COMPOSITION OF CELLULAR MEMBRANES
IN THE PANCREAS OF THE GUINEA PIG

IV. Polyacrylamide Gel Electrophoresis and
Amino Acid Composition of Membrane Proteins

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ABSTRACT
Two methods of polyacrylamide gel electrophoresis (the acid method of Eytan and Ohad and the Na dodecylsulfate (SDS) disc method of Maizel) have been used for analyzing the proteins of gel fractions isolated from the guinea pig pancreatic exocrine cells and in particular the proteins bound to the membranes involved in the synthesis, intracellular transport, and discharge of secretory enzymes: rough (RM) and smooth microsome (SM) membranes, zymogen granule (ZG) membranes, and plasma membranes (PM). Since in the two systems the electrophoretic mobility of proteins depends on different factors (size, shape, and net charge of molecules in the acid system; size only in the SDS system) a deeper insight into the protein composition of the fractions could be obtained. The gel patterns of RM, SM, and ZG membranes turned out to be accounted for mainly by segregated secretory enzymes (in rough microsomes also by ribosome proteins) and thus were found to share most of the bands. In contrast, with highly purified membrane fractions different patterns were obtained: RM and SM membrane proteins turn out to contain a large number of different proteins with molecular weights varying between ~150,000 and 15,000 daltons. The pattern of ZG membranes was greatly different in the two systems: only two bands were separated by the acid method and as many as 23 by the SDS method. PM gave a rather complex pattern in either system. Both ZG membranes and PM were found to contain a large proportion of low molecular weight proteins. Nothing appears in common between the proteins of SM membranes (primarily of Golgi origin) and those of ZG membranes, while the latter and PM exhibit a certain degree of similarity. By amino acid analysis we found only slight differences: relative to the other fractions: RM membranes were higher in basic amino acids and ZG membranes contained a larger amount of methionine. Taken together with recent data on lipid composition and enzyme activities of the same fractions, these results indicate that the membranes of the pancreatic exocrine cells are chemically and functionally distinct, and hence do not mix randomly with one another during the transport of secretory products.

1 Abbreviations used: RER, rough-surfaced endoplasmic reticulum; GC, Golgi complex; ZG, zymogen granule(s); RM, rough microsome(s); SM, smooth microsome(s); PM, plasma membrane; DOC, Na deoxycholate; TEMED, tetramethylenediamine; SDS, Na dodecylsulfate; RNA, ribonucleic acid; PLP, phospholipid; DFP, diisopropylfluorophosphate; TLCK, 1-chloro-3-tosylamido-7-amino heptanone; TPCK, N-tosyl-L-phenylalanyl-chloromethyl ketone; TCA, trichloroacetic acid.
The content of the latter is ultimately discharged in the glandular lumen by exocytosis (1-6).

Transfer of secretory proteins from one cell compartment to the next most probably requires repeated fusions and fissions of the limiting membranes (5, 6). Little information is available at the present time about such membrane interactions.

In previous papers we have reported that the membranes involved in the process, i.e., the membranes of rough microsomes (RM) (=RER), of smooth microsomes (SM) (primarily Golgi vesicles and cisternae), of ZG, and the plasma membranes (PM) can be isolated as highly purified fractions from homogenates of guinea pig pancreas (7, 8). The comparative study on the lipid composition (9) and enzymic activities (10) of these fractions led us to the conclusion that considerable differences exist among the various types of membranes, thus suggesting that they do not mix randomly with one another during the transport of secretory products.

In this paper the investigation was extended to membrane proteins, studied by electrophoresis on polyacrylamide gel as well as by amino acid analysis, the purpose being to find out whether, and to what extent, further differentiation exists among the various membranes under study.

**MATERIALS AND METHODS**

**Animals**

Male albino guinea pigs, weighing 500–600 g (gift of Sigurth Drug Co., Milan, Italy), were starved for 18–20 hr with water ad lib., then stunned by a blow over the head. Pancreases were quickly excised and immersed in ice-cold 0.3 M sucrose (Special Enzyme Grade from Mann Research Labs. Inc., New York).

**Cell Fractionation Procedures**

Details on the homogenization and on most of the cell fractionation procedures used are given in references 7 and 8.

**RM AND SM**: The procedure developed by Tartakoff in Palade's Laboratory has been used (11). Total microsomal pellets (obtained as in reference 7) were resuspended by hand in 1.3 M sucrose; 1.5 ml samples of this suspension were applied to a discontinuous gradient set up by layering in a Spinco SW 41 centrifuge tube the following solutions: 1.5 ml of 2 M sucrose; 3.5 ml of 1.35 M sucrose; 1.5 ml of microsomal suspension; 2 ml of 1.2 M sucrose; 0.3 M sucrose to volume. Centrifugation at 40,000 rpm for 5 hr yielded bands at the 0.3–1.2 M sucrose and 1.35–2 M sucrose interfaces. By chemical, enzymological, and morphological criteria these bands were found to be analogous with those separated by the original continuous gradient procedure of Jamieson and Palade (5), thus containing highly purified SM and RM (upper and lower band, respectively).

**RM AND SM MEMBRANES**: The procedure, which is described in detail in reference 8, involves, in sequence, incubation of RM and SM in 0.2 m KCl, 5 mm MgCl2, 0.5 mm puromycin (12), pH 6, for 4 hr at 4°C, recovery of partially purified membranes by density gradient centrifugation, and finally washing with 0.2 m NaHCO3 buffer, pH 7.8. The first step releases a subfraction (indicated hereafter as KCl-puromycin-released), which contains most of the ribosomes as well as a large proportion of the secretory proteins and absorbed cell sap proteins. By the second step we obtained a second subfraction, indicated as NaHCO3-extracted, which is accounted for nearly exclusively by secretory proteins, and a final pellet which contains highly purified membranes.

