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GLYCOPROTEIN SYNTHESIS IN THE ADULT RAT PANCREAS

IV. SUBCELLULAR DISTRIBUTION OF MEMBRANE GLYCOPROTEINS

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Summary

Zymogen granule membranes from the rat exocrine pancreas displays distinctive, simple protein and glycoprotein compositions when compared to other intracellular membranes. The carbohydrate content of zymogen granule membrane protein was 5—10-fold greater than that of membrane fractions isolated from smooth and rough microsomes, mitochondria and a preparation containing plasma membranes, and 50—100-fold greater than the zymogen granule content and the postmicrosomal supernate. The granule membrane glycoprotein contained primarily sialic acid, fucose, mannose, galactose and *N*-acetylglucosamine. The levels of galactose, fucose and sialic acid increased in membranes in the following order: rough microsomes < smooth microsomes < zymogen granules.

Membrane polypeptides were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The profile of zymogen granule membrane polypeptides was characterized by GP-2, a species with an apparent molecular weight of 74 000. Radioactivity profiles of membranes labeled with [^{3}H]glucosamine or [^{3}H]leucine, as well as periodic acid-Schiff stain profiles, indicated that GP-2 accounted for approx. 40% of the firmly bound granule membrane protein. Low levels of a species similar to GP-2 were detected in membranes of smooth microsomes and the preparation enriched in plasma membranes but not in other subcellular fractions. These results suggest that GP-2 is a biochemical marker for zymogen granules.

Membrane glycoproteins of intact zymogen granules were resistant to neuraminidase treatment, while those in isolated granule membranes were readily degraded by neuraminidase. GP-2 of intact granules was not labeled by expo-

sure to galactose oxidase followed by reduction with NaB^3H_4. In contrast, GP-2 in purified granule membranes was readily labeled by this procedure. Therefore GP-2 appears to be located on the zymogen granule interior.

Introduction

Recent evidence suggests that glycoproteins are widely distributed among subcellular membranes [1,2]. In secretory tissues, membranes of the smooth microsomes and rough microsomes [3,4], the Golgi complex [5], as well as storage granules [6], possess glycoproteins as firmly bound constituents. In this context, the formation and fate of storage granules of the exocrine pancreas, zymogen granules, are of particular interest. Little is known of the molecular events by which zymogen granules are formed. Zymogen granules are the final product of translational and post-translational events [7]. Thus biochemical analyses of this organelle should shed light on its formation and function. In previous studies in this series we observed that a glycoprotein galactosyltransferase, enriched in smooth microsomes and membranes of Golgi-rich fractions, was not detectable in zymogen granules from rat pancreas [8,9]. A preliminary study suggested that zymogen granule membranes from rat and five other mammalian species were enriched in a characteristic glycoprotein [6]. Furthermore, endogenous galactosyltransferase acceptors in Golgi-rich membranes resembled components of zymogen granule membranes [10]. To further define the relationships among the zymogen granule membrane and other membranes implicated in its formation and function, we have compared carbohydrate and glycoprotein compositions of the major subcellular fractions from rat pancreas. We also report the results of studies of the surface orientation of glycoproteins of zymogen granule membranes.

Materials and Methods

Preparation of subcellular fractions. Male Sprague-Dawley rats (Spartan Research, Haslett, Mich.) weighing 200—300 g were maintained on commercial chow and fed water ad libitum. Rats, fasted overnight, were stunned and decapitated. Pancreases were excised and homogenized in 0.3 M sucrose (ultrapure, Schwarz/Mann) containing 0.10 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.) [8]. Homogenates were centrifuged at 500 × *g* for 10 min. The pellet was resuspended in 0.3 M sucrose and the suspension was recentrifuged. A 700 × *g* particulate fraction, enriched in plasma membranes, was prepared from the resulting pellet according to the procedure of Meldolesi et al. [11] with the following modifications. The pellet was suspended by two strokes of a hand homogenizer in 1.58 M sucrose and 1% (w/v) Dextran, (Pharmacia Dextran 500). The suspension was overlaid with 0.3 M sucrose and the discontinuous gradient was centrifuged at 192 000 × *g* for 60 min. Material at the interface was collected. Collagenase and hyaluronidase treatments [11] were omitted. The 700 × *g* particulate fraction contained smooth membrane fragments, as judged by electron microscopic examination performed as previously described [8]. Nuclei were prepared from isolated acinar cells [12] by the procedure devised to

obtain liver nuclei [13]. Zymogen granules were pelleted by centrifugation at 1000 × *g* for 30 min and washed by repeated resuspension and recentrifugation. Mitochondria, smooth and rough microsomes were prepared as described previously [8]. The postmicrosomal supernatant represented material not pelleted by centrifugation for 90 min at 83 000 × *g*.

The following procedure was employed to prepare zymogen granule membranes. All operations were performed at 4°C. Steps 1 and 2 represent modifications of the procedure of Meldolesi et al. [11].

Step 1. Washed zymogen granules from 10 g (wet weight) of pancreas were suspended in 3 ml of 0.17 M NaCl containing 0.66 mg/ml soybean trypsin inhibitor and lysed by the addition of 10 ml of 0.2 M $NaHCO_3$, pH 8.2. After 45 min the suspension was centrifuged 45 min at 195 000 × *g* (SW41 rotor, Beckman Instruments, Inc.). The pellet contained ZGM-1, and mitochondria contaminants. The supernate, designated the zymogen granule content, was dialyzed against 50 mM NaCl and 50 μM phenylmethyl sulfonylfluoride on ice. The preparation was lyophilized and stored at −80°C.

Step 2. Granule membranes after lysis were separated from the more dense mitochondria by the use of a discontinuous sucrose gradient. The pellet obtained from Step 1 was gently resuspended in 4 ml of 1 M sucrose using a Dounce homogenizer (Kontes Glass Co.). The suspension was overlayed with 9 ml of 0.3 M sucrose without soybean trypsin inhibitor and centrifuged for 45 min at 195 000 × *g* (SW41 rotor). The granule membranes, designated ZGM-2, appeared at the 0.3—1 M sucrose interface and were collected.

Step 3. Approx. 1 ml of the granule membrane suspension from step 2 was diluted with 8 ml of 0.25 M NaBr and sonicated 10—15 s (maximum setting, Biosonik II sonicator with a needle probe (Bronwill Scientific, Rochester, N.Y.). The final volume was approx. 13 ml per 500 μg membrane protein. The suspension was centrifuged 45 min at 195 000 × g, SW41 rotor), and the supernatant fraction was removed. The membrane pellet, ZGM-3, was frozen on solid CO_2 and stored at −80°C. This modification was used in later experiments. Aliquots of the zymogen granule lysate were placed directly over a discontinuous gradient containing 0.3 M sucrose over 1.0 M sucrose. The discontinuous gradient was centrifuged as described. The $NaHCO_3$ layer was removed and the membranes collecting at the 0.3 —1.0 M sucrose interface, ZGM-2, were treated as described above. The yield of ZGM-3 was doubled and the purity was comparable to Step 3.

