COMPOSITION OF CELLULAR MEMBRANES IN THE PANCREAS OF THE GUINEA PIG

I. Isolation of Membrane Fractions

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ABSTRACT
The subcellular components involved in the synthesis, transport, and discharge of secretory proteins in the guinea pig pancreatic exocrine cell have been isolated from gland homogenates by differential and gradient centrifugation. They include rough and smooth microsomes derived respectively from the rough endoplasmic reticulum and Golgi periphery, a zymogen granule fraction consisting mainly of mature zymogen granules and a smaller population of condensing vacuoles, and a plasmalemmal fraction. Membrane subfractions were obtained from the particulate components by treatment with mild (pH 7.8) alkaline buffers which extract the majority (>95%) of the content of secretory proteins, allowing the membranes to be recovered from the extracting fluid by centrifugation. The purity of the fractions was assessed by electron microscopy and by assaying marker enzymes for cross-contaminants. The rough and smooth microsomes were essentially free of mitochondrial contamination; the smooth microsomes contained <15% rough contaminants. The zymogen granule fraction and its derived membranes were free of rough microsomes and contained <3% contaminant mitochondria. The plasmalemmal fraction was heterogeneous as to origin (deriving from basal, lateral, and apical poles of the cell) and contained varying amounts of adherent fibrillar material arising from the basement membrane and terminal web. The lipid and enzymatic composition of the membrane fractions are described in the following reports.

INTRODUCTION
Studies carried out in our laboratory on the pancreatic acinar cell in relation to the synthesis, intracellular transport, and discharge of secretory proteins (1–6) have led to the general conclusion that secretory products are initially segregated within the cisternae of the rough-surfaced reticulum (RER)1 (4, 7), and subsequently transported to their site of discharge through a series of functionally interconnected membrane-bounded compartments. Specifically, this series involves, in sequence, the cisternae of the RER—including

1 The following abbreviations are used in this paper: BTEE, benzoyl-L-tyrosine ethyl ester; EDTA, ethylenediaminetetraacetate; ER, endoplasmic reticulum; PLP, phospholipid; RER, rough-surfaced endoplasmic reticulum; RNA, ribonucleic acid; TCA, trichloroacetic acid; Tris, (hydroxymethyl)aminomethane; ZG, zymogen granule.
its transitional elements, the small peripheral vesicles and the condensing vacuoles of the Golgi complex, and finally the zymogen granules (ZG), the last component representing the end point in the maturation process of condensing vacuoles (3, 5, 6). The content of these granules is ultimately discharged into the glandular lumina by exocytosis.

Since it is clear that the membranes bounding each compartment involved in transport play a central role in the process and since little is known about them at present, we decided to investigate some of their chemical and enzymatic properties.

In this paper we describe and evaluate procedures for the isolation of membrane fractions from homogenates of guinea pig pancreas, while in the companion articles, we will present data on the lipid composition and enzyme complement of these fractions.

**METHODS AND MATERIALS**

**Animals**

Male albino guinea pigs weighing 550-650 g, obtained from The Rockefeller University colony, were starved 18-20 hr with water given ad libitum, then stunned by a blow over the head. Their pancreases were rapidly removed and immersed in 0.3 M sucrose at 0°C.

**Cell Fractionation Procedures**

The isolation of cell fractions and their membranes will be described in detail under Results. Here we give only the two homogenization procedures used.

**ZYMOCEN GRANULES:** Pancreases of two to three animals were trimmed free of fat and mesentery, minced with scissors, and homogenized in 10 volumes (w/v) of 0.3 M sucrose (special enzyme grade from Mann Research Labs Inc., New York), using three strokes of a Teflon-glass Brendler-type homogenizer (Arthur H. Thomas Co., Philadelphia, Pa., type C, clearance 0.005-0.007 inch), driven by a motor at 3000 rpm.

**ROUGH AND SMOOTH MICROSOMES AND PLASMA MEMBRANES:** Pancreases of five animals were minced as above, passed through a stainless steel tissue press, and homogenized in 10 volumes (w/v) of 0.3 M sucrose by means of 30 strokes in a hand homogenizer fitted with a rubber pestle (8). The homogenate was filtered through 110-mesh nylon cloth.

All operations were carried out at 0°-4°C.

**Electron Microscopy**

Zymogen granule pellets were fixed for 2 hr in the centrifuge tube with 1% OsO₄ in either 0.3 M sucrose (unbuffered, pH ~5), or 0.1 M cacodylate, or phosphate buffer (pH 7.4).

Smooth and rough microsomes were fixed either as pellets with 1% OsO₄ in cacodylate buffer (0.1 M, pH 7.4) or in suspension by diluting the bands collected from sucrose gradients with 1% OsO₄ in water or in cacodylate buffer (0.1 M, pH 7.4). The final sucrose concentration was ~1 M for smooth microsomes and ~1.3 M for rough microsomes. The suspensions were centrifuged and the fixation of the pellets continued for 2 hr in the same OsO₄ solution.

Zymogen granule membranes, rough and smooth microsomal membranes, and plasma membranes were fixed as pellets with either 1% OsO₄ in cacodylate buffer (0.1 M, pH 7.4) or 2% glutaraldehyde, followed by 1% OsO₄, both in the same buffer.

In all cases, the OsO₄ fixation was followed by staining of the pellets in block with 0.5% Mg uranyl acetate (K & K Laboratories Inc., Plainview, N.Y.) in 0.9% NaCl.

After rapid dehydration, fine strips were obtained from each pellet by cutting perpendicularly to its surface; the strips were embedded in Epon (9) in such a way as to allow thin sections to be cut parallel to the axis of sedimentation through the entire depth of the pellet. The sections were doubly stained with uranyl acetate and lead citrate and examined in a Hitachi HU 11C, a Philips EM 300, or Siemens Elmiskop 1 electron microscope. In all cases, the entire depth of the pellets was systematically examined.