Partially purified RM and SM membranes can be obtained by simply washing RM and SM with 0.2 m NaHCO3 buffer, pH 7.8 (7). Such treatment releases the bulk of secretory proteins and absorbed cell sap proteins (7, 8); however, ~50% of the RNA is recovered in these membrane subfractions (7).

**ZG AND ZG MEMBRANES**: ZG have been isolated by differential centrifugation as described in reference 7. The fraction is known to be 95–98% pure. ZG membranes have been obtained by lysis of isolated ZG in 0.2 m NaHCO3 buffer, pH 7.8 and purification of the recovered membranes by gradient centrifugation (7).

**PM**: The PM fraction was isolated as described in reference 7. As discussed in detail previously, such a fraction contains substantial amounts of adherent fibrillar material arising from the basement membrane and the terminal web. In order to remove such a contamination, isolated plasmalemmal preparations were resuspended in Krebs-Ringer bicarbonate solution, pH 7.4, containing 0.05% collagenase (either crude or purified) and 0.1% hyaluronidase, and incubated at 37°C for 10 min. At the end of the incubation the preparations were diluted with ice-cold Krebs-Ringer bicarbonate solution and centrifuged at 48,000 rpm for 45 min in a Spinco 50 Ti rotor. The supernatant was discarded; the pellet was washed twice by resuspension in Krebs-Ringer bicarbonate solution and recentrifugation as described, to remove enzyme molecules adsorbed onto membrane fragments.

**RIBOSOMES**: Two isolation procedures have been used with identical results. In procedure No. 1, the KCl-puromycin-released fraction obtained during the purification of RM membranes (see above) was diluted with glass distilled water to a sucrose concentration of 0.3 M and the ribosomes were sedimented
by centrifugation at 48,000 rpm for 90 min in a Spinco 50 Ti rotor. In procedure No. 2, total microsomal pellets (7) were resuspended in 0.3 M sucrose and cleared with Na deoxycholate (DOC) (0.2%, final concentration [5]). Centrifugation of these preparations at 48,000 rpm for 90 min in a Spinco 50 Ti rotor yielded pellets of partially purified ribosomes which were carefully resuspended in 2 ml of 2 M sucrose, transferred to Spinco SW 41 tubes, overlayed to volume with 0.3 M sucrose, and centrifuged at 40,000 rpm for 90 min. The band at the 0.3–2 M sucrose interface was aspirated and discarded; the ring-shaped pellet was resuspended in the 2 M load, diluted with glass distilled water to 0.3 M sucrose, and centrifuged at 48,000 rpm for 90 min in a Spinco 50 Ti rotor. The ensuing pellets, containing ribosomes, were washed once with 0.5 M KCl–5 mM MgCl₂. By both methods preparations of highly purified ribosomes were obtained, as shown both by electron microscopy and by chemical analysis (RNA: protein = 0.9; phospholipid (PLP):protein = 0.006).

**GEL ELECTROPHORESIS:** Several polyacrylamide gel systems have been used (14–18). However in this paper we have included only the results obtained by two methods: the acid method of Eytan and Ohad (17), which is a recent modification of the procedure originally developed by Takayama et al. (14), and the Na dodecylsulfate (SDS) disc gel method in discontinuous buffer system described by Maizel (18). These procedures were found to have distinct advantages over the others, particularly with respect to reproducibility and resolution of the bands.

In preliminary experiments the cell fractions containing membranes were delipidated before electrophoresis by treatment with aqueous acetone as described by Fleischer et al. (19): 95–99% of the PLP were extracted by the treatment. Since, however, no distinct changes in the electrophoretograms appeared, delipidation was routinely omitted.

In the Eytan and Ohad acid method proteins were dissolved in a mixture of phenol, acetic acid, and water (4:2:1; v/v) containing 1% of the nonionic detergent Nonidet P-40 and 0.5% of B-mercaptoethanol. Polyacrylamide gels containing 7.5% acrylamide, 35% acetic acid, 5% urea, 0.2% Nonidet P-40, 0.2% bisacrylamide, 0.06% tetramethylenediamine (TEMED), and 0.37% of NH₄ persulfate were prepared in glass tubes with an internal diameter of 5 mm and a length of 75 mm and loaded with 50–150 µg of protein in 0.1 ml of solvent mixture containing 13% sucrose. 10% acetic acid was used as electrode buffer and electrophoresis was carried out at room temperature at 1.5 ma per tube for 4.5–5 hr. Gels were stained with 1% amido black in 7% acetic acid for 2–4 hr and the excess dye was removed electrophoretically.

In separate runs samples of the different preparations were mixed with 10 µg of ribonuclease and then applied to the gels. The rate of migration of the major bands of the preparations relative to the enzyme was thus estimated. Ribonuclease could not be included in the routine runs since it contains some aggregates that overlap the bands of the cell fractions.