Other particulate fractions were suspended by sonication in 0.2 M $NaHCO_3$ containing 0.10 mg soybean trypsin inhibitor/ml to a final concentration of 5 mg protein/ml. After 45 min on ice, the particulate fractions were collected by centrifugation and extracted with 0.25 M NaBr as described above.

Preparation of radioactive subcellular fractions. 3—6 adult rat pancreases were trimmed free of connective tissue, sectioned with a Steadie-Riggs tissue slicer (A.H. Thomas Co., Philadelphia), and placed on sterile filters (HAWP, Millipore Corp., Bedford, Mass.). The filters were floated in a medium containing by volume 90% (v/v) Eagle's minimal essential medium, 5% (v/v) fetal calf serum (Grand Island Biological Co., N.Y.) supplemented with soybean trypsin inhibitor (0.1 mg/ml). The following radioactive precursors were obtained from New England Nuclear; each was added singly to incubation media: D-[6-^{3}H]-

glucosamine · HCl (7.3 Ci/mmol), 25 μCi/ml; D-[1-^{14}C]glucosamine · HCl (55 mCi/mmol), 2 μCi/ml; L-[U-^{14}C]leucine (331 mCi/mol), 2.8 μCi/ml. After incubation, tissues were rinsed four times in 0.3 M sucrose and fractionated as described above. To determine bound radioactivity, aliquots of subcellular fractions were precipitated with 10% (w/v) trichloroacetic acid. The precipitates were collected on filters (Whatman, GF/C) and extracted with lipid solvents according to published procedures [14]. Precipitated protein was solubilized by heating in 0.5 ml 1% (SDS) and 0.1 M NaOH at 90°C. The samples were neutralized and counted in a toluene/Triton X-100 scintillation fluid [15] using a Packard liquid scintillation spectrometer. The counting efficiency was 84% for ^{14}C and 38% for ^{3}H. In dual label experiments, tritium counts were corrected for spill-over of ^{14}C. The counting efficiencies in this case were 67 and 30%, respectively. The soluble leucine pool of adult tissue slices equilibrated within 30 min and the rate of [^{14}C]leucine incorporation into acid-precipitable products was linear for at least 4 h.

Polyacrylamide gel electrophoresis. Membrane pellets were dissolved directly in protein solvent buffer (1% SDS, 10 mM Tris · HCl, pH 8.0, 5 mM EDTA, 2% 2-mercaptoethanol) by heating at 90°C for 5 min after brief sonication. Polyacrylamide gel electrophoresis in Tris/acetate, pH 7.4, containing 1% SDS was performed in 9% acrylamide gels, 0.5 cm or 0.6 cm × 11 cm, essentially as described by Fairbanks et al. [16] except that dimethyldichlorosilane-coated gel tubes were employed. The acrylamide to bisacrylamide ratio was 36 : 1. A constant potential gradient of 10 V/cm was employed, and the current did not exceed 5 mA/gel. A constant length of each electrophoretogram was obtained by allowing the tracking dye, pyronin B, to migrate 8.7 cm from the origin. Gels were placed in two changes of 10% trichloroacetic acid for several days, then were developed with either the periodic acid-Schiff stain according to Fairbanks et al. [16], with the omission of isopropyl alcohol, or Coomassie Blue [17]. The gels were scanned at 550 nm (Coomassie Blue) or 560 nm (periodic acid-Schiff) using a Gilford 240 spectrophotometer equipped with a linear transport. Gels containing radioactive samples were fractionated using a Savant Autogel Divider (Savant Instruments, Inc.). Fractions were collected in scintillation vials containing 1 ml of 0.1 M NaOH and 1% SDS. The vials were capped and stored at 37°C for 3 days. The samples were then neutralized and counted. In some experiments, zymogen granule membranes were fractionated in 9% acrylamide gels containing 1% SDS using a pH 2.4 buffer system [18]. In other cases, electrophoresis in 8% acrylamide slab gels in 0.1% SDS was performed as described by Laemmli [19].

Analytical procedures. Protein samples were precipitated with 10% trichloroacetic acid (w/v) and solubilized in 0.1 M NaOH before analysis by a minor modification of the Lowry et al. [20] procedure using bovine serum albumin as a standard. RNA content was determined by the orcinol procedure [21]. The standard was yeast RNA (Sigma Chemical Co.). Phospholipids were extracted in chloroform/methanol (2 : 1, v/v) and the phosphorous content was determined [22]. Amylase was assayed by the method of Bernfeld [23] and cytochrome *c* oxidase was assayed as given by Wharton and Tzagaloff [24]. Hexosamine analysis of [^{3}H]glucosamine-labeled zymogen granules was performed using column chromatography [25]. Samples were hydrolyzed in 4 M HCl under N_2 for 4 h at 100°C.

For carbohydrate analyses, membrane fractions were extracted with chloroform/methanol/diethylether (2 : 1 : 0.1, v/v). Methanolysis for 8 h was performed as described [26]. After re-*N*-acetylation, trimethylsilyl ethers of methylglycosides were prepared [27]. These were analyzed by gas-liquid chromatography by the method of Bhatti et al. [26]. Sialic acid content was also determined by the thiobarbituric acid method [28].

In some experiments surface sialyl residues of zymogen granules and granule membranes were exposed to neuraminidase (Type VI, *Clostridium perfringens*, Sigma Chemical Co., 0.040 units/mg protein assayed using bovine submaxillary mucin). In other instances, zymogen granules and subfractions were treated with galactose oxidase (Sigma Chemical Co., 124 units per mg) and then were reduced with NaB^3H_4 (New England Nuclear, 100 Ci/mol).

Results

Purification of zymogen granule membranes

Our initial study indicated that rat zymogen granule preparations were minimally contaminated by smooth microsomes and Golgi-rich membranes as judged by glycoprotein galactosyltransferase activity [9]; and by rough microsomes, as judged by electron microscopic observations [8]. Table I further extends these observations. In accord with morphological studies, zymogen granules contained little RNA (1.4 μg per mg protein) compared to rough microsomes. On the other hand, zymogen granule membranes and microsomal membranes were highly enriched in phospholipids. Similar data were obtained by other workers [11]. Note that zymogen granules contained appreciable sialic acid preferentially associated with the membranes.

The problem of purity of secretion granule membranes is a recurrent one [29—32]. We earlier reported that Golgi membranes were dissimilar from zymogen granule membranes, based upon comparisons of galactosyltransferase activities and polypeptide compositions [9]. Since interpretation of membrane

TABLE I

RNA, PHOSPHOLIPID AND SIALIC ACID IN SUBCELLULAR FRACTIONS FROM ADULT RAT PANCREAS

Subcellular fractions were prepared, and the RNA and phospholipid phosphorous content were analyzed as described in Materials and Methods. Sialic acid, as *N*-acetylneuraminic acid, was determined using the thiobarbituric acid procedure ± S.E. for 3—6 determinations.