**Enzyme Assays**

The assays used were: for cytochrome c oxidase, Cooperstein and Lazarow (10); for succinate dehydrogenase, Arrigoni and Singer (11); for α-amylase, Bernfeld (12); for ribonuclease, Kalnitzky (13); and for chymotrypsin, Hummel (14), after overnight activation by trypsin (15).

**Analytical Procedures**

Lipids were extracted from homogenates or resuspended cell fractions with 20 volumes of chloroform-methanol 2:1 (v/v), and purified according to Folch et al. (16). Phospholipid phosphorus was determined according to Ames (17); values for phospholipid phosphorus were converted to phospholipid using a factor of 25.

All proteins were precipitated with cold 10% trichloroacetic acid (TCA) (final concentration). The precipitate was recovered by centrifugation, dissolved
in 1.0 M NaOH, and its protein content measured according to Lowry et al. (18), using bovine plasma albumin as standard. This procedure probably may lead to an underrecovery of glycoproteins but the error is expected to be small.

To determine RNA, portions of cell fractions were precipitated with cold 10% TCA (final concentration), washed three times with 5% TCA in the presence of bovine plasma albumin as coprecipitant, and extracted with 5% TCA at 90°C for 20 min; RNA was measured in the extract by the orcinol reaction (19) with purified (20) yeast RNA as standard.

**Materials**

The chemicals used were obtained from the sources indicated below: horse heart cytochrome c (grade III), phenazine methosulphate and commercial RNA (Sigma, grade VI), from Sigma Chemical Co., St. Louis, Mo.; trypsin, chymotrypsin, and benzoyl-L-tyrosine ethyl ester (BTEE), from Worthington Biochemical Corp., Freehold, N.J.; 2,6-dichlorophenolindophenol, from Calbiochem, Los Angeles, Calif. All other chemicals were reagent grade.

**RESULTS**

**Isolation and Characterization of Zymogen Granules**

**RATIONALE:** ZG fractions have been isolated from the pancreas of several mammalian species, e.g., the dog (21), ox (22), mouse (23), and guinea pig (5, 24). These fractions were contaminated to varying degrees, primarily by microsomes and mitochondria, as indicated by the presence of variable amounts of RNA (range 5 [21]-160 [24], µg/mg protein) and by unusually high phospholipid concentrations. In some cases, the type of contamination was established by the electron microscopical survey of the pellets (5, 22, 24).

Since in the present investigation we were interested in studying the membranes of zymogen granules, it was necessary to develop a procedure that yields a granule fraction reasonably free of the major contaminants noted above. Accordingly, our approach concentrated on purity at the expense of recovery. After the isolation of satisfactory fractions, the granules were lysed and their membranes recovered by floatation in a sucrose density gradient, which results in a further purification of the membranes.

**ISOLATION OF ZYMOGEN GRANULES:** 6-ml samples of a homogenate prepared as indicated under Methods were centrifuged in 12-ml glass conical tubes for 12 min at 1000 gmax in a swinging bucket International centrifuge (International Equipment Co., Needham Heights, Mass.) (size 2, model K, yoke No. 240). The supernatant was aspirated, the pellets were carefully re-suspended by means of a Teflon rod in 5 ml of 0.3 M sucrose, and the suspension was centrifuged at 180 gmax for 12 min to sediment cell debris, erythrocytes, nuclei, and plasma membranes. The resulting supernatant (~3 ml) was removed by means of a Pasteur pipette, filtered through 110-mesh nylon cloth, and transferred to round-bottomed 15-ml Corex tubes which were centrifuged for 3.5 min at 1000gmax (same centrifuge) to yield a tightly packed white pellet of zymogen granules, covered by a loose tan layer rich in mitochondria. This top layer was removed by swirling the tube twice with 1 ml of 0.3 M sucrose. Rinsing removed ~55% of the proteins, ~40% of the chymotrypsin, and >95% of the cytochrome c oxidase and succinate dehydrogenase activities of the original pellet. Zymogen granule recovery could be increased by resuspending the pellet of the 180 g X 12 min centrifugation in 3 ml of 0.3 M sucrose and repeating the isolation procedure.

**MORPHOLOGY AND BIOCHEMISTRY OF ISOLATED ZYMOGEN GRANULES:** A representative low-power view of the bottom two-thirds of a zymogen granule pellet prepared as above is shown in Fig. 1. More than 90% of the components are large electron-opaque, spherical zymogen granules, and ~5% are condensing vacuoles identifiable in the pellet as in the cell by virtue of their scalloped, somewhat irregular profiles. Such vacuoles are more frequent in the upper one-third of the pellet. About 2% of all particles comprising the fraction are mitochondria; their frequency increases toward the top of the pellet, consistent with the gross stratification seen prior to rinsing. A few intracisternal granules still contained within rough-surfaced membranes (25) are also occasionally seen.

At higher magnification the zymogen granules and condensing vacuoles are bounded by a single unit membrane ~90 A thick in normal sections (Fig. 4 a). When fixed in unbuffered 1% OsO4-0.3 M sucrose, most granules have a well preserved content and an intact limiting membrane; some of them, however, show blebs, or myelin figures protruding from their surface (Fig. 3). In addi-
tion, membrane pieces, apparently peeled off from damaged granules (Figs. 1 and 2), and granule "ghosts" partially or totally devoid of content are seen. After fixation in OsO4 in either cacodylate or phosphate buffer, many more damaged granules are found and even those apparently well preserved have limiting membranes affected by multiple interruptions, or focal losses in density (Fig. 4 b).