For the SDS disc gel electrophoresis, the proteins were dissolved in a mixture containing: 1% SDS; 6 M Tris-phosphoric acid buffer, pH 6.7; 1% B-mercaptoethanol; 8% sucrose, and 1% bromophenol blue, the latter serving as a tracking dye in the electrophoretic run. Samples containing 20–150 µg of protein were heated for 2 min in boiling water before loading onto the gels. Two types of resolving gels differing in pore size were prepared by mixing the following chemicals to the concentrations specified below: acrylamide and bisacrylamide (either 10 and 0.33% or 7.5 and 0.25%, respectively); Tris-HCl buffer, pH 8.9 (0.375 M); SDS (0.1%); TEMED (0.05%); and NH₄ persulfate (either 0.025 or 0.05%).

The spacer gel was ~2 cm long and contained acrylamide 3%; bisacrylamide 0.09%; Tris-phosphoric acid buffer pH 6.7, 50 mM; SDS, 0.1%; TEMED 0.05%; NH₄ persulfate 0.1%. 50 mM Tris-glycine buffer, pH 8.5, was used as electrode buffer and electrophoresis was carried out at room temperature at 2 ma per tube for 4.5–5.5 hr. Gels were fixed overnight with 10% trichloroacetic acid (TCA) in 25% isopropanol, rinsed twice with large volumes of 10% acetic acid in 25% isopropanol, and stained with 0.05% Coomassie blue in 10% acetic acid–25% isopropanol. Excess dye was removed by soaking the gels in several changes of 10% acetic acid–10% isopropanol.

Calibration of the SDS gel system was carried out by running separately the following protein markers of known molecular weight: collagenase A (mol wt 103,000 (20); bovine serum albumin (mol wt 68,000 (21); ovalbumin (mol wt 43,000 (21); carboxypeptidase A (mol wt 34,000 (22); trypsin (mol wt 23,000 (21); ribonuclease (mol wt 14,000 (21); chymotrypsin C and B chains (mol wt 13,000 and 11,000, respectively (21). For both the 7.5 and the 10% gels the results obtained were analogous with those reported by Maizel (18). Densitometric tracings of stained gels were obtained with a Joyce-Loebl Chromoscan MK II Densitometer. In order to improve the resolution of the tracings of the specimen: record ratio was set at 1:3. Thus, all tracings shown in this paper are expanded 3-fold in width relative to the gels.
Amino Acid Analysis

Samples were hydrolyzed in 6 M HCl in sealed ampoules under vacuum at 110°C for 18 hr. The hydrolysate was dried and redissolved in 0.2 M citrate buffer, pH 2.2; the amino acids were analyzed in a Unichrom-Beckman automatic analyzer according to the method of Spackman et al. (23) as modified by Dévény (24).

Analytical Procedures

The following assays were used: protein, Lowry et al. (25); RNA, the orcinol procedure (26) on hot 5% TCA extracts of fractions. Lipids were extracted overnight with 20 vol of 2:1 chloroform-methanol at 4°C under nitrogen and purified according to Folch et al. (27). Lipid phosphorus was estimated as described by Ames (28).

Enzyme Assays

The assays used were: for trypsin and chymotrypsin, Hummel (29); for carboxypeptidase A, Folk and Schirmer (30). These assays were carried out both immediately after the isolation of the fraction and after activation of the zymogens by trypsin (30, 31). Lipase, α-amylase, and RNase were assayed as described previously (7, 9).

Electron Microscopy

The procedures used for the electron microscope study of membrane pellets are described in detail in reference 7.

Materials

The chemicals used were obtained from the sources indicated below: acrylamide, bisacrylamide, and TEMED, Eastman Kodak Co., Rochester, N.Y.; crude collagenase (type I), hyaluronidase (type I), ovalbumin (type V), benzoyl-L-tyrosine ethyl ester (TTEE), tosyl-L-arginine methyl ester (TAME), hypatryl-L-phenylalanine and SDS, Sigma Chemical Co., St. Louis, Mo.; ribonuclease (type R), purified collagenase (type CLSPA), carboxypeptidase A (type COA), trypsin (type TRL), and chymotrypsin (type CDI), Worthington Biochemical Corp., Freehold, N. J.; bovine serum albumin (fraction V): Pentex Biochemical, Kankakee, Ill.; amido black, Merck A. G., Darmstadt, Germany; Coomassie Brilliant Blue R 250, Colab Labs, Inc., Glenwood, Ill.; puromycin hydrochloride, Nutritional Biochemicals Corporation, Cleveland, Ohio; diisopropylfluorophosphate (DFP), British Drug House, Poole, England. 1-chloro-3-tosylamido-7-aminotennapane (TLCK), and N-tosyl-L-phenylalanlylcholoromethyl ketone (TPCK) were the kind gift of Dr. E. N. Shaw, Brookhaven National Laboratories, Upton, N. Y.

RESULTS

Isolation of Membrane Fractions

In previous studies we have developed procedures for the purification of rough and smooth microsome and ZG membranes. The purification was achieved by removing from both microsome fractions and ZG the nonmembrane components while leaving the structure of the membranes apparently unaffected (7, 8). Since the purification of membrane fractions is a necessary prerequisite to the work presented in this paper, we have summarized in Table I some of our previous

Table I

| Membranes | Protein | PLP | RNA | α-amylase | Chymotrypsinogen | RNase | Lipase | Adsorbed protein* |
|-----------|--------|-----|-----|-----------|------------------|-------|--------|-------------------|
| RM membranes | 29.8† | 70.8† | 4.6† | 1.3‡ | 0.5§ | 0.5§ | — | 2.5§ |
| SM membranes | 34.6‡ | 71.0‡ | 3.4‡ | 0.6‡ | 0.4§ | 0.7§ | — | 2§ |
| ZG membranes | 1.1‖ | 52.3‖ | — | 0‖ | 0‖ | — | — | 0.1‖ |

* Adsorbed protein is defined as the TCA-insoluble radioactivity recovered from the fractions isolated from nonradioactive pancreas tissue homogenated in radioactive postmicrosomal supernatant. The latter was obtained by centrifuging, at high speed, homogenates of pancreas slices labeled in vitro with 14C-L-leucine. For details see reference 8.