Fraction	μg RNA per mg protein	μg phospholipid phosphorous per mg protein	mg protein per g wet weight tissue *	nmol sialic acid per mg protein
Zymogen granules	1.4 ± 1.0	12 ± 3	4.5	8.1 ± 4
Zymogen granule membranes	<1	828 ± 85	<0.1	100 ± 25
Mitochondria	n.d.	114 ± 20	14.5	13 ± 4
Microsomes	220 ± 16	156 ± 22	34.6	15 ± 4
Microsomal membranes	400 ± 35	350 ± 55	10	30 ± 8
Smooth microsomes	65 ± 9	213 ± 11	2.7	41 ± 15
Rough microsomes	330 ± 30	117 ± 10	9.5	<1

n.d., not determined.

* From ref. 8.

TABLE II

LEVELS OF AMYLASE AND CYTOCHROME *c* OXIDASE IN ZYMOGEN GRANULES AND GRANULE SUBFRACTIONS

Amylase and cytochrome *c* oxidase activities were measured as described in Materials and Methods. Values are the means of the number of experiments given in parentheses.

Fraction	Protein (%)	Amylase		Cytochrome *c* oxidase	
		Total activity (%)	Specific activity (units per mg protein × 10^3)	Total activity (%)	Specific activity (units per mg protein × 10^3)
Intact zymogen granules	100	100	101 (4)	100	1.0 (5)
Membranes after $NaHCO_3$ extraction (ZGM-1)	3.8	2.2	8.0 (2)	157	10.3 (2)
Membranes from discontinuous gradient (ZGM-2)	1.4	1.2	12.1 (3)	16	4.7 (3)
Membranes after NaBr extraction (ZGM-3)	0.54	0.1	0.8 (3)	0.4	0.2 (5)

data relied upon the purity of zymogen granule membranes, this aspect was studied in detail. Table II summarizes the levels of amylase and cytochrome *c* oxidase, a marker for mitochondrial membranes, at each stage of purification of zymogen granule membranes. The granule content fraction contained 96% of the protein and 98% of the amylase activity of intact zymogen granules. The granule ghosts, ZGM-1, accounted for 4% of the granule protein, 98% of the cytochrome *c* oxidase activity and 2% of the granule amylase activity.

The difference in density between ZGM-1 and mitochondria was exploited to separate these two fractions [14]. Mitochondria sedimented through 1 M sucrose, while granule membranes, ZGM-2, collected at the 1—0.3 M sucrose interface. Table II demonstrates the efficacy of this procedure in the removal of cytochrome *c* oxidase. However, amylase activity remained at a high level and a further purification step was needed. Chaotropic agents at concentrations of 2—4 M disaggregate membrane complexes [33], and we considered the possibility that the use of such agents at a lower concentration might selectively elute adsorbed contaminants without effecting intrinsic membrane polypeptides. Extraction of ZGM-2 with 0.25 M NaBr reduced the specificic activity of amylase in the purified granule membranes, ZGM-3, to less than 1% of the level of zymogen granules. Other studies demonstrated that NaBr treatment did not inactivate amylase activity [36]. Furthermore, the released activity could be recovered in the NaBr supernatant. The final yield of granule membrane was 0.5—1.0% of the total granule protein.

Estimates of membrane contamination by adsorbed soluble proteins based upon enzymic activities can be inaccurate [30,31]. We therefore performed the mixing experiment summarized by Table III. Zymogen granule membranes were isolated from a mixture of non-radioactive intact granules and granule content labeled with [^{3}H]leucine. As judged from the specific radioactivity of the granule content and the amount of radioactivity bound to ZGM-3, adsorbed secretory protein represented approx. 6% of the purified zymogen granule

TABLE III

ADSORPTION OF SECRETORY PROTEIN TO ZYMOGEN GRANULE MEMBRANES

Non-radioactive zymogen granules (10.1 mg) were suspended in 10 ml of 0.2 M $NaHCO_3$ containing granule content (2.30 mg, $27.3 \cdot 10^4$ dpm) prepared from tissue slices incubated in the presence of [^{3}H]-leucine as described in Materials and Methods. After storage at 4°C for 30 min to lyse the granules, ZGM-3 was isolated and the amount of bound radioactivity was measured.

Fraction	Protein * (μg)	Radioactivity * (dpm)	Specific radioactivity (dpm/μg protein)
Zymogen granule content	12 300 ± 20	273 400 ± 100	22.2
Zymogen granule membranes (ZGM-3)	100 ± 3	135 ± 6	1.4

* ±S.E.

membrane protein. Similar binding studies were performed with microsomes and radioactive postmicrosomal supernatant or $NaHCO_3$ extracts. The results indicated that approx. 10% of the microsomal membrane protein was adsorbed secretory protein (data not given). The specific activity of cytochrome *c* oxidase in mitochondrial membranes, after sequential extractions with 0.2 M $NaHCO_3$ and 0.25 M NaBr, was 21 munits per mg protein, while the specific activity of cytochrome *c* oxidase in purified granule membranes prepared in this manner was 0.2 munits per mg protein. By this criterion we estimate that the mitochondrial contamination in ZGM-3 was approx. 1%.

Identification of zymogen granule membrane polypeptides

The removal of soluble secretory and mitochondrial proteins and the enrichment of granule-membrane-associated polypeptides was monitored by SDS-polyacrylamide gel electrophoresis. When the granule content was analyzed, eight components were observed (Fig. 1A). The major component, Band II, accounted for approx. 50% of the stain intensity and had an apparent molecular weigh of 52 000 as judged by comparison with proteins of known molecular weight. This agrees well with the molecular weight of amylase from adult rat pancreas [34]. Analyses of zymogen granule membranes at each purification stage (Figs. 1B—1D) document the loss of the adsorbed secretory proteins during purification. The majority of Coomassie Blue stain intensity in zymogen granule ghosts corresponded to secretory proteins, particularly Band II. Subsequent extraction with 0.25 M NaBr removed polypeptides with mobilities identical to Bands I—VIII of the granule content.

Several polypeptides were enriched in the particulate fraction at each stage of granule membrane purification. These are designated Bands 1—10 in order of increasing electrophoretic mobility. Note that Band 2 was the predominent polypeptide species. Its relative stain intensity in ZGM-3 was 6-fold greater than in ZGM-1. Three species of the granule content, Bands I—III, were likely contaminants. Electrophoresis in SDS gels containing 12% acrylamide and in acetic acid/urea [35] fractionated Band 4 into two peaks of equal stain intensity [36]. We conclude that this region is a mixture of Band 4 (a membrane protein) and Band II (a secretory protein). Band 10, containing low molecular weight polypeptides, was present in the membrane fraction at each stage of purification. Its intensity in ZGM-3 was variable. When purified granule mem-

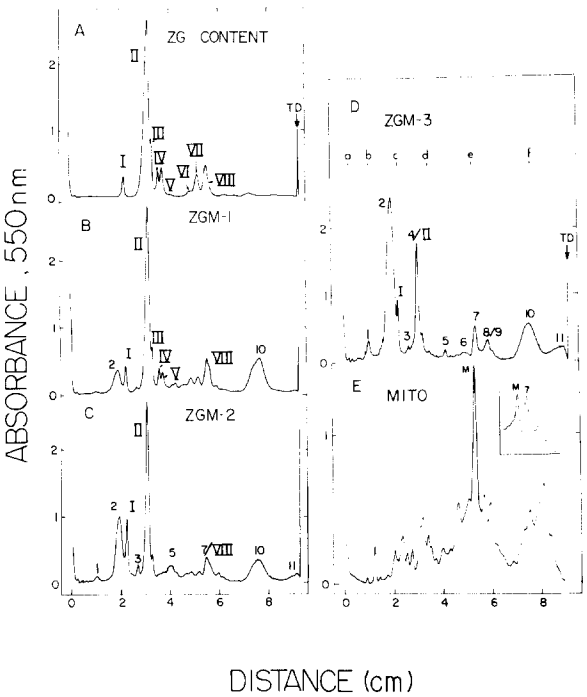

Fig. 1. Electrophoretic analysis of zymogen granule subfractions and mitochondrial membranes. Zymogen granules were isolated and fractionated as described in Table I. Samples were solubilized in electrophoresis sample buffer and electrophoresed in 1% SDS-9% acrylamide gels. The following amounts of protein were applied: A, 31 μg zymogen granule content; B, 51 μg ZGM-1, C, 36 μg, ZGM-2; D, 42 μg ZGM-31, E, 65 μg mitochondrial membranes; Inset, 42 μg ZGM-3 plus 33 μg mitochondrial membrane. The gels were stained with Coomassie Blue and scanned. The protein bands of the granule content are numbered I—VIII. Protein bands of zymogen granule membrane subfractions are numbered 1—11 according to increasing mobility. M denotes the major mitochondrial polypeptide species and TD marks the distance of migration of the tracking dye. Duplicate gels were calibrated with the following standards a, thyroglobulin (160 000); b, phosphorylase *a* (94 000); c, serum albumin (68 000); d, aldolase (40 000); e, α-chymotrypsinogen (25 000) and f, cytochrome *c* (12 400).

branes were incubated with trypsin, the intensity of GP-2 diminished and concomitantly Band 10 increased [36]. Possibly Band 10 represents hydrophobic portions of membrane components that remain membrane associated after limited proteolysis. Band 11 had the mobility and staining properties of a mixture of sphingomyelin, phosphatidylcholine and glycolipids and probably represents the membrane lipids.

Since mitochondria represent the most likely particulate contamination, we considered the possibility that Band 7 of zymogen granule membranes represented a mitochondrial membrane polypeptide. If this were the case, mitochondrial contamination would be greater than estimated by cytochrome *c* oxidase levels. The predominant mitochondrial membrane protein, designated Band M, migrated with an apparent molecular weight 26 000 and accounted for 10—15% of the stain intensity of the mitochondrial polypeptide stain profile (Fig. 1E). To determine whether Band 7 represented Band M, a mixture of purified granule membranes and mitochondrial membranes was subjected to

electrophoresis. A small amount of mitochondrial membrane was added to ZGM-3, so both components were of equal stain intensity. As shown by Fig. 1E (Inset), Band 8 migrated more rapidly than Band M. This indicates that mitochondrial contamination of ZGM-3 was negligible.

Glycoprotein composition of zymogen granule membranes

Preliminary studies of rat zymogen granule membranes suggested that Band 2 was a glycoprotein, according to its periodic acid-Schiff-staining properties [6]. Four periodic acid-Schiff-positive species were detected in polyacrylamide gels of purified zymogen granule membranes (Fig. 2). The first three corresponded to glycoproteins and are designated as GP-1, GP-2 and GP-3, in order of increasing electrophoretic mobility. GP-1 (estimated molecular weight 120 000) corresponds to the minor species Band 1. GP-2 (molecular weight 74 000) is the predominant glycoprotein and corresponds to Band 2. GP-3 (molecular weight 52 000) corresponds to Band 4. We utilized a second strategy to confirm the presence of glycoproteins in granule membranes. Subcellular fractions were isolated from tissue slices incubated with radioactive glucosamine or leucine. Analyses performed with the amino acid analyzer indicated that more than 85% of the radioactivity in zymogen granules, prepared from tissues incubated with [^{3}H]glucosamine for 4 h, eluted with D-glucosamine. As given by Fig. 2, the radioactivity profile of zymogen granule membranes from tissue slices labeled with [^{3}H]glucosamine corresponded to [^{14}C]leucine-labeled granule membrane polypeptides. These data indicate that Bands 1, 2 and 4 are equivalent to GP-1, GP-2 and GP-3. Band 10, a putative degradation product, was not labeled with [^{3}H]glucosamine, nor was it periodic acid-Schiff positive.

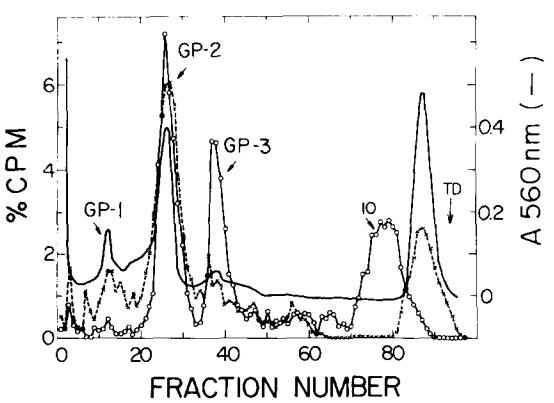

Fig. 2. Electrophoretic analysis of zymogen granule membrane glycoproteins. ZGM-3 (400 μg, 672 cpm) from tissue slices incubated with [^{14}C]leucine was mixed with ZGM-3 (50 μg, 2150 cpm) from tissue slices labeled with [^{3}H]glucosamine and electrophoresed in 1% SDS-9% acrylamide. The gel was fractionated and the distribution of radioactivity was determined. The results are expressed as percent of total cpm. —o—, ^{14}C; —x—, ^{3}H. A replicate gel containing 40 μg ZGM-3 was stained by the periodic acid-Schiff procedure and scanned (solid line). The major periodic acid-Schiff-positive bands are designated GP-1, GP-2 and GP-3 according to increasing mobility. The position of Band 10, a low molecular weight granule membrane polypeptide, is denoted.

Molecular weight and relative abundance of zymogen granule membrane polypeptides

The molecular weights of polypeptides associated with granule membranes were estimated by comparing electrophoretic mobilities of polypeptides of known molecular weight (Table IV). Values for polypeptides judged to be integral membrane components, GP-1, GP-2 and GP-3, are respectively 120 000, 74 000 and 52 000.

To limit the uncertainty of molecular weight estimates, mobilities of granule membrane polypeptides relative to protein standards were analyzed in several polyacrylamide gel systems. Increasing the acrylamide concentration in SDS-polyacrylamide gels may reveal inconsistencies in estimates of molecular weights of glycoproteins [37]. However electrophoresis in gels containing 5.6, 7, 9 or 12.5% acrylamide did not alter these molecular weight estimates.

The homogeneity of GP-2 was studied in another experiment. Zymogen granule membranes were solubilized in phenol/acetic acid/urea and were electrophoresed as described in Materials and Methods. The acetic acid/urea buffer system [35] does not employ SDS and electrophoretic migration according to polypeptide size is enhanced by imposing a uniform positive charge [38]. The major membrane band had an apparent molecular weight of 72 000 compared to protein standards. Membrane proteins in unstained gels were located by aligning with duplicate stained gels. The region of the unstained gel corresponding to the major granule membrane band was cut out from the acetic/urea gels, eluted from the gel section, and re-electrophoresed in 1% SDS at pH 7.4. This purified material had the same mobility as GP-2 (74 000 molecular weight).