The ZG fraction accounts for ~3.3 mg protein/g wet tissue, contains only traces of RNA, and has a consistently low phospholipid/protein ratio (Table I). As expected from findings on other species (21, 22), the fraction also contains digestive enzymes in higher concentration than the starting homogenate: specific activity figures calculated on a protein basis (SApr)2 show, for instance, 2.4- and 5.5-fold increases in concentration for α-amylase and chymotrypsin, respectively. The apparently greater enrichment in chymotrypsin activity relative to amylase probably is due either to incomplete chymotrypsinogen activation by trypsin in the total homogenate, or to preferential loss of amylase from granules (21), rather than to an actually higher concentration of the proenzyme in zymogen granules.

The SApr's of the ZG fraction for mitochondrial enzymes are extremely low: 2.4-3% of the SApr's of a crude mitochondria fraction (Table I). Expressed on a phospholipid basis (SApl), they appear to represent ~20-25% of the corresponding value for the mitochondrial fraction. The latter is, however, highly contaminated by microsomes (note the large RNA and digestive enzyme content of the fraction), which may account for 50% of its particles. Hence, the actual contamination of the granule fraction by mitochondria on a phospholipid basis is likely to be <10%.

**EXTRACTION OF GRANULE CONTENT AND ISOLATION OF MEMBRANES:** To extract secretory proteins contained in granules, we took advantage of the finding that isolated zymogen granules (21, 22) release their contents upon suspension in isotonic buffers at a pH >7.2. Hence, we resuspended the ZG fraction in 0.17 M NaCl (~8 mg of protein/3 ml) and diluted the suspension with 7 ml of 0.2 M Na bicarbonate, pH 7.8. In some experiments, 0.2 M Tris-HCl buffer (pH 7.8) was used instead of bicarbonate, with similar results. The treated suspension was immediately centrifuged for 30 min at 30,000 rpm (Spinco No. 50 rotor), which yielded a pellet (labeled ZG pellet I) containing the membranes of the granules, a few unextracted granules, and the majority of contaminating mitochondria. The bulk of the proteins, including nearly all the α-amylase and chymotrypsinogen, were recovered in the supernate (Table II).

To separate zymogen granule membranes from mitochondria and unextracted granules, the ZG pellet I was carefully resuspended in 3 ml of 1.0 M sucrose, placed in a Spinco SW 39 tube (Beckman Instruments, Inc., Fullerton, Calif.), and overlaid with 0.5 ml of 0.3 M sucrose. Centrifugation at 39,000 rpm for 60 min yielded a band at the interface, and a pellet (ZG pellet II), which accounted for the bulk of the mitochondria and unextracted granules as shown by its morphology and by its high cytochrome c oxidase and chymotrypsin activities (Table III).

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**Figure 1** Zymogen granule fraction. This representative field in a sectioned pellet shows that the fraction primarily consists of zymogen granules (z) recognizable by the circular shape of their profiles and the homogeneity of their dense content. Differences in diameter reflect differences in granule size as well as differences in the position of the section close to the center (z1) or to the periphery (z2) of the granules. The limiting membrane of most zymogen granules is apparently unchanged (z3); in some cases it has formed tubular (z4) or rounded (z5) myelin figures. The thin membranes seen among the granules are tubule-like (m1) or bubble-like (m2) myelin figures whose insertion on zymogen granules was missed by the section. Note that dense thickenings appear only in myelin figures (arrow), not in zymogen granule membranes. A few condensing vacuoles (cv), recognizable by their irregular or angular profiles, are present in this field which also demonstrates the few contaminants, e.g., intracisternal granules (ic) and mitochondria (mt) found in zymogen granule fractions. X 12,000.
FIGURE 2  Zymogen granule fraction. This figure illustrates, at a higher magnification, the limiting membranes (lm) of the zymogen granules, the tubule-like ($m_1$) or bubble-like ($m_2$) myelin figures formed at their expense, and the difference between the circular profiles of zymogen granules (z) and the festooned profile of a condensing vacuole (cv) near full conversion into a zymogen granule. × 30,000.

FIGURE 3  Zymogen granule fraction. Pellet fixed in 1% OsO$_4$ in 0.1 M PO$_4$ buffer (pH 7.4). The micrograph demonstrates the uneven thickness of the limiting membrane (lm) and the formation of myelin figures (m) at its expense. z, zymogen granules. × 90,000.
Figure 4  Limiting membranes of isolated zymogen granules (Figs. 4a and 4b) and of zymogen granule ghosts (Figs. 4c and 4d). Note the regular layered structure of the membrane (lm) fixed in 1% OsO₄ in 0.3 M sucrose, the focal thickenings (arrows) and thinnings of the membranes fixed in 1% OsO₄ in 0.15 M phosphate buffer, pH 7, the layered structure of the ghost membrane (lm) (4c) (4d), and its rupture in Fig. 4d (arrow). Fig. 4a, X 162,000; Figs. 4b and c, X 167,000; Fig. 4d, X 175,000.

The band, which contained the zymogen granule membranes, was collected, diluted with distilled water to 0.3 M sucrose, and centrifuged at 50,000 rpm for 60 min in the Spinco No. 50 rotor. This pellet is referred to hereafter as ZG membrane fraction or, for convenience, as “ZG membranes.” As shown by the representative field in Fig. 5, it exclusively consists of membranes, often in the form of empty vacuoles of the size expected for zymogen granule ghosts. Smaller empty vesicles are numerous, however, especially at the top of the pellet; sometimes they appear to be contained within, or continuous with, the ghosts. The small vesicles are probably the result of the fragmentation of the ghosts during the extraction procedure. The limiting membranes of vacuoles and vesicles have the usual unit membrane structure (Fig. 4c, d), and quite often appear discontinuous. The pellet contains no mitochondria or other recognizable contaminants; it comprises, however, in its bottom layers a small amount of flat membrane sheets, with free edges.