† Reference 8
‡ Reference 7.
§ Unpublished.

Values are expressed as percentages. 100% = the level found in RM, SM, and ZG, respectively.
FIGURE 1  Plasmalemmal fraction. The preparation consists primarily of large sheets of membrane either single, paired (p), or arranged in vacuoles (v). The membranes are usually well preserved but in some cases they exhibit a globular substructure (arrows). Single sheets are often associated with parallel sheets of basement membrane (bm, right); paired membranes are seen joined together by recognizable desmosomes (am) and are often in contact with fine fibrillar material (ff) originating from cytoplasmic filaments associated with the terminal web. The double arrow points to a contaminating rough microsomal vesicle. ×35,000.

FIGURE 2  Plasmalemmal fraction after collagenase plus hyaluronidase treatment. The fraction contains large empty vacuoles and membrane fragments with free edges. These at some points (arrows) appear continuous with a filamentous material which apparently consists of partially disrupted membranes. A recognizable occluding zonula is marked oz. × 65,000.
biochemical data as well as some recent results on this matter. It is evident that a large proportion of the PLP originally present in RM, SM, and ZG are recovered in the membrane fractions whereas the removal of nonmembrane components, i.e., ribosomes, secretory proteins, and adsorbed cell sap proteins, appears virtually complete. These conclusions are strengthened by the morphological study of the same fractions, reported in detail in references 7 and 8.

The PM fraction is known to contain sheets and fragments of membranes often joined together by recognizable tight junctions and desmosomes, with attached fibrillar material originating from the basement membrane and terminal web (7) (Fig. 1). In order to remove the contaminating fibrillar material, preparations of isolated PM were resuspended and treated with a mixture of collagenase and hyaluronidase. After digestion the fraction appears to contain primarily small vesicles and membrane pieces with free edges; recognizable isolated tight junctions are also seen, while most of the contaminating material is no longer evident (Fig. 2). Morphologically, the preparations digested with the purified collagenase were indistinguishable from those treated with the crude enzyme.

**Gel Electrophoresis**

The gel electrophoretograms of the isolated fractions and the corresponding densitometric tracings are shown in Figs. 3-10. In particular RM, SM, and their subfractions are shown in Figs. 3 and 4 (acid gels), 5 and 6 (10% SDS gels); ZG, PM, and their subfractions in Figs. 7 and 8 (acid gels), 9 and 10 (10% SDS gels). In order to obtain a direct comparison of the different gel patterns in the tracings, we have labeled with the same number the bands of analogous mobility.

The proteins of the RM fraction were resolved in 23 bands in the acid gel (Figs. 3 and 4A) and in

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3 This, of course, does not imply that the bands of different fractions, labeled by the same number, necessarily contain the same protein(s). For both acid and SDS gels the numbers are those used for labeling progressively the gel of RM.
FIGURE 4

A

B

C

D

E

F

G

H

I

RM membranes

SM membranes

RM; NaHCO₃-extracted proteins

SM; NaHCO₃-extracted proteins

RM; KCl-puromycin-released proteins

SM; KCl-puromycin-released proteins

Ribosomes

FIGURE 4
FIGURES 5 and 6. SDS disc gel system. Typical electrophoretograms (Fig. 5) and densitometric tracings (Fig. 6) of the proteins of rough microsomes, smooth microsomes, and subfractions isolated therefrom. Preparations isolated in five to eight separate experiments were analyzed in duplicate or triplicate.

A = RM, ~ 100 µg; B = RM, membrane subfraction, ~ 100 µg; C = RM, NaHCO₃-extracted subfraction, ~ 120 µg; D = RM, KCl-puromycin-released subfraction, ~ 150 µg; E = ribosomes, ~ 80 µg; F = SM, ~ 80 µg; G = SM, membrane subfraction, ~ 80 µg; H = SM, NaHCO₃-extracted subfraction, ~ 100 µg; I = SM, KCl-puromycin-released subfraction, ~ 150 µg; L = split gel: RM membranes (left) ~ 30 µg and SM membranes (right) ~ 20 µg.

more than 50 bands in the SDS gel (Figs. 5 and 6A). In the acid gel most of the fast-moving bands (Nos. 13-19) appear to be contributed predominantly by ribosomes. Hence, they are present in pure ribosome preparations (Figs. 3 and 4E) and in the KCl-puromycin-released subfraction (containing ribosomes, secretory, and adsorbed proteins (8)) (Figs. 3 and 4D) and absent from the subfractions containing either secretory proteins (NaHCO₃-extracted) (8) (Figs. 3 and 4C) or membranes (8) (Figs. 3 and 4B).