TABLE IV

MOLECULAR WEIGHT AND RELATIVE ABUNDANCE OF POLYPEPTIDES IN PURIFIED ZYMOGEN GRANULE MEMBRANES

Molecular weights were estimated by comparison with electrophoretic mobilities of proteins of known molecular weight as described in Fig. 1. Stained gels were scanned and the percent relative abundance was estimated by cutting out and weighing peaks from the scans. Band II is lipid and its stain intenssity contribution was not included in estimating relative abundance. Radioactive ZGM-3 was prepared from tissues incubated with radioactive precursors as described in Materials and Methods. Samples were electrophoresed as given in Fig. 1. The gels were fractionated and counted. The radioactivity in each peak is expressed as the percentage of total recovered cpm.

Component	Apparent molecular weight	Relative abundance (%)			
		Coomassie Blue stain (n = 12)	[^{3}H]Leucine (n = 2)	Periodic acid-Schiff stain (n = 4)	[^{3}H]Glucosamine (n = 2)
Band 1 (GP-1)	120 000	2.6 ± 1.1	3.0 ± 0.8	13.4 ± 1.5	11 ± 2
Band 2 (GP-2)	74 000	38.2 ± 5.9	37.4 ± 1.8	63.2 ± 4.0	54 ± 4
Band 4 (GP-3) plus Band II	52 000 *	17.9 ± 4.9	18.0 ± 2.8	6.9 ± 3	10.0 ± 1.5
Band 7	25 000	5.1 ± 1.8	7.6 ± 1.2	n.d.	n.d.
Band 8	22 000	2.5 ± 0.9	3.7 ± 0.6	n.d.	n.d.
Band 10	12 000–14000	12.5 ± 7.9	19.3 ± 8.3	n.d.	n.d.

n.d., not detected.

* Gel electrophoreses at pH 2.4 in the 9% polyacrylamide or at pH 7.4 in 12% polyacrylamide separated Band 4 into two components with apparent molecular weights of 53 000 and 56 000.

Table IV also presents estimates of relative abundance for the predominant granule membrane proteins based upon stain intensity and radioactivity profiles. The relative abundance of zymogen granule membrane polypeptides estimated by protein stain closely correlated with values obtained from the radioactivity profiles of granule membranes isolated from pancreatic slices incubated with [^{3}H]leucine. Similarly, the relative amounts of GP-1, GP-2 and GP-3 estimated by periodic acid-Schiff stain intensity corresponded to the distribution of radioactivity from membranes labeled with [^{3}H]glucosamine. We conclude that GP-2, the most abundant protein of the granule membrane, is a glycoprotein representing 35—40% of the integral protein of zymogen granule membranes.

Carbohydrate compositions of pancreatic subcellular membranes

Gas liquid chromatography was utilized to determine the carbohydrate content of the rough microsomal membranes (derived from rough endoplasmic reticulum [11]), smooth microsomes (derived from the Golgi complex [9]) and 700 × *g* pellet (enriched in plasma membranes [11]). Zymogen granule membrane after lipid extraction contained 44% carbohydrate by weight (Table V). This value was 4-fold greater than smooth microsomal membranes and 7-fold greater than rough microsomal membranes. The 700 × *g* particulate fraction contained significantly more carbohydrate than smooth microsomal membranes. The granule content contained less than 0.3% carbohydrate; individual sugars were present at 1 and 2% of the levels found in zymogen granule membranes.

Mannose is a constituent of inner core regions of a major class of mammalian

TABLE V

CARBOHYDRATE COMPOSITION OF SUBCELLULAR FRACTIONS FROM RAT PANCREAS

Subcellular fractions were prepared as described in Materials and Methods. The carbohydrate compositions of membrane fractions after lipid extraction and of soluble fractions were determined by gas-liquid chromatography. The results are expressed as nmol carbohydrate per mg protein ± S.E. for 4—6 different preparations. The values of glucuronic acid and xylose were <0.5 nmol per mg protein for each fraction.

Sugar	Fraction					
	Zymogen granule membranes	Smooth microsomal membranes	Rough microsomal membranes	Membranes from 700 × *g* pellet	Post-microsomal supernatant	Zymogen granule content
Fucose	551 ± 33	89 ± 6	25 ± 3	169 ± 10	<0.5	2.7 ± 0.5
Mannose	525 ± 24	157 ± 20	128 ± 9	205 ± 18	26 ± 2	5.7 ± 0.5
Galactose	472 ± 29	130 ± 6	26 ± 2	214 ± 7	15 ± 1	2.6 ± 0.5
N-Acetylglucosamine	336 ± 19	50 ± 17	13 ± 4	174 ± 19	2 ± 0.3	4.0 ± 0.3
N-Acetylgalactosamine	52 ± 10	8 ± 3	<0.5	<0.5	<0.5	<0.5
Sialic acid	102 ± 18	21 ± 4	<0.5	<0.5	<0.5	1.2 ± 0.2
Glucose	418 ± 33	132 ± 10	122 ± 18	518 ± 29	53 ± 4	<0.5
Ribose	<0.5	<0.5	44 ± 8	<0.5	<0.5	<0.5
mg carbohydrate per mg protein	0.44	0.11	0.063	0.23	0.017	0.0022
nmol fucose per nmol mannose	1.0	0.56	0.19	0.82	0.03	0.48
nmol galactose per nmol mannose	0.90	0.82	0.20	1.0	0.57	0.46

oligosaccharides, while fucose and sialic acid are restricted to terminal positions of oligosaccharide chains. Galactose is often the penultimate sugar. It is therefore of interest to compare ratios of fucose, sialic acid and galactose to mannose for each subcellular fraction. Each of these ratios increased in the following order: rough microsomal membranes < smooth microsomal membranes < zymogen granule membranes. Membranes from the 700 × *g* pellet contained relatively high levels of fucose, mannose, galactose and *N*-acetylglucosamine, but no detectable sialic acid or *N*-acetylgalactosamine. The ratios of fucose/mannose of galactose/mannose for granule content protein were less than the corresponding ratios for granule membranes. These data and previous electrophoretic analyses [42] suggest that different glycoproteins were present in the granule content and the granule membranes.

All fractions contained glucose at levels roughly equivalent to the mannose content. Repeated extraction with 0.25 M NaBr and recentrifugation diminished the overall percentage of carbohydrate in membranes, but it did not alter the ratio of glucose to mannose. Xylose and glucuronic acid were not detected, and the level of *N*-acetylgalactosamine was quite low.

Electrophoretic analysis of pancreatic membrane polypeptides

Comparison of Coomassie Blue profiles of membranes of mitochondria, nuclei, rough and smooth microsomes, and the 700 × *g* pellet with zymogen

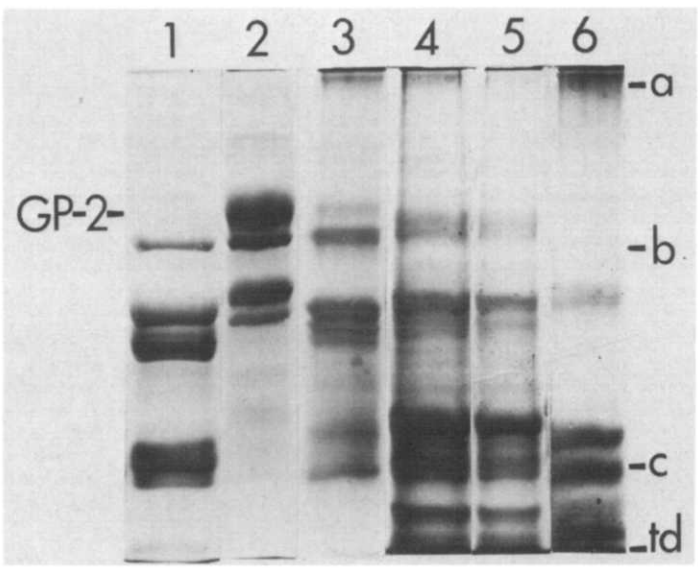

Fig. 3. Electrophoretic analysis of polypeptides of pancreatic membranes and zymogen granule content. Membrane fractions and zymogen granule content were prepared, solubilized, and electrophoresed on slab gels as described in Materials and Methods. The electrophoretograms were stained with Coomassie Blue. The following amounts of protein were analyzed: lane 1, 100 μg of the granule content; lane 2, 100 μg zymogen granule membranes; lane 3, 165 μg membranes from the 700 × *g* pellet; lane 4, 150 μg of rough microsomal membranes; lane 5, 125 μg of smooth microsomal membranes; lane 6 150 μg of nuclear membranes. The positions of GP-2 and molecular weight markers are indicated (a, thyroglobulin, b, bovine serum albumin; c, chymotrypsin). td denotes the position of the tracking dye.

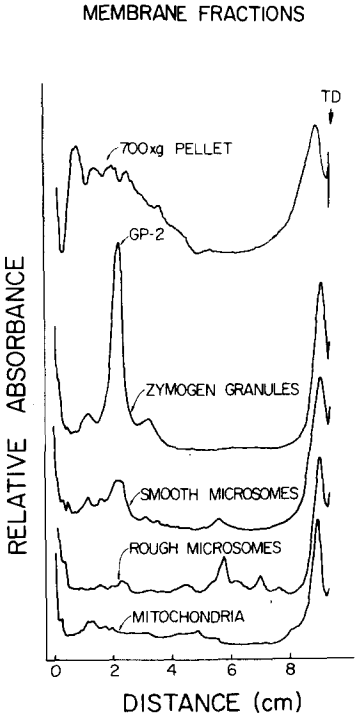

Fig. 4. Electrophoretic analysis of glycoproteins of pancreatic membranes. Procedures for the preparation of membranes are given in Materials and Methods. Samples were solubilized and electrophoresed on 0.6 cm (diameter) gels as given in Fig. 1. The amounts of protein analyzed were: membranes from the 700 X *g* pellet, 250 μg; zymogen granule membranes, 40 μg; smooth microsomal membranes, 200 μg; rough microsomal membranes, 200 μg; mitochondrial membranes, 200 μg. Electrophoretograms were developed by the periodic acid-Schiff stain procedure and scanned at 560 nm. The results are expressed as relative absorbance. The positions of GP-2 and the tracking dye, TD, are given.

granule membranes suggests that GP-2 represented less than 5% of the protein stain profile of the major membrane classes in the rat pancreas (Fig. 3). Similar results were obtained with another protein stain, Amido Black (unpublished observations). Smooth and rough microsomal membranes exhibited similar polypeptide stain profiles, as has been reported for liver microsomal subfractions [39]. The membrane polypeptide profiles were more complex than the profile of the granule membrane polypeptides. The predominant polypeptides of all membranes except zymogen granule membranes exhibited molecular weights ranging between 12 000 and 52 000.

Scans of periodic acid-Schiff-stained gels of the membrane fractions are presented in Fig. 4. As anticipated from the data presented in Table V, zymogen granule membranes were most intensely stained by periodic acid-Schiff. While one band in membranes of the 700 X *g* pellet corresponded to GP-2, the most intensely stained species migrated more slowly than GP-2. Rough and smooth microsomal membranes contained rather different periodic acid-Schiff-stain profiles, aside from the intensely stained lipid region. Smooth microsomal membrane preparations exhibited a major periodic acid-Schiff-positive band that corresponded in mobility to GP-2. The presence of several bands in the

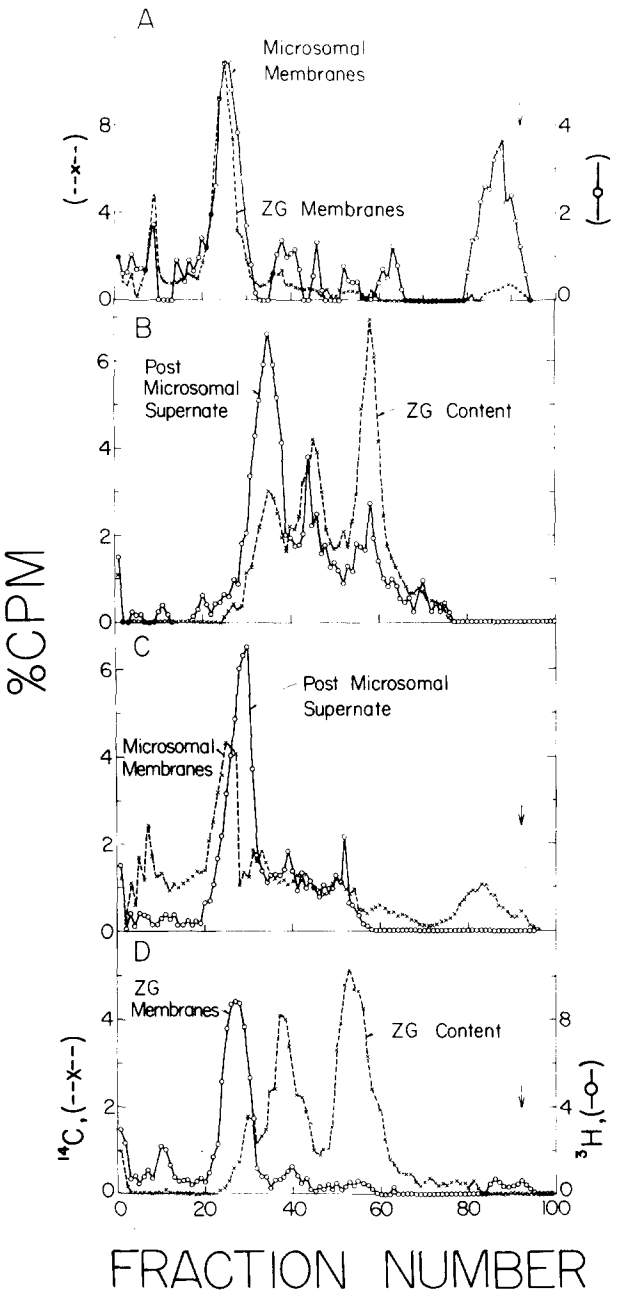

Fig. 5. Comparison of glycoproteins of pancreatic fractions labeled with radioactive D-glucosamine in vitro. Tissue slices were incubated in medium containing either D-[^{3}H]glucosamine or D-[^{14}C]glucosamine, then subcellular fractions were prepared according to Materials and Methods. Mixtures were applied to 0.6 cm (diameter) polyacrylamide gels and electrophoresed. The gels were fractionated and the fractions were counted. To normalize the radioactivity profiles, the results are expressed as the percentage of radioactivity recovered. (A) ZGM-3 (25 μg, 2730 cpm) labeled with [^{3}H]glucosamine plus microsomal membranes (470 μg, 1140 cpm) labeled with [^{14}C]glucosamine. (B) Postmicrosomal supernatant (700 μg, 830 cpm) labeled with [^{14}C]glucosamine plus granule content (25 μg, 650 cpm) labeled with [^{3}H]glucosamine. (C) Postmicrosomal supernatant (600 μg, 1000 cpm) labeled with [^{14}C]glucosamine and microsomal membranes (35 μg, 1800 cpm) labeled with [^{3}H]glucosamine. (D) Granule content (300 μg, 1100 cpm) labeled with [^{3}H]glucosamine and ZGM-3 (50 μg, 540 cpm) labeled with [^{14}C]glucosamine. Arrows indicate the position of the tracking dye.

low molecular weight region of rough microsomal membranes was distinctive. The periodic acid-Schiff stain profile of mitochondrial membranes was characterized by a minor component with a relative mobility significantly less than GP-2. Glycoproteins were not detected in nuclear membranes. These results illustrate the striking differences between glycoprotein composition of granule membrane and the composition of other intracellular membranes.

Polyacrylacmide gels of zymogen granule content or the phostmicrosomal supernate did not exhibit periodic acid-Schiff-positive bands. This paucity of periodic acid-Schiff-positive material is in agreement with data presented in Table II. To increase the sensitivity of the electrophoretic procedure for detection of glycoproteins, subcellular fractions were prepared from tissues incubated with [^{14}C]- or [^{3}H]glucosamine. Fig. 5A demonstrates that smooth microsomal membranes contained a glucosamine-labeled species migrating like GP-2. This confirms the similarity noted in Fig. 4. Fig. 5B demonstrates that both zymogen granule content and the postmicrosomal superantant contained similar glucosamine-labeled polypeptides. To determine whether the soluble glycoproteins comigrated with GP-2, dual-labeled gels were analyzed (Figs. 5C and 5D). These results suggest that soluble, glucosamine-labeled polypeptides of the granule content and the postmicrosomal supernatant did not comigrate with GP-2. We conclude that GP-2 is localized in zymogen granule membranes.

Assessment of proteolytic degradation of membrane polypeptides

Procedures such as lipid extraction with chlorofrom/methanol (2 : 1, v/v), dissolution in 2-chloroethanol, prolonged dialysis, and heating in solvent buffer failed to alter electrophoretograms of membrane polypeptides [36]. In a preceding paper of this series, evidence was presented that suggested that isolation of subcellular fractions in the presence of soybean trypsin inhibitor minimized degradation [8]. Electrophoretograms of purified granule membranes prepared in the presence of 0.1 mM phenylmethyl sulfonylfluoride, a serine protease inhibitor, were similar to those in which soybean trypsin inhibitor was employed. To determine whether the membrane protein profiles would be altered by storage, microsomes were stored at 0°C with the postmicrosomal supernatant containing either 0.1 mM phenylmethyl sulfonylfluoride or 0.25 mg/ml soybean trypsin inhibitor. After 18 h microsomes were collected. Electrophoretograms of microsomal membranes stored with soybean trypsin inhibitor did not differ significantly from the zero time profiles. On the other hand, incubation of microsomes at 37°C for 6 h led to profound changes in the membrane profiles.

Surface organization of zymogen granule membrane-glycoproteins

The orientation of membrane glycoproteins is important in considering potential functions of these constituents during secretion, as well as possible mechanisms of zymogen granule formation. In an initial experiment (Table IV) intact zymogen granules and granule ghosts were subjected to treatment with neuraminidase. 70% of glycoprotein-bound sialic acid of granule membranes was susceptible to neuraminidase, while sialic acid of intact zymogen granules was resistant to this treatment.

A second strategy was employed. Glycoproteins of cell surfaces with ex-

TABLE VI

ASYMMETRIC DISTRIBUTION OF SIALIC ACID ON INTACT ZYMOGEN GRANULES AND GRANULE MEMBRANES

Solutions containing either 5 mg of zymogen granules or 0.37 mg of ZGM-1 were incubated in 0.3 M sucrose containing 0.064 unit of neuraminidase for 40 min at 27° C. Where noted, the enzyme was heated for 10 min at 95°C prior to addition. The particulate fractions were collected by centrifugation at 93 000 × *g* for 1 h. The fractions were washed by resuspension in distilled water and recentrifugation. Carbohydrate analysis after lipid extraction was then performed using gas-liquid chromatography. Values are expressed as percent ± S.D. for three determinations based upon values of 6 nmol *N*-acetylneuraminic acid for granule membranes (Table V).

Fraction	Addition	Sialic acid
Intact zymogen granules	Neuraminidase	100 ± 10
	Heated enzyme	100 ± 10
Zymogen granule membranes	Neuraminidase	40 ± 18
	Heated enzyme	100 ± 11

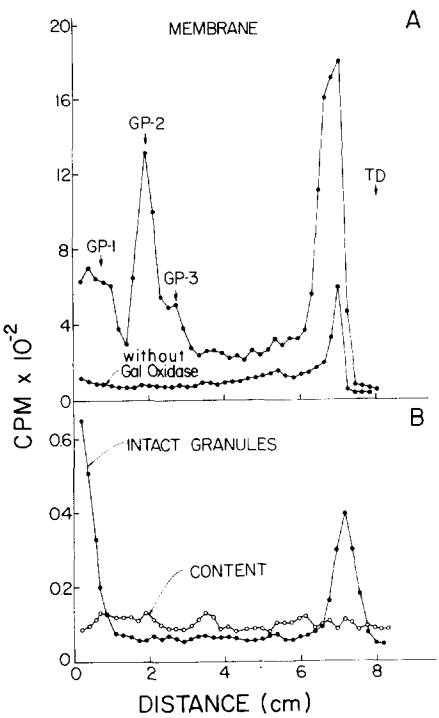

Fig. 6. Electrophoretic analysis of intact zymogen granules and granule subfractions labeled with NaB^3H_4 following galactose oxidase treatment. Granule fractions (30—50 μg) were incubated in 0.17 M NaCl containing 1 mM sodium phosphate, pH 7.2, galactose oxidase (15 units/ml) and soybean trypsin inhibitor (10.25 mg/ml). Intact granules were incubated in a medium containing in addition 0.3 M sucrose. Intact zymogen granules were recovered by centrifugation at 1000 × *g* and were washed several times in 0.3 M sucrose. The zymogen granule content was precipitated with 10% trichloroacetic acid. Zymogen granule membranes were recovered by centrifugation at 100 000 × *g* for 1 h. Samples were electrophoresed in polyacrylamide gels containing 1% SDS. Gels were fractionated and counted as described in Materials and Methods. (A) Zymogen granule membranes. The positions of GP-1, GP-2, GP-3 and the tracking dye (TD) are indicated by arrows. (B) Intact granules (closed circles); zymogen granule content (open circles).

posed galactosyl moieties can be oxidized by galactose oxidase and reduced by treatment with NaB^3H_4 [40]. In the experiment summarized by Fig. 6, intact zymogen granules, zymogen granule content or granule ghosts were reduced by NaB^3H_4, with or without prior exposure to galactose oxidase. The high molecular weight component, GP-1, and glycolipids were the only species in intact zymogen granules that were labeled by this procedure (Fig. 6A). None of the glycosylated species of the granule content was labeled (Fig. 6B). In contrast, GP-1, GP-2 and GP-3 were labeled by sequential treatment of the granule ghosts with galactose oxidase and NaB^3H_4. Little radioactivity was incorporated into the fraction without prior incubation with galactose oxidase. The results suggest that GP-2 is inaccessible to membrane-impermeable reagents.

Discussion

In this study, zymogen granule membranes from rat pancreas were judged to be 90% pure by the following criteria: (A) Glycoprotein galactosyltransferase, a pancreatic Golgi membrane marker, could not be detected in ZGM-3 [9]. (B) Active amylase represented no more than 1% of ZGM-3. (C) Cytochrome *c* oxidase activity and analysis of polypeptide profiles indicated that mitochondrial membranes represented less than 1% of the granule membrane protein. (D) Mixing experiments demonstrated that 6% of the granule membrane protein represented secretory protein. (E) Ribose, an indication of contamination by rough microsomes, was not detected in ZGM-3.

Sugars characteristic of mucopolysaccharides, such as xylose and glucuronic acid, were not detected in rat zymogen granules, nor were these sugars detected in dog zymogen granules [41]. Elsewhere we have reported that the rat pancreas in vitro can incorporate inorganic $^{35}SO_4^{2-}$ into heparan sulfate [42]. The very low levels of heparan sulfate in zymogen granules make it unlikely that the concentration of basic secretory proteins depends exclusively upon a charge neutralization mechanism with this acidic mucopolysaccharide [43].

RNAase from rat pancreas is not glycosylated, nor are the major enzymes and proenzymes from rat pancreas [34]. The paucity of carbohydrate in the secretory proteins from rat pancreas was verified by gas chromatographic analyses. The minor glycoproteins of the granule content were elctrophoretically distinguishable from granule membrane glycoproteins; their identity remains to be established.

We earlier suggested that Band 2 (GP-2) is a glycoprotein and is, moreover, the predominant protein of mammalian zymogen granule membranes [6]. The present study supports these proposals. (A) The major periodic acid-Schiff-positive bands in stained electrophoretograms of zymogen granule membranes corresponded to the major polypeptide. (B) Both radioactive leucine and glucosamine were incorporated into GP-2. (C) GP-2 was degraded by treatment of zymogen granule membranes with trypsin. We recently isolated and partially characterized GP-2 from dog pancreas [41]. GP-2 accounts for 45% of the granule membrane protein from dog pancreas and 35—40% of the granule membrane protein from rat pancreas. Thus, GP-2 may serve an important structural role in rat and dog granules.

Comparisons of the different pancreatic membrane classes were under-

taken to gain insights into their relationships with zymogen granule membranes. Studies of guinea pig pancreas had suggested that the granule membrane was distinct from microsomal subfractions [11,29]. However, granule membrane polypeptides were insufficiently resolved from contaminants to detect intrinsic membrane components [29,30]. Our previous studies showed that rat zymogen granule membranes were less complex than membranes of Golgi-rich fractions (their putative precursor) and indicated that GP-2 accounted for less than 5% of the protein of Golgi membrane-rich fractions from rat pancreas [9]. In the present study, electrophoretic analyses demonstrate that the preponderance of membrane polypeptides of mitochondria, microsomal subfractions, nuclei and the 700 × *g* pellet possessed molecular weights less than that of GP-2. Only smooth microsomal membranes exhibited a glycoprotein profile resembling that of ZGM-3.

An endogenous galactosyltransferase of smooth microsomes and Golgi membrane-rich fractions can glycosylate glycoproteins that possess the same electrophoretic mobilities as GP-1, GP-2 and GP-3 [10]. The endogenous acceptors are antigenically similar to the granule membrane glycoproteins and we proposed that relatively low levels of precursors of the zymogen granule membrane are present in Golgi membranes. None the less, the marked differences in enzymic activities and polypeptide compositions between zymogen granule membranes and membranes derived from the Golgi complex implicate a mechanism by which granule membrane constituents are segregated from Golgi membrane glycosyltransferases. The molecular basis for this segregation remains an intriguing question for future investigation.

Our data are in accord with a multi-site mechanism for membrane glycoprotein synthesis. Thus, glycoproteins of rough microsomal membranes contained the lowest level of carbohydrate and the lowest ratios of galactose, fucose and sialic acid relative to mannose. Smooth microsomal membranes (derived from the Golgi complex [9]) contained substantially higher levels of carbohydrate than rough microsomal membranes and were enriched in these terminal sugars, while zymogen granules (produced by the Golgi complex) exhibited the highest level of glycosylation.

The high levels of terminal glycose moieties in zymogen granules suggest that a battery of glycosyltransferases associated with the Golgi complex participates in the glycosylation of zymogen granule membranes, analogous to the biosynthesis of membrane glycoproteins of the intestinal mucosa [44]. Evidence that the Golgi complex participates directly in zymogen granule membrane glycoprotein synthesis is our observation that a Golgi membrane-associated glycoprotein galactosyltransferase can glycosylate endogenous acceptors resembling GP-1, GP-2 and GP-3 [10].

Several components of isolated zymogen granule membranes could be modified by neuraminidase or galactose oxidase treatment. However, GP-2 and GP-3 in intact zymogen granules were resistant to these enzymes. Caution must be used in interpreting these results. GP-2 and GP-3 would be inaccessible if localized on the cisternal (inner) surface of zymogen granules. Alternatively, glycosyl moieties of the granule surface were unmasked during granule lysis. We feel that the former explanation is more likely, since exposure of the granules at pH 8.2 is a mild treatment, not be expected to cause a dramatic reorganiza-

tion of intrinsic membrane components. If GP-2 is on the inner granule membrane, fusion of storage granule membranes with the apical cell surface [7] would result in the appearance of GP-2 on the outer cell surface. Supporting the prediction is the observation that a periodic acid-Schiff-positive band resembling GP-2 was detected in membranes of the 700 × *g* pellet, shown by other investigators to be enriched in plasma membranes [11]. Furthermore, data to be published elsewhere indicate that exocytosis is accompanied by the appearance of zymogen granule membrane polypeptide antigens on the acinor cell surface (O'Donnell, Jr., J.J. and Ronzio, R.A., in preparation).

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