STUDIES OF EXOCYTOSIS IN ACINAR CELLS OF ADULT RAT PANCREAS

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ABSTRACT

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By

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Exocytosis is the final step in secretion in mammalian cells. The process by which this occurs in the exocrine pancreas involves the fusion of the zymogen granule membrane with the plasma membrane, resulting in discharge of the secretory product. The aim of this research was to establish biochemical evidence for the fusion of the zymogen granule membrane to the plasma membrane during secretogogue-stimulated secretion.

A fraction enriched in plasma membrane (700 x g pellet) was prepared from homogenized tissue by discontinuous gradient centrifugation, with subsequent extraction with 0.25 M NaBr. The membranes of this fraction were compared with other intracellular membranes by SDS polyacrylamide gel electrophoresis. The gels were stained for protein with Coomassie blue, and for carbohydrate by the periodic acid-Schiff procedure. The zymogen granule membrane profile was determined to be unique with respect to the other intracellular membranes. With the exception of smooth microsomal membranes, no protein which comigrated with GP-2, the major glycoprotein in zymogen granule membrane, was detected in the various intracellular membrane fractions. The species detected in smooth microsomes represented only 3% of the total membrane protein.

Fluorographic analysis of membrane proteins labeled with ^{125}I were analyzed during secretogogue-stimulated secretion. No changes in the labeling profiles were detected between nonsecreting and secreting cells. The general specificity of the labeling technique prevented detection of the appearance of zymogen membrane proteins on the cell surface during secretion.

Studies involving the use of iodinated Staphylococcal Protein A, coupled with rabbit antisera directed against determinants of the zymogen granule membrane, revealed a tenfold increase in the appearance of zymogen granule membrane antigenic determinants on the cell surface during the first 2 hours of secretogogue-stimulated secretion. A decrease in zymogen granule membrane antigenic determinants to the original level was detected during the third hour. This study presents preliminary biochemical evidence for the fusion of zymogen granule membrane with the plasma membrane during secretogogue-stimulated secretion in the rat exocrine pancreas.

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OF ADULT RAT PANCREAS

By

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ABBREVIATIONS

FMA	fluorescein mercuric acetate
RER	rough endoplasmic reticulum
KRB	Kreb's Ringer bicarbonate solution
EDTA	ethylenediaminetetraacetate
BPA	bovine plasma albumin
DMSO	dimethylsulfoxide
SDS	sodium dodecyl sulfate
TEMED	tetramethylethylenediamine
BHT	butylated hydroxytoluene
Con A	concanavalin A
PBS	phosphate buffered saline
GP	glycoprotein
ZGC	zymogen granule content

LITERATURE REVIEW

Secretion with Exocrine Pancreas

The basic process by which secretory cells synthesize, transport, and export products was elucidated through a series of experiments by Palade and co-workers.[1-4] The studies involved electron microscopy and cell fractionation experiments which led to the accepted theory of the secretory process summarized below:

Step I. Amino acids are incorporated into the digestive enzymes, proenzymes and zymogens on the ribosomes attached to the outer surface of the membranes of the rough endoplasmic reticulum.

Step II. After completion of peptide synthesis, the proteins are sequestered in the intercisternal spaces of the rough endoplasmic reticulum. It has been proposed[5] that the nascent peptide grows within a channel or groove in the large subunit of the ribosome and eventually penetrates the endoplasmic reticulum membrane through a dynamic or continuously opened orifice. Recognition of this orifice has been described as the signal hypothesis and is summarized as follows: The essential feature of the signal hypothesis is the occurrence of a unique sequence of codons, located immediately to the right of the initiation codon, which is present only in those mRNAs whose translation products are to be transferred across a membrane. No other mRNAs contain this unique sequence. Translation of this signal sequence of the nascent chain from within a space in the large ribosomal subunit triggers attachment of the ribosome to the membrane, thus providing the topological conditions for the transfer of nascent chain across the membrane.[6] The polypeptide is able to enter the cisternal spaces directly upon completion.

Step III. The proteins are transferred from the rough endoplasmic reticulum to the transitional elements of the rough endoplasmic reticulum (part rough surfaced, part smooth surfaced), where these membranes bud off and migrate to the Golgi complex. These transitional vesicles are concentrated at the Golgi complex by fusion with preexisting condensing vacuoles or with each other. Condensing vacuoles lie near the Golgi complex and probably arise by pinching off at the apical peripheri of the Golgi complex. Within these vacuoles condensation occurs; this may involve concentration of the vacuole contents by loss of water and interacting of the protein with other components, such as polysaccharides.[7] The final result of this condensation is the appearance of mature zymogen granules.

Step IV. Mature zymogen granules migrate to the cell apex where they fuse with the plasma membrane releasing their content into the extracellular spaces when stimulated by hormone. This final step has been termed exocytosis.