The pattern of distribution shown by bands 5-11, which are much more evident in the NaHCO₃-extracted and KCl-puromycin-released subfractions than in ribosomes and membranes, suggests that they are mainly accounted for by secretory proteins, with a minor ribosome contribution to bands 7, 8, 10, and 11. Band 12 is apparently contributed in similar proportion by secretory and ribosome proteins. The pattern of distribution shown by bands 5-11, which are much more evident in the NaHCO₃-extracted and KCl-puromycin-released subfractions than in ribosomes and membranes, suggests that they are mainly accounted for by secretory proteins, with a minor ribosome contribution to bands 7, 8, 10, and 11. Band 12 is apparently contributed in similar proportion by secretory and ribosome proteins. The pattern of distribution shown by bands 5-11, which are much more evident in the NaHCO₃-extracted and KCl-puromycin-released subfractions than in ribosomes and membranes, suggests that they are mainly accounted for by secretory proteins, with a minor ribosome contribution to bands 7, 8, 10, and 11. Band 12 is apparently contributed in similar proportion by secretory and ribosome proteins.

These criteria are sometimes insufficient for the identification of all the bands because (a) the release of secretory proteins during KCl-puromycin treatment of microsomes seems "nonparallel", i.e., some of these proteins (particularly those of low molecular weight) are apparently extracted more efficiently than others and therefore do not appear in the NaHCO₃ extract. This finding seems to correlate well with previous observations indicating that some enzymes, such as amylase, can be easily released from pancreatic particles (2, 32) whereas others, such as lipase, are more tightly bound (32-34); (b) as far as the adsorbed proteins present in the fractions are concerned, we do not know either their number or their actual amount.
Figure 6
FIGURE 7

FIGURES 7 and 8. Acid system of Eytan and Ohad. Typical electrophoretograms (Fig. 7) and densitometric tracings (Fig. 8) of zymogen granules, plasma membranes, and of the subfractions isolated therefrom. Preparations isolated in either 12 (ZG and ZG subfractions) or three (PM and PM subfractions) separate experiments were analyzed in duplicate or triplicate. A = ZG, ~150 µg; B = ZG, membrane subfraction, ~50 µg; C = ZG, NaHCO3-extracted subfraction, ~150 µg; D = PM, ~150 µg; E = PM after collagenase + hyaluronidase treatment, ~150 µg.

The membrane subfraction is less clearly defined although very reproducible. Proteins are spread rather evenly in the slow-moving region with visible peaks appearing in positions 2, 3, 5, 7, 8, and 10–11. By comparing such a pattern with that of total RM, we can tentatively suggest that the membranes contribute, in different proportion, to most of the slow-moving bands.

Also in the SDS gels (Figs. 5 and 6) the fast-moving bands (Nos. 38–51) appear predominantly contributed by ribosomal proteins, with sizable contributions by membrane (bands 38 and 42) as well as by secretory proteins (bands 47–48). Ribosomal proteins appear also to contribute to bands 23, 25, 28–34, and to predominate in band 31. Bands 9, 14, 20, 22, and 36 appear primarily accounted for by secretory proteins. At variance with the situation found with the acid gel the RM membranes (Figs. 5 and 6B) appear very well resolved in 40 different bands. In the RM pattern the RM membrane proteins appear to contribute substantially to bands 13, 36, 18, 19, 22, 25, 31, and 42–43.

Thus their identification in the gels is practically impossible; (c) the rate of migration of a protein can be influenced by the other proteins present in the preparation: for instance a minor component trailing a large band will be retarded and its mobility will increase upon removal of the latter. Some of these doubts have been clarified simply by comparing the gel patterns of RM, SM, and ZG, taking advantage of the fact that these fractions are extensively characterized, i.e., all fractions are known to contain secretory proteins, although in different proportion; ribosomes are abundant in RM, sparse in SM, and absent from ZG; there is much adsorbed protein in both microsome fractions and very little in ZG. Furthermore, possible changes of the rate of migration of single bands depending on the load have been investigated by running routinely two to four different samples of each preparation.

In the SDS gels of RM and SM as well as in those of the corresponding membrane subfractions (Figs. 5 and 6A, 6B, 6F, and 6G), band 1 is primarily ac-
By both electrophoretic techniques we found that the pattern of SM is very similar to that of RM. In the acid gels (Figs. 3 and 4F) the similarity is particularly evident in the region of the slow-moving bands 5–12 (corresponding to the secretory proteins). As expected, bands 13–19, identified as predominantly ribosomal, are much less evident than in RM. Also the pattern of SM membranes (Figs. 3 and 4G) is very similar to that described for RM membranes. Membranes seem to contribute to all slow-moving bands found in SM and particularly to bands 2, 3, and 4. Small peaks were found in positions 5, 7, 10, and 11.

In the SDS gels (Figs. 5 and 6F) the situation is analogous to that described with the acid gels. The bands of secretory proteins 9, 11, 14, 16, 20, 22, 25, 30, 34, and 36 are prominent and ribosomal bands are less evident than in RM. SM membranes (Figs. 5 and 6G) appear distinctly resolved in 37 bands. Some of these, i.e. 11, 14, 19, 22, 30,
FIGURES 9 and 10  SDS disc gel system. Typical electrophoretograms (Fig. 9) and densitometric tracings (Fig. 10) of the proteins of zymogen granules, plasma membranes, and of the subfractions isolated therefrom. Preparations isolated in either six (ZG and ZG subfractions) or three (PM and PM subfractions) separate experiments were analyzed in duplicate or triplicate. A = ZG, ~ 100 µg; B = ZG, membrane subfraction, ~ 80 µg; C = ZG, NaHCO₃-extracted subfraction, ~ 100 µg; D = PM, ~ 100 µg; E = PM after treatment with pure collagenase + hyaluronidase, ~ 100 µg; F = PM after treatment with crude collagenase + hyaluronidase, ~ 100 µg.

