDGEMENTS

We are grateful to Dr A. Loyter and Dr R. G. Kulka for reviewing this manuscript. We thank Miss Peggy Gairon from the Hadassah School of Medicine for performing the amino acid analyses.

This work was supported by a grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S.A. (No. 5 RO 1 AM 10451-08 BIO).

One of us (N.K.) was the recipient of U.S. Public Health Service Special Fellowship 1971-1972.

REFERENCES

1 Schramm, M. (1967) Annu. Rev. Biochem. 36, 307–320
2 Abrahams, S. J. and Holzman, E. (1973) J. Cell Biol. 56, 540–558
3 Lagunoff, D. (1973) J. Cell Biol. 57, 252–259
4 Satir, B., Schooley, C. and Satir, P. (1973) J. Cell Biol. 56, 153–176
5 Amsterdam, A., Ohad, I. and Schramm, M. (1969) J. Cell Biol. 41, 753–773
6 Palade, G. E. (1959) in Subcellular Particles (T. Hayashi, ed.), pp. 64–80, The Ronald Press Company, New York
7 Amsterdam, A., Schramm, M., Ohad, I., Salomon, Y. and Selinger, Z. (1971) J. Cell Biol. 50, 187–200
8 Heuser, J. E. and Reese, T. S. (1973) J. Cell Biol. 57, 315–344
9 Ceccarelli, G., Hurlbut, W. P. and Mauro, A. (1973) J. Cell Biol. 57, 499–524
10 Laemmli, U. K. (1970) Nature 227, 680–685
11 Kirshner, N., Wallach, D., Sharoni, Y. and Schramm, M. (1973) Anal. Biochem. 52, 589–594
12 Robinovitch, M. R., Sreebny, L. M. and Smuckler, E. A. (1968) J. Biol. Chem. 243, 3441–3446
13 Batzri, S., Selinger, Z., Schramm, M. and Robinovitch, M. R. (1973) J. Biol. Chem. 248, 361–368
14 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
15 Bernfeld, P. (1955) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. I, pp. 143–158
16 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
17 Loening, U. E. (1967) Biochem. J. 102, 251–257
18 Robinovitch, M. R., Keller, P. J., Iversen, J. and Kauffman, D. L. (1974) J. Dental Res. 53, 264
19 Meldolesi, J. and Cova, D. (1972) J. Cell Biol. 55, 1–18
20 Ellison, S. A. (1967) in Handbook of Physiology (Code, C. F., ed.), Section 6, Vol. II, pp. 531–559, American Physiological Society
21 Oppenheim, F. G., Hay, D. I. and Franzblat, C. (1971) Biochemistry 10, 4233–4238
22 Hokin, L. E. (1955) Biochim. Biophys. Acta 18, 379–388

23 Singer, S. J. and Nicolson, G. L. (1972) Science 175, 720–731
24 Rosenberg, S. A. and Guidotti, G. (1969) J. Biol. Chem. 244, 5118–5124
25 Mangos, J. A., McSherry, N. R. and Arvanitakis, S. N. (1973) in Fundamental Problems of Cystic Fibrosis and Related Diseases (Mangos, J. A. and Talamo, R. C., eds), pp. 221–228, Intercontinental Medical Book Corporation, New York
26 Ben-Zvi, R., Ph. D. thesis submitted to the Hebrew University of Jerusalem, 1965
27 Wallach, D. and Schramm, M. (1971) Eur. J. Biochem. 21, 433–437
28 Salomon, Y., Babad, H. and Schramm, M. (1968) Israel J. Chem. 6, 127p
29 Meldolesi, J. (1974) in Advances in Cytopharmacology, Vol. 2, in the press, Raven Press, New York
30 Winkler, H., Schöpf, J. A. L., Hörtnagl, H. and Hörtnagl, H. (1972) Naunyn-Schmiedeberg's Arch. Pharmacol. 273, 43–61
31 Jamieson, J. D. and Palade, G. E. (1967) J. Cell Biol. 34, 597–615
32 Castle, J. D., Jamieson, J. D. and Palade, G. E. (1972) J. Cell Biol. 53, 290–311
33 Sreebny, L. M., Johnson, D. A. and Robinovitch, M. R. (1971) J. Biol. Chem. 246, 3879–3884
34 MacDonald, R. J. and Ronzio, R. A. (1972) Biochem. Biophys. Res. Commun. 49, 377–382