In the second stage of the study Palade et al. defined the metabolic requirements of the transportation of secretory proteins. The transport of completed proteins to the Golgi complex from rough endoplasmic reticulum is not caused by a continuous accumulation of newly synthesized proteins. Addition of cycloheximide (10^{-4} M) inhibits protein synthesis by 95% but did not inhibit transfer of completed proteins from the rough endoplasmic reticulum to the Golgi complex. It was further shown that this stage of the secretory process was not energy dependent as it was unaffected by glycolytic inhibitors (fluoride, iodoacetate), respiratory inhibitors (N_2, cyanide, Antimycin A), and inhibitors of oxidative phosphorylation (dinitrophenol, oligomycin).[4] The next steps in the secretory pathway are energy dependent. Concentration of transitional elements into mature zymogen granules requires energy in the form of ATP, generated by oxidative phosphorylation. Hokin and Schramm[8] had shown earlier that the final step of secretion, exocytosis, is dependent on respiratory energy.

Meldolisi[9] coupled *in vitro* studies with *in vivo* experiments to elucidate protein turnover in membranes. For *in vitro* studies pancreas slices were pulse labeled for 3 minutes with L(4,5 ^{3}H) leucine followed by a 150-minute chase, with an excess of unlabeled leucine. The specific activity of the membrane proteins remained unchanged during the chase incubation, the specific activity of the membranes being 1/15 to 1/30 of those of the secretory proteins. This implied a different rate of synthesis between membrane and secretory protein. The *in vivo* experiments utilized a double label technique developed by Schimke.[10] L(4,5 ^{3}H) leucine was injected 4 1/2 or 6 1/2 days and L(U-^{14}C) leucine was injected 13 hours before sacrifice. By comparing ^{14}C/^{3}H ratios, relative protein turnover can be determined, as a high turnover would result in a relative high ^{14}C/^{3}H ratio, whereas slow turnover would result in a low ^{14}C/^{3}H ratio. The double isotope ratios implied that membrane proteins of rough, smooth (Golgi complex), and zymogen granule membranes had half-lives of 3-6 days. In a separate study secretory proteins of the rat pancreas were reported to have half-lives of 10-20 hours.[11] Cellular membranes of the guinea pig pancreas have considerable differences in their composition.[12] Comparison of polypeptide profiles has shown that the rough microsomal, smooth microsomal membranes, and plasma membranes contain ∿40 polypeptide species with molecular weights ranging from 150,000-15,000. Although the polypeptide profiles are somewhat similar, the membranes have different lipid composition and have characteristic enzymic activities. This suggests that the membranes do not mix randomly with one another during secretion. Results of studies of rat pancreas support these hypotheses.[13,14] Characterization of the Golgi complex membranes (smooth microsomal membranes) showed that the Golgi membranes contain a glycoprotein galactosyltransferase which is not present in significant amounts in other cellular membranes. Glycoproteins are a major component of zymogen granule membranes as well as some of the secretory proteins of the granule content.[15] The absence of galactosyltransferase in the zymogen granule implies the involvement of the Golgi complex in post-translational events (i.e., glycosylation) involving zymogen granules.

Studies of intracellular membranes involved in secretion have shown the path to be a discontinuous one.[9,16] There is no distinct continuity of the membranes, nor are there distinct limits to the different cell compartments. Interactions between the membranes must also be controlled since the membranes retain their identity throughout the secretory process. In addition, the fact that membrane protein synthesis and turnover proceed at rates slower than those of secretory proteins suggests that acinar pancreatic cells possess mechanisms for the recycling or continuous use of these membranes. This brief survey suggests that in studying secretion not only should the synthesis and discharge of secretory products be considered, but also the movement, interaction, and function of the membranes involved in the secretory process. In considering the function of the zymogen granule, I have focused on the process of exocytosis, which involves the interaction of the zymogen granule membrane with the plasma membrane. The result of this interaction is the fusion of the zymogen granule with the plasma membrane and subsequent release of the secretory proteins.

Exocytosis

Electron microscopy studies provide evidence for granule fusion in many secretory tissues, as is shown by Table 1.[17] These morphological observations usually entail a series of electromicrographs, first showing the granule in close proximity with the plasma membrane, with another picture showing an invagination in the plasma membrane consistent with the size of the secretory granule. The results are equivocal. One problem is that a static analysis (electron micrographs) is being used to study a dynamic process (exocytosis). There is an uncertainty in the interpretation of the electron-micrographic image of the membranes being fused together rather than separated by a very small gap. Another limitation is that the fixation of the membranes for electron microscopy may significantly affect their structure.[40] Any change in ionic strength, ionic species or temperature can affect the membranes and the attractive forces between the membranes.[41] Reduction in the numbers of zymogen granules within a relatively short time after secretory stimulus is applied also supports the theory of membrane fusion. Perhaps the best evidence to date for fusion of secretory granule membranes with plasma membrane comes from studies on the change in the surface area of the secretory cell after secretion.[43] These studies have shown that the surface area and hence the plasma membrane increases more than 300% during secretion. Freeze fracture studies show pits on the surface of

Table 1. Membrane fusion in secretion and exocytosis

Tissue	Diameter of secretory granule	Substance secreted	Electron-microscopic demonstration of fusion	Reference
Adrenal medulla	0.2	Catecholamines	+	18
Neurohypophysis	0.15	Oxytocin	+	19
		Vasopressin	+	20
Adenohypophysis	0.25	Melanotropin	+	21
	0.1	Thyrotropic hormone	+	22
	0.25	Luteinizing hormone	+	23
	0.3	Growth hormone	+	24
	0.6	Prolactin	+	25
	0.2	Adrenocorticotropin	+	26
Pancreas	0.3	Insulin	+	27
	1.0	Digestive enzymes	+	31
Salivary gland (parotid)	1.0	Amylase	+	32
Polymorphonuclear leukocytes	1.0	Lysosomal enzymes	+	34
Mast cells	0.4	Histamine	+	35
Platelets				
(β-granules)	0.3	β-Glucuronidase	+	36
(dense granules)	0.1	5-Hydroxytryptamine	+	37
Cholinergic synapses	0.05	Acetylcholine	+	38
Adrenergic synapses	0.08	Noradrenaline	+	39

pancreatic B cells during secretion.[42] To demonstrate unequivocally the fusion of secretory granule membrane to the plasma membrane, two biochemical requirements should be met:

1. There must be a biochemical marker for the zymogen granule membrane.
2. There must be a method for detecting the appearance of this zymogen granule membrane marker on the cell surface during secretogogue-stimulated secretion.

The rat exocrine pancreas fulfills the first requirement and is capable of providing a situation which fulfills the second.

The zymogen granule membrane has been well characterized.[13-15,44] The zymogen granule membrane has a simpler protein composition than other intracellular membranes.[15,46] When fractionated by SDS polyacrylamide gel electrophoresis, the zymogen granule membrane consists of 9 protein species. The zymogen granule membrane is characterized by a glycoprotein which has a molecular weight of ∿74,000, which accounts for ∿40% of the membrane protein. This glycoprotein has been designated GP-2. A species similar to GP-2 has been detected in smooth microsomal membranes. This species accounted for less than 3% of the membrane protein. GP-2 has not been detected in the rough or mitochondrial membranes nor in the zymogen granule content or the postmicrosomal supernatant.[14]

Ronzio and Morhlok[45] have developed an antiserum specific for rat zymogen granule membrane. This antiserum pre-absorbed with zymogen granule content was characterized by double diffusion in 0.5% agarose. The serum did not cross-react with zymogen granule content nor with solubilized microsomes of 100,000 x g supernatant fractions from rat spleen, lung, liver, kidney, stomach, or small intestine.

These results imply that zymogen granule membrane antigens are tissue specific. At the equivalence point antigranule membrane serum precipitated 70-80% of the total granule membrane.

In studying endogenous galactosyltransferase acceptors, antisera against zymogen granule membrane specifically precipitated 35-40% of the total radioactivity in the microsomal, smooth microsomes and fractions of the Golgi complex labeled with UDP(^{14}C) galactose. Unlabeled zymogen granule membrane competed for binding with the antisera, whereas liver microsomal membranes did not.

Analysis by polyacrylamide gel electrophoresis showed that the antisera precipitated three bands. These bands comigrated with GP-1, GP-2, and GP-3 of the zymogen granule membrane profile. This suggests that zymogen granule membrane precursors may be glycosylated at the Golgi complex.

Studies on rat zymogen granules[46] have shown that glycoproteins of intact zymogen are resistant to neuraminidase treatment, whereas in isolated zymogen granule membranes glycoproteins were easily degraded by neuraminidase. In addition glycoproteins, especially GP-2, in zymogen granules were not labeled by treatment with galactose oxidase followed by reduction with NaB^3H_4, whereas isolated zymogen granule membranes were labeled during this procedure. These results imply that glycoproteins, GP-2 in particular, are located on the inner (cisternal) side of the zymogen granule membrane.

In summary, the zymogen granule membrane has a unique polypeptide composition. GP-2, a glycoprotein of 74,000 molecular weight, represents 40% of the granule membrane protein and GP-2 is localized inside zymogen granules. Thus, GP-2 is an appropriate marker to follow the path of the zymogen granule through the final steps in secretion.

Isolation and characterization of the plasma membrane of the exocrine pancreas would provide the best and least ambiguous way to fulfill the second requirement for the study of exocytosis. Comparison of isolated plasma membranes before and after hormone induced secretion should detect appearance of any zymogen granule membrane marker after secretion.

Plasma Membrane Isolation and Characterization

Morphologically plasma membranes are characterized in electron micrographs as long smooth strands of membrane or smooth membrane vesicles.[47,48] Electron micrographs can be used to monitor the relative contamination due to cell debris and intracellular membranes which are morphologically distinct, i.e., rough endoplasmic reticulum. Electron micrographs cannot delineate between smooth membranes from different organelles, i.e., Golgi complex, zymogen granules, and plasma membranes.

Meldolisi[48] has isolated plasma membranes from homogenized guinea pig pancreas tissue and describes