31, and 43 exhibit a mobility analogous to that found with RM membranes (Figs. 5 and 6B). The exact correspondence between the rate of migration of these bands in RM and SM membranes was also checked by means of split gels (Fig. 5L). However, some distinct differences also appear: hence, bands 2 and 8 are present in the membranes of SM and absent from those of RM; the opposite situation was found for bands 6-7, 17, 25, and 38. The comparison between the SM and SM membrane patterns suggests that membrane proteins are the major component of bands 15, 2, and 8 of the SM gel and give a sizable contribution to bands 11, 18, 19, 22, 28-31, and 42-43.

The procedure for the purification of microsome membranes involves a high ionic strength step which is known to result not only in the detachment of ribosomes and extraction of segregated proteins, but also in the solubilization of a small proportion of membrane protein (8). In order to estimate the influence of the latter process on the electrophoretic pattern of membranes, we studied gels of partially purified membrane subfractions, isolated by washing RM and SM with 0.2 M NaHCO₃ buffer, pH 7.8, without previous high salt-puromycin treatment. Such subfractions contain only trace amounts of secretory proteins; however, their RNA:protein ratio is as high as in the original microsome fractions (7). In both gel systems we found that the patterns given by the partially purified membrane subfractions were very similar to those of pure membranes except for the presence of some fast-moving bands which,
however, are probably due, at least in part, to ribosomal proteins (not shown). Hence, even if it is possible that fast-moving proteins are extracted by the high salt-puromycin treatment during membrane purification, a large scale removal seems unlikely. With this limitation in mind, the patterns of highly purified membranes shown in Figs. 3, 4, 5, 6B, and 6G, will be considered representative of RM and SM membrane proteins.

In both electrophoretic systems the pattern of ZG (Figs. 7-10A) is made up virtually exclusively by secretory proteins and is practically undistinguishable from that of the NaHCO3-extracted subfraction (Figs. 7-10C). This is not surprising since it is known that in ZG most of the proteins are secretory, with ZG membranes accounting for only 1–2% (Table I and reference 7). The gel pattern of the latter is much different and is also different from that found with RM and SM membranes. Hence, in the acid system the bulk of the protein is separated in the fast-moving band 21 and in a small band in position 18-19; very small bands were also found in positions 8, 10, and 11 (Figs. 7 and 8B). The SDS gel system revealed a much
larger complexity of the protein moiety of the ZG membrane, since 23 different bands were resolved (Figs. 9 and 10B). The major component appears to have a small molecular weight, running just behind the tracking dye; other major peaks were found in positions 21, 26, 27, 31, 35, and 36.

In one experiment we attempted to establish a correlation between the acid and the SDS patterns of ZG membranes. The major band of the acid gel (identified by briefly staining a gel electrophoresed in parallel) was eluted and rerun in the SDS system. For elution, gel slices containing ~50 µg of protein were squashed and soaked overnight at room temperature in 1% SDS, 6 mm Tris-phosphoric acid buffer, pH 6.7, 1% mercaptoethanol, and 0.1 mm DFP. The eluate was dialyzed against 0.1% SDS, 0.6 mm Tris-phosphoric acid buffer, 0.1 mm DFP, then lyophilized, redissolved, and applied to a 10% SDS gel. Nine bands appeared, the major of them being found in positions 20, 35, 36, 38, and 50. Even if it is impossible to draw any certain conclusion from this experiment (elution was probably incomplete; degradation of protein during elution and dialysis cannot be excluded), nevertheless it seems to be indicated that the fast-moving band of the acid gel is largely heterogeneous.

The acid gels obtained from the PM exhibit 19 discrete bands, with the major peaks appearing in positions 3, 5, 8, 9, 15, 17-18, and 20-21. After digestion with hyaluronidase + either crude or purified collagenase, we observed a disappearance of the major band (No. 8) and a decrease of bands 17-18.

In the SDS gels the bulk of the protein of the PM preparations separated into two large bands moving just behind the front. This suggests that they have a low molecular weight. Smaller peaks containing larger proteins were also present, the most important being found in positions 3, 12, 19, 24, 25, and 34 (Figs. 9 and 10D). Digestion with hyaluronidase + collagenase yielded relatively small changes. With both collagenase preparations a peak, most likely due to adsorption of hyaluronidase, appeared in position 30, while peak 24 was greatly reduced. The treatment with crude collagenase also resulted in the decrease of band 3 and in the appearance of a double peak in position 9.

Even if the interpretation of these data is open to a certain degree of uncertainty we can tentatively suggest that the bands which are unaffected by treatment with hyaluronidase + purified collagenase might be specific for the PM while the others might be accounted for, at least partially, by the proteins of the fibrillar material removed by the treatment.

### Amino Acid Composition

The amino acid composition of the membranes of RM, SM, and ZG is given in Table II. Only slight differences were found. Relative to the other fractions, RM membranes were slightly higher in basic amino acids (arginine, histidine, and lysine) whereas ZG membranes had a higher content of methionine and also of proline, serine, and threonine, and a slightly lower content of isoleucine and valine.