As shown in Table III, ZG membranes are rich in phospholipids (phospholipid/protein ratio ≈0.5) and are apparently free of granule content as shown by the absence of chymotrypsin activity. The final purification step eliminated a further two-thirds of the cytochrome oxidase activity reducing the mitochondrial contamination by twofold (on a phospholipid basis) compared to the initial ZG fraction.

Isolation and Characterization of Rough and Smooth Microsomes

The isolation of rough and smooth microsomes was carried out as described by Jamieson and Palade (5), with minor modifications. The
### Table I

**Grass Chemistry and Enzyme Activities of Total Homogenates, Zymogen Granule, and Mitochondrial Fractions Prepared from Guinea Pig Pancreas**

|                     | Protein | RNA | PLP | α-amylase* | Chymotrypsin* | Cytochrome oxidase* | Succinate dehydrogenase* |
|---------------------|---------|-----|-----|------------|---------------|---------------------|-------------------------|
|                     | mg/g tissue wet wt | μg/mg protein | μg/mg protein | mg/mg protein | μmoles/mg protein | μmoles/mg protein | μmoles/mg PLP |
| Total homogenate    | 144.0 (20) | 55.0 (2) | 77.1 (4) | 6.0 (3) | 3.6 (2) | 16.4 (10) | 8.3 (5) |
| 126.0–162.0         | 50.0–60.0 | 71.0–86.0 | 5.8–6.2 | 3.3–3.9 | 12.7–22.4 | 6.9–9.5 |
| ZG fraction         | 3.3 (16) | 3.5 (10) | 13.5 (10) | 14.5 (3) | 20.1 (8) | 2.9 (10) | 195.0 (7) |
| 2.2–4.5             | 0.5–10.0 | 9.0–19.0 | 10.4–17.5 | 16.5–23.1 | 1.0–4.6 | 170.0–280.0 | 2.0–6.0 | 152.0 (4) |
| Mitochondrial       | 2.0 (9) | 108 (1) | 114.0 (5) | 2.9 (1) | 3.4 (3) | 118.0 (6) | 992.0 (6) |
| fraction†           | 1.3–2.6 | 90.0–140.0 | 1.6–4.8 | 100.0–155.0 | 890.0–1030.0 | 51.4–88.0 | 430.0–712.0 |

Values given are averages. Number of experiments is shown in parentheses. Ranges are in italics.

* The figures represent: amylase, mg maltose released/min at 30°C; chymotrypsin, μmoles BTEE split/min at 25°C; cytochrome oxidase, μmoles cytochrome c oxidized/min at 25°C; succinate dehydrogenase, μmoles succinate oxidized/min at 25°C; the values are normalized per mg protein or mg phospholipid (PLP) as indicated.

† The mitochondrial fraction was isolated by different centrifugation according to Jamieson and Palade (6) and is contaminated by both microsomes and zymogen granules.
TABLE II
Per Cent Distribution of Protein, Phospholipid, and Enzymes among Subfractions Obtained upon Alkaline Treatment of Isolated Zymogen Granules

|                 | Protein | Phospholipid | α-amylase | Chymotrypsin | Cytochrome oxidase |
|-----------------|---------|--------------|-----------|--------------|-------------------|
| ZG fraction     | 100     | 100          | 100       | 100          | 100               |
| ZG pellet I (membranes) | 5.9 (4) | 78.0 (12)    | 1.2 (3)   | 1.1 (4)      | 75.0 (3)          |
|                 | 5.1-7.2 | 62.0-87.0    | 0.8-2.0   | 0.4-1.5      | 70.0-78.0         |
| ZG supernate (content) | 91.8 (5) | 9.3 (12)     | 98.7 (3)  | 99.6 (5)     | 0 (3)             |
|                 | 84.0-97.5 | 3.9-18.0     | 96.0-102.0| 96.0-103.0   |                   |
| Recovery        | 97.8    | 87.3         | 99.9      | 100.6        | 75.0              |

Values given are averages. Number of experiments is shown in parentheses. Ranges are shown in italics.

TABLE III
Distribution of Protein, Phospholipid, and Enzyme Activities among Subfractions Obtained by Gradient Centrifugation of ZG Pellet I*

|                 | Protein | PLP | Cytochrome oxidase† | Chymotrypsin† |
|-----------------|---------|-----|---------------------|--------------|
|                 | %       | µg/mg protein | % mülules/mg PLP | % µmules/mg protein |
| ZG membranes    | 18.3 (6) | 67.0 (11) | 460.0 (5) | 32.0 (8) | 97.0 (10) | 0 | 0 |
|                 | 9.0-33.0 | 58.0-85.0 | 375.0-570.0 | 16.0-35.0 | 53.0-136.0 |
| ZG pellet II    | 81.7 (6) | 33.0 (11) | 60.5 (5) | 68.0 (8) | 502.0 (9) | 100.0 | 2.3 |
|                 | 66.0-91.0 | 15.0-42.0 | 42.0-101.0 | 45.0-84.0 | 278.0-770.0 |

Values given are averages. Number of experiments is shown in parentheses. Ranges are shown in italics.

* Since in these experiments recovery was not evaluated systematically, data expressed as percentage refer to the total material recovered from the gradient rather than to material applied thereon. When determined, the recovery of protein and PLP was ~70%.
‡ See Table I.