### Control Experiments

Since pancreatic homogenates are known to contain a great deal of proteolytic zymogens which are specifically concentrated in some of the cell fractions under study (RM, SM, and ZG), we considered the possibility that these zymogens

| Amino acid | RM | SM | ZG |
|------------|----|----|----|
| Alanine    | 5.32 | 5.60 | 5.53 |
| Arginine   | 7.69 | 6.58 | 6.81 |
| Aspartic acid | 9.22 | 10.77 | 8.66 |
| Glutamic acid | 11.97 | 12.48 | 9.87 |
| Glycine    | 4.52 | 4.50 | 5.28 |
| Histidine  | 4.19 | 3.95 | 2.86 |
| Isoleucine | 4.23 | 4.41 | 2.87 |
| Leucine    | 10.82 | 11.92 | 11.22 |
| Lysine     | 10.60 | 6.86 | 8.74 |
| Methionine | 0.76 | 0.79 | 2.91 |
| NH₃        | 2.24 | 2.01 | 3.14 |
| Phenylalanine | 5.76 | 6.42 | 5.80 |
| Proline    | 2.63 | 2.86 | 3.74 |
| Serine     | 6.21 | 5.89 | 8.56 |
| Threonine  | 3.12 | 3.54 | 2.78 |
| Tyrosine   | 3.95 | 4.12 | 6.26 |
| Valine     | 6.73 | 7.28 | 4.95 |

Aspartic and glutamic acids include asparagine and glutamine, respectively; tryptophane, cysteine, and cystine were not determined.
could become activated during the relatively long times needed for cell fractionation, thereupon causing artifactual degradation of the proteins of the fractions. Three different approaches have been used to investigate this problem. First of all, the activity of three pancreatic proteolytic enzymes, trypsin, chymotrypsin, and carboxypeptidase A, was assayed in all cell fractions. The results were clear-cut since in two separate experiments we could not find any activity in freshly isolated fractions, whereas after incubation at 4°C with trypsin we found high activity in RM, SM, and ZG as well as in all KCl-puromycin-released and NaHCO3-extracted subfractions.

In another series of experiments samples of the fractions were aged at +4°C for 24-48 hr before electrophoretic analysis. In most cases no changes were found in the electrophoretic pattern of the fractions; blurring of the bands and appearance of fast-moving components were seen only occasionally in KCl-puromycin-released and NaHCO3-extracted subfractions after 48 hr of aging. Other samples of the fractions were precipitated with ice-cold TCA immediately after their isolation, in order to minimize the danger of proteolysis and then analyzed in the SDS system (18). Again, no changes were observed.

Finally we studied fractions isolated from pancreatic homogenates prepared in the presence of protease inhibitors and maintained in the presence of inhibitors throughout the time of the isolation. In the presence of DFP (35) (0.5 mm) the fractionation of the pancreatic homogenate was severely upset and we were able to carry out the isolation of only a total microsome fraction and of its subfractions. In contrast homogenates prepared in the presence of the combination of two inhibitors, one specific for trypsin (TLCK, 1 mm) (36) and the other for chymotrypsin (TPCK, 1 mm) (37) can be successfully fractionated according to the usual scheme, yielding fractions morphologically and biochemically identical to their normal counterparts. When the proteins of these DFP and TLCK + TPCK fractions were analyzed by gel electrophoresis we found that in both systems their patterns were practically undistinguishable from those of the corresponding fractions isolated without inhibitors. Taken as a whole, these experiments indicate that in our experimental conditions proteolytic zymogens are not activated and, therefore, extensive digestion of cell proteins does not occur. Thus, the gel patterns of isolated fractions presented in this paper most likely are representative of the situation present in the intact cell structures.

**DISCUSSION**

Electrophoresis on polyacrylamide gel has recently been used by several investigators for analyzing comparatively the proteins of cellular membranes isolated from different sources (14-16, 38-46). However, in many of these studies fractions of pure membranes, such as PM, were compared with fractions containing large proportions of nonmembrane proteins, such as microsomes, mitochondria, and nuclei (40-42, 45, 46); in some others, either only some classes of these nonmembrane proteins were removed (15) or a direct demonstration of total extraction was not given (43); finally, in other studies the membranes analyzed were not interconnected functionally (39).

In the present paper, we have studied the proteins of purified membranes obtained from RM, SM, and ZG, and of the PM, isolated from the pancreas of the guinea pig. As demonstrated in this paper, the proteins of the various fractions under study contain only trace amounts of nonmembrane contaminants. In both of our systems the characteristic electrophoretograms of total RM, SM, and ZG turned out to be mainly due to nonmembrane proteins: secretory proteins, which, with minor changes in electrophoretic mobility, can be recognized in all these fractions.

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6 An exact quantitative description of the patterns of secretory proteins recovered in the different cell fractions is beyond the scope of this work and cannot be deduced from the data given in Figs. 3-10 since we did not take in account critically the possible role of several factors such as the extraction of some segregated enzymes during isolation of cell fractions and the exact balance between the amount of microsomal secretory proteins extracted by the KCl-puromycin treatment and that extracted by the NaHCO3 treatment.

Nevertheless, we have the impression that the electrophoretic pattern of secretory proteins of the various cell fractions is not identical in both the acid gel (increased importance of bands 8 and 11 and disappearance of band 9 from RM to ZG) (Figs. 3, A and 7, A) and the SDS gel (appearance of band 24 in ZG) (Fig. 9A). This could be the result of chemical changes of some of these proteins (glycosylation?) occurring during their intracellular transport.
and ribosome proteins, present in RM. Since it is likely that a similar situation would occur also with analogous fractions isolated from different sources, we believe that the quantitative removal of nonmembrane proteins should be considered a necessary prerequisite to the study of the gel pattern of membrane proteins. Furthermore, RM, SM, ZG membranes, and plasma membranes are functionally interconnected since they are known to bound the cell compartments involved in the synthesis, intracellular transport, and discharge of secretory enzymes (1-7, 9, 10). Studies on the composition of these membranes could therefore shed some light on the cellular mechanisms regulating the functional processes in which they are involved.

In our work the proteins of these membranes have been analyzed by means of two different polyacrylamide gel electrophoresis systems, the rationale being that the factors determining the relative electrophoretic mobility of proteins are not the same in the two systems. Hence, in acid gels the rate of migration of a protein depends on its molecular size, shape, and net charge, whereas in the SDS gel the rate of migration depends primarily on size only since the anionic detergent virtually eliminates conformational and charge density differences (18, 47). By the use of these two systems a much deeper insight into the composition of the protein moiety of pancreas cytoplasmic membranes could therefore be obtained.

In both gel systems the electrophoretograms of RM and SM appear similar in many respects: in the acid gel the proteins are spread rather evenly in the slow-moving region while in the SDS system they appear well resolved in a large number of bands distributed throughout the whole gel. These results clearly indicate that both microsomal membranes contain a large number of proteins having a molecular weight of between ~150,000 and 15,000 daltons. The complex protein composition of these membranes seems to correlate well with previous results indicating a large spectrum of membrane-bound enzyme activities in RM and SM isolated from different tissues (10, 48, 49). On the other hand, the observation that the RM and SM membrane gel patterns have several bands in common but also exhibit clear differences is in line with our previous results showing that these fractions share, in similar proportion, several enzyme activities; yet, several other enzymes were detected in only one of the two fractions and were absent from the other (10).

With ZG membranes the patterns obtained by the two electrophoretic methods were drastically different: only two bands in the acid system and as many as 23 bands in the SDS system. Even if drawing an exact correlation between these two patterns is impossible we have evidence that at least the major band of the acid gel is largely heterogeneous and accounts for many of the bands resolved by the SDS system. Thus, ZG membranes also seem to contain many proteins. However, relative to the microsomal membranes, their composition seems more simple and the molecular weight of their proteins distinctly smaller: the major component has a molecular weight of ~12,000 daltons, and all the others are smaller than 70,000 daltons. It should be noted that in our previous studies we found very little enzyme activity in ZG membranes (10) and that Winkler et al. (50) and Amsterdam et al. (51) found very simple gel patterns in the membrane proteins of secretory granules isolated from the adrenal medulla and the parotid gland. Also the amino acid composition of the membranes of secretory granules appears to be somewhat peculiar, as shown by the high concentration of proline in the salivary glands (51) and the sizable amount of methionine in the pancreas.

The finding that the gel pattern of ZG membranes does not have anything in common with that of SM membranes was unexpected since SM are primarily accounted for by GC vesicles and cisternae, and since morphological data, obtained with various cell systems, strongly suggest that secretory granules derive their membranes from those of the GC (2, 6, 52). Furthermore, our recent kinetic experiments with pancreas tissue slices incubated in vitro, in which membrane proteins were labeled with radioactive amino acids, suggest that ZG membranes are not synthetized as such but rather derive from preexisting structures (53). This point cannot be explained satisfactorily at present and deserves further inquiry.

By treating the isolated PM fraction with collagenase and hyaluronidase we have tried to identify which of the bands could be accounted for by PM proteins and which depend on the presence of contaminating fibrillar material (7). The indications obtained, however, are only tentative, due to the fact that the fibrillar material was not removed completely and that some of the changes observed are due to adsorption of the enzymes (particularly hyaluronidase) onto the membranes. Notwithstanding these limitations it should be
noted that the acid gel pattern of PM appears rather complex, being composed of 19 bands. In the SDS system the bulk of the PM protein turns out to have a low molecular weight, and several other larger components were also observed. These data seem in agreement, at least partially, with previous observations on PM fractions isolated from other sources (39, 40, 42-46, 54) as well as with their large spectrum of enzyme activities (10, 55).

In both electrophoretic systems we could detect a certain degree of similarity between ZG membranes and PM. This observation might be of interest since the granule membrane is known to fuse with and to be incorporated into the apical part of the PM during discharge of secretory proteins by exocytosis (2). However, the PM fraction contains portions of plasmalemma derived not only from the apex but also from the rest of the cell surface (7) which apparently is not connected functionally with the ZG membrane. Therefore, any conclusion relating the gel pattern of the ZG membrane to the PM is open to a large degree of uncertainty.

Taken as a whole together with our previous data on the lipid composition and enzyme activity (9, 10), our results demonstrate that a clear distinction can be made among the various types of cytoplasmic membranes of the guinea pig pancreas. Thus, RM and SM membranes have similar but not identical gel patterns and share some enzyme activities; however their lipid composition and the distribution of other enzymes are different (9, 10). SM and ZG membranes and PM have similar lipid composition (9). However, their enzyme activities overlap only partially (10) and their gel patterns are quite different.

These observations further support the conclusion previously put forth (9, 10) that the transport of secretory proteins from RER to the extracellular space through the GC and ZG must be carried out in vivo by a mechanism which does not lead to membrane mixing. In other words, the interactions of the different membranes should not be random, i.e., any membrane patch fusing with a membrane of different type is not definitely incorporated into the latter but is eventually removed, thus permitting each of the membranes involved in the secretory process to preserve its characteristic composition.

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