Homogenate, obtained as described under methods, was centrifuged at 1000 g_m for 12 min in an International centrifuge, size 2, model K; yoke No. 240. The ensuing pellet was used for the isolation of plasma membranes while the supernatant was collected and centrifuged at 12,000 rpm for 15 min in the Spinco No. 40.3 rotor. The upper two-thirds of the resulting supernatant was removed with a Pasteur pipette and centrifuged at 40,000 rpm for 60 min in the same rotor to sediment a total microsomal fraction. The new supernatant was discarded and the microsomal pellet resuspended in 0.3 M sucrose by means of a Teflon pestle to a concentration of ~6 mg protein/ml. Samples of 0.2-0.3 ml of this suspension were layered in Spinco SW 39 tubes on top of a 5 ml linear sucrose density gradient ranging from 1.04 to 2.0 M, and the tubes were centrifuged for 5 hr at 38,000 rpm. Of the three major bands only the upper and the lower were collected, diluted with distilled water to 0.3 M sucrose, and centrifuged for 60 min at 50,000 rpm in the Spinco No. 50 rotor; the resulting pellets were either fixed in situ for electron microscopy or resuspended in distilled water for biochemical assays. Some bands were fixed in suspension as described under Methods.

MORPHOLOGY AND BIOCHEMISTRY OF ROUGH AND SMOOTH MICROSOMAL FRACTIONS: The morphology and the biochemistry of rough and smooth microsomal fractions do not differ significantly from those described previously.
Figure 5 Pellet of zymogen granule membranes. The granule ghosts retain a closed circular profile, hence a spherical, possibly closed form (g1), or are distorted by flattening (g2). Some are torn open (g3), and others appear in grazing section (g4). The small vesicles (v) and small pieces of membranes (f) seen among and sometimes within the ghosts, probably represent ghost membrane fragments. × 46,000.
by Jamieson and Palade (5, 6). The rough microsomal fraction is homogeneous throughout the whole depth of the pellet and consists of vesicles of various sizes, each bounded by a unit membrane carrying attached ribosomes, and each containing a finely fibrillar material (Fig. 6). The smooth microsomal fraction primarily consists of small, spherical vesicles, with an admixture of small flattened cisternae and larger vesicles all bounded by a unit membrane. A filamentous content is usually present within these vesicles (Fig. 7). The lower one-fourth of the pellet contains, in addition, large flat pieces of membranes with free edges as well as a few contaminating rough microsomes. When rough and smooth microsomes are fixed in suspension in buffered OsO₄ most of the vesicles appear broken and devoid of content. In contrast, when fixed in suspension with unbuffered OsO₄ in concentrated sucrose, the microsomes are better preserved: their membranes appear intact and their filamentous content is retained (Figs. 6 and 7) often in compacted form (Fig. 7). Small dense droplets are frequently associated with both rough (Fig. 6) and smooth (Fig. 7) microsomal vesicles and appear to be either continuous with, or embedded in, their unit membranes (Fig. 8). The significance of this observation will be discussed in the companion paper which deals with the lipid composition of pancreatic membranes.

The gross chemistry and enzyme activity of total, rough, and smooth microsomes are shown in Table IV. Since the isolation procedure was developed to enhance purity at the expense of yield, protein recovery is low, especially for rough microsomes. The rough microsomes have a much lower phospholipid/protein ratio than smooth microsomes, but their RNA content is more than sevenfold higher.

Both fractions are virtually free of mitochondrial contamination, as shown by their extremely low cytochrome c oxidase activities. Both contain α-amylase and ribonuclease, two enzymes which from work on other species, as well as on the guinea pig (4, 24), are known to be contained within microsomal vesicles. The S₅₀₀ of these enzymes is approximately the same in both fractions, and is similar to that of the homogenate.

**Extraction of Content from Smooth and Rough Microsomes and Isolation of Membranes:** The content of microsomes and microsomal subfractions, like that of zymogen granules, can be extracted with isotonic alkaline NaCl-NaHCO₃. Isolated rough and smooth microsomes were, therefore, resuspended by means of a Teflon pestle in 3 ml of 0.17 M NaCl, diluted with 7 ml of 0.2 M NaHCO₃, pH 7.8, and immediately centrifuged at 50,000 rpm for 60 min (Spinco No. 50 rotor). The pellets so formed will be referred to as rough and smooth microsomal membranes, the corresponding supernatants being designated rough and smooth microsomal contents, respectively.

As shown in Figs. 9 and 10, the pellets contain only membranes, usually organized in closed vesicles, which are bounded by unit membranes and have no visible content. All rough vesicles appear stripped of recognizable ribosomes; their outer surface is covered, however, by a fine fibrillar material which could be the result of partial ribosomal degradation. Dense, membrane-associated droplets are less frequent in smooth microsomal membranes than in unextracted smooth microsomes; in rough microsomal membranes they are practically absent.

Table V gives the distribution of chemical constituents and enzyme activities among pellets (membranes) and supernatants (contents). About 50% of the proteins and nearly all phospholipids are recovered in the membrane pellets. The rest of the proteins, and nearly all the activity of the segregated enzymes (assayed for α-amylase and RNase) are found in the supernatants, along with ~50% of the RNA of rough microsomes.

We can conclude that alkaline treatment extracts practically all the content of rough and smooth microsomes and detaches a significant number of ribosomes from rough-surfaced membranes, thereby making possible the isolation of partly purified membranes. Rough microsomal membranes have a phospholipid/protein ratio of ~0.19; after correction for the presence of ribosomal protein it rises to ~0.28. Smooth microsomal membranes have a higher phospholipid/protein ratio of ~0.46, which is close to that found in zymogen granule membranes. About 35% of the proteins of the original rough microsomal fraction appear to be membrane

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3 For convenience only. In fact, this supernatant also contains ~50% of the degraded ribosomes from rough microsomes as well as degraded or fragmented membranes.
Isolation and Characterization of Plasma Membrane

RATIONALE: Plasma membrane fractions previously have been isolated from tissues, such as liver (for a review see reference 26 and also references 27–31), kidney (32), intestine (32, 33), bladder (34), as well as from single cells, such as those of HeLa (35) and L (36) cell cultures, and Ehrlich ascites tumors (37), but most of the published work concerns cell membrane fractions prepared from liver. With few exceptions, the methods designed to isolate enzymatically active fractions are derived from the procedure originally employed by Neville (27). This involves gentle homogenization of the tissue to avoid extensive fragmentation of plasma membranes, repeated low-speed centrifugation of the homogenate to sediment plasma membranes along with nuclei and cell debris, separation of plasma membranes from the other components of the nuclear pellet by gradient centrifugation, and purification of membranes either by repeated washing or by further gradient centrifugation.

For the pancreas we followed the same general procedure, but the results were less satisfactory than for liver for the following reasons: First, as will be discussed later, fibrillar material firmly adheres to the fragments of plasma membrane thereby increasing their density and making the separation from contaminating organelles by density gradient floatation much less effective. Second, we could not use divalent cations in the extraction fluids to prevent leakage of the nuclear DNA (26, 38) (which leads to gel formation and extensive contamination of fractions derived from the original nuclear pellet) because they promoted particle aggregation leading to losses associated (after correcting for ribosomes); the corresponding figure for smooth microsomes is \( \sim 50\% \).

Figure 6 Rough microsome fraction. The fraction primarily consists of rough microsomes of varied size and content density. Most of them have a homogeneous content of moderate density (\( m_1 \)), some are partly extracted (\( m_4 \)), and finally, a few appear swollen and almost completely extracted (\( m_6 \)) probably on account of damage incurred during tissue homogenization. A few rough microsomes show highly dense thickening of their limiting membranes (arrows); such appearances are more frequently encountered in the upper quarter of the pellets. The fraction contains a few contaminating particles tentatively identified as damaged mitochondria (\( mt \)) (outer membrane probably lost). \( \times 43,000 \).

Figure 7 Smooth microsome fraction. The fraction primarily consists of smooth microsomes with a small percentage of partly rough and partly smooth vesicles (\( r \). For most smooth microsomes the content is denser (\( m_1 \)) than for rough microsomes (Fig. 6), but partially (\( m_2 \)) or fully (\( m_3 \)) extracted vesicles are also seen scattered throughout the field. The arrows indicate focal thickenings of the limiting microsomal membranes similar to those seen in rough microsomes. \( \times 43,000 \).
of plasma membrane fragments to the original nuclear pellet. And finally, ethylenediaminetetraacetate (EDTA) could not be used to wash the fraction because in the case of the pancreas it causes a much more rapid and extensive fragmentation of the isolated plasma membranes than in the case of the liver (26, 38).

The final procedure employed was as follows:

**ISOLATION OF PLASMA MEMBRANES:** 6-ml samples of total homogenates, obtained as described under Methods, were centrifuged in 12-ml glass conical tubes at 1000g max for 12 min in an International centrifuge, size 2 (model K, yoke No. 240). The supernatant was discarded and the pellet was washed twice with 0.3 M sucrose and once with 1 ml of 0.17 M NaCl plus 3 ml of 0.2 M NaHCO₃, pH 7.8. Under the sedimentation conditions used, the latter treatment which releases the content of both microsomes and zymogen granules, is critical for reducing the contamination of the fraction to an acceptable degree.

**MORPHOLOGY AND BIOCHEMISTRY OF PLASMA MEMBRANE FRACTION:** The morphology of the plasma membrane fraction is shown in Figs. 11a and 12. Most of the membranes appear as packed sheets intermingled with layers or masses of fibrillar material. The membranes have the usual unit membrane structure (Fig. 13), measure ~100 Å in thickness, and occur either as relatively large single sheets with free margins, as large vacuoles, or finally, as paired sheets held together by recognizable junctional elements, such as occluding zonules and desmosomes. The latter, however, are partly disorganized usually by loss of their intercellular plaques (Figs. 11b and 12).

The fibrillar material mentioned above appears to derive from two different sources: in the lower one-fourth of the pellet it consists of fragments of basement membrane layers which apparently firmly adhere to pieces of basal plasma membrane (Fig. 11a); in the rest of the pellet, it is represented by bundles or masses of fibrils occasionally associated with recognizable desmo-

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**Table IV**

| Protein | PLP | RNA | Cytochrome oxidase* | α-amylase* | RNase |
|---------|-----|-----|---------------------|------------|-------|
|         | µg/g tissue | µg/mg protein | µg/mg protein | mµmoles/mg protein | mg/mg protein |
| **Total microsomes** | 106.0 (5) | 262.0 (2) | 5.4 (2) | 55.4 (2) |
| | 87.0-120.0 | 254.0-270.0 | 0-1.25 (5) | 5.0-5.8 | 55.0-55.8 |
| **Rough microsomes** | 9.0 (4) | 98.6 (5) | 361.0 (3) | 5.9 (2) | 61.2 (2) |
| | 7.5-11.0 | 87.0-106.0 | 340.0-380.0 | 0-1.0 (5) | 5.0-6.8 | 57.4-65.0 |
| **Smooth microsomes** | 6.0 (4) | 248.0 (3) | 52.0 (3) | 6.7 (2) | 49.0 (2) |
| | 4.6-7.2 | 180.0-305.0 | 33.0-80.0 | 0-1.1 (5) | 6.3-7.1 | 46.0-52.0 |

Values given are averages. Number of experiments is shown in parentheses. Ranges are shown in italics.

* The figures represent: cytochrome oxidase, mµmoles cytochrome c oxidized/min at 25°C; α-amylase, mg maltose/min at 30°C, both per mg protein.

† 1 unit of RNase activity is equivalent to the activity of 1 µg of bovine pancreatic RNase.

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FIGURE 9 Rough microsomal membranes. The fraction consists of empty (v1), distorted (v2), and frequently ruptured (v3) microsomal vesicles (ghosts). Their limiting membranes have a fuzzy outer aspect which only in places still bear ribosomal remnants (r). The arrows point to membrane fragments “exploded” by the distention of their middle layer. $\times 60,000$.

FIGURE 10 Smooth microsomal membranes. The fraction contains swollen, empty, smooth-surfaced vesicles (ghosts) (v1), often broken over a large sector of their circumference (v2). Most vesicle profiles are circular (v1, v2); a few appear elongated (v2). Membrane fragments (f) cut at various angles are seen scattered among the vesicles. Dense focal thickenings (arrows) are still visible on a few vesicular membranes. $\times 60,000$. 
TABLE V

*Distribution of Chemical Components and Enzyme Activities in Subfractions Obtained from Alkaline-Treated Rough and Smooth Microsomes*

|                     | Protein | PLP | RNA | α-amylase | RNase† |
|---------------------|---------|-----|-----|-----------|--------|
| Rough microsomes    | %       | µg/mg protein | %     | µg/mg protein | %     | mg/mg protein | %     | U/mg protein |
| 100                 | 98.6 (5) | 360.0 (2) | 5.9 (2) | 61.0 (2) |
| Rough microsomal    | %       | µg/mg protein | %     | µg/mg protein | %     | mg/mg protein | %     | U/mg protein |
| membranes           | 51.4 (3) | 97.0 (4) | 190.4 (4) | 43.3 (2) | 316.0 (2) | 1.8 (2) | 0.18 (2) | 0.8 (2) | 1.1 (2) |
| Rough microsomal    | %       | µg/mg protein | %     | µg/mg protein | %     | mg/mg protein | %     | U/mg protein |
| supernatant         | 41.8 (5) | 2.0 (4) | 4.8 (4) | 54.0 (2) | 525.0 (2) | 96.0 (2) | 14.2 (2) | 95.5 (2) | 170.5 (2) |
| Recovery            | %       | µg/mg protein | %     | µg/mg protein | %     | mg/mg protein | %     | U/mg protein |
| 93.2                | 99.0    | 97.2 | 97.8 | 96.2 |

| Smooth microsomes   | %       | µg/mg protein | %     | µg/mg protein | %     | mg/mg protein | %     | U/mg protein |
| 100                 | 248.0 (5) | 43.0 (2) | 6.7 (2) | 43.0 (2) |
| Smooth microsomal   | %       | µg/mg protein | %     | µg/mg protein | %     | mg/mg protein | %     | U/mg protein |
| membranes           | 50.5 (5) | 99.5 (5) | 438.0 (5) | 16.0 (2) | 12.5 (2) | 1.7 (2) | 0.23 (2) | 1.0 (2) | 1.2 (2) |
| Smooth microsomal   | %       | µg/mg protein | %     | µg/mg protein | %     | mg/mg protein | %     | U/mg protein |
| supernatant         | 43.8 (4) | 2.0 (4) | 12.0 (3) | 73.0 (2) | 51.0 (2) | 98.3 (2) | 14.8 (2) | 108.5 (2) | 105.0 (2) |
| Recovery            | %       | µg/mg protein | %     | µg/mg protein | %     | mg/mg protein | %     | U/mg protein |
| 94.3                | 101.5 | 89.0 | 100 | 109.5 \n
Values given are averages. Numbers in parentheses show number of experiments. Ranges are shown in italics.

* See Table 1.
† See Table IV.
FIGURE 11 a  Plasmalemmal fraction. Field in the bottom quarter of the pellet. At this level the preparation consists primarily of large single sheets of cell membranes (cm) often associated with parallel sheets of basement membrane (bm) which indicate that they are coming from the basal region of the exocrine cells. × 35,000.

FIGURE 11 b  Plasmalemmal fraction. Field in the upper three-quarters of the pellet. At this level the fraction mainly is comprised of membranes either paired (p) or organized in large vacuoles (v). A recognizable occluding zonule is marked oz. The fine fibrillar material (ff) interspersed among the membranes comes from cytoplasmic filaments associated with either adhering zonules or adhering maculae (desmosomes). × 38,000.
FIGURE 12  Plasmalemmal fraction. Detailed view of a series of exploded desmosomes. The intercellular discs are lost and the adjacent cell membranes (arrows) are no longer parallel. The intracellular dense plaques (dp) and the associated masses of fine filaments (ff) are still recognizable. Paired membranes appear at p. X 66,000.

FIGURE 13  Plasmalemmal fraction. At this magnification, the stratified structure of the cell membrane is visible wherever the membrane fragments cm are normally sectioned. X 185,000.

TABLE VI

| Protein Content | PLP Content | Cytochrome Oxidase Activity |
|-----------------|-------------|-----------------------------|
| mg/g tissue wet wt | µg/mg protein | µmol/mg protein |
| Plasma membrane fraction | 0.202 (10) | 199.0 (9) |
| 0.170-0.250 | 170.0-232.0 | 91.3 (8) |

Values given are averages. Numbers in parentheses show number of experiments. Ranges are given in italics.

* See Table I.

somes (Figs. 11 b and 12) and identified on this account as fragments of the terminal web of the apical region of the exocrine cells. Taken together the fibrillar material accounts for as much as 50% of the structured components of the pellets. Except for a few lysosomes, or lysosomal residues (crystals), no other subcellular component can be recognized in this fraction.

The gross chemistry and enzyme activity of the plasma membrane fraction is shown in Table VI. The protein yield is very low; the phospholipid/protein ratio is also low, being
~ one-half of that observed in most of the plasma membrane preparations from other tissues (26, 31, 36, 39, 40). This most likely is due to the massive contamination by basement membranes and terminal web fragments which are known or expected to contain protein but not phospholipids (41). As shown by the SAₐ₃ for cytochrome c oxidase, our plasma membrane fraction also contains a mitochondrial contamination which accounts for <8% of its protein.

DISCUSSION

We have described in this paper the isolation of several cell fractions, e.g. rough and smooth microsomes, zymogen granule, and plasma membrane fractions, from pancreatic homogenates (guinea pig). Furthermore, we have demonstrated that treatment of isolated zymogen granules and microsomes with an isosmotic alkaline buffer releases practically all the content of these organelles, and permits the isolation of their membranes.

Cell Fractionation Procedures

Our isolation procedures are largely derived from methods already reported in the literature. The procedure for zymogen granule separation is based on that developed by Hokin for the dog pancreas (21) and modified by Jamieson and Palade for the guinea pig pancreas (5). Our zymogen granule fraction compares favorably with the best preparations reported in the literature: it contains only a trace of RNA which mainly is due to its limited content of intracisternal granules surrounded by rough-surfaced membranes; it has the lowest phospholipid/protein ratio so far recorded, and contains ~2-3% mitochondria, as determined both by counting organelles in micrographs which monitor the entire depth of the pellet, and by assaying the SAₐ₃ of the fraction for mitochondrial enzymes.⁴

Because of the high concentrations of digestive enzymes in the pancreatic homogenate, the possibility exists that mitochondrial marker enzymes may become inactivated during the isolation of the fractions, thus making unsure the quantitative estimation of mitochondrial cross-contamination. This does not seem to be the case, however, since pancreatic homogenates aged at 4°C for 24 hr retain ~90% of their cytochrome oxidase and ~83% of their succinate dehydrogenase activities.

The corresponding SAₐ₃ suggests that mitochondrial membranes account for ~10% of the membranes present in the zymogen granule fraction. Using a different approach, Greene et al. (22) estimated that an even larger percentage (~50%) of the phospholipids recovered in their zymogen granule fraction from bovine pancreas was attributable to contaminants.

Our rough and smooth microsomal fractions essentially are identical to those isolated by Jamieson and Palade (5). A few minor modifications were made in order to eliminate mitochondrial contamination. The rough microsomal fraction is homogeneous and consists of resealed vesicular fragments of RER cisternae. The smooth microsomes represent primarily vesicles and cisternae of the Golgi complex, as shown by Jamieson and Palade (5). In the companion articles we will discuss additional evidence bearing on the identification of the latter fraction.

The plasma membrane fraction is less satisfactory on account of its low yield and heavy contamination by adhering fibrillar material derived from both the terminal web of the apical pole of the cells and the basement membrane that covers their basal aspects. The ratio of basal to lateral to luminal cell membrane fragments is difficult to establish in our fraction, and could be quite different from the corresponding value in the intact cell. This point deserves further inquiry since there is reason to assume that in glandular epithelia (as already demonstrated in the liver) interstitial membranes are not equivalent to luminal membranes (42-44), and since only the latter are of interest in relation to the mechanism of discharge of zymogen granules. Notwithstanding the limitations mentioned, we assume that the cell membrane fraction obtained is still useful for a comparative study of membrane enzymes and, especially, of membrane lipids.

Preparation of Membrane Fractions

As shown first by Hokin (21) and studied later in detail by Greene et al. (22), isolated zymogen granules release their content when resuspended in alkaline buffers. A similar observation was made by Jamieson and Palade in the case of pancreatic microsomes (5). Taking advantage of these observations, we investigated quantitatively the extraction of the content of pancreatic organelles, with the aim of isolating purified granule
membranes, and were able to demonstrate that alkaline treatment releases almost the entire content from both microsomes and zymogen granules as judged by the quantitative recovery of segregated enzymes in the extract (supernate) after removing the membranes by sedimentation. In the case of the ZG fraction this approach was particularly successful because, after the extraction of the granular content, the membrane fraction could be freed of most of its contaminating mitochondria by gradient flotation. To make them comparable to the other membranes, the plasmalemmal fractions were also subjected to alkaline treatment.

The effect of alkaline buffers on isolated pancreatic organelles cannot be explained satisfactorily at present. Since it is immediate and does not depend on temperature (in the range of 4°-25°C), it may result from changes in the molecular architecture of the membranes rather than from the activation of certain membrane-associated enzymes. Alternatively, the state of aggregation of the segregated enzyme molecules may be altered at an alkaline pH, leading to aggregation of the segregated enzyme molecules. From the analysis of the granular content, the membrane fraction of the plasma membranes could not be calculated accurately at present. Since it is immediate and does not depend on temperature (in the range of 4°-25°C), it may result from changes in the molecular architecture of the membranes rather than from the activation of certain membrane-associated enzymes. Alternatively, the state of aggregation of the segregated enzyme molecules may be altered at an alkaline pH, leading to aggregation of the segregated enzyme molecules.

Membrane subfractions isolated from zymogen granule and smooth and rough microsomes fixed in OsO4 buffered at pH 7.4 with respect to those fixed in unbuffered OsO4-sucrose solutions (pH 5), probably is due to a certain degree of swelling and extraction.

The poorer preservation of both zymogen granules and microsomes and resulting in their lysis. The poorer preservation of both zymogen granules and microsomes fixed in OsO4 buffered at pH 7.4 with respect to those fixed in unbuffered OsO4-sucrose solutions (pH 5), probably is due to a certain degree of swelling and extraction.

Membrane subfractions isolated from zymogen granule and smooth and rough microsomes, as well as plasma membranes, have distinctive gross properties. For instance, the phospholipid/protein ratio of rough microsomal membranes (after correction for the presence of residual ribosomes) is only 0.28, whereas the corresponding ratio for smooth microsomal membranes and zymogen granule membranes is ~0.45. The phospholipid/protein ratio of plasma membranes could not be calculated because of the high and indeterminate contamination by extraneous fibrillar material. Membrane proteins account for 35%, 50%, and 2%, respectively, of the total proteins found in the original rough microsomes, smooth microsomes, and ZG fraction. Other differences found by investigating the lipid composition and enzyme activity of the isolated fractions will be described in detail in the companion articles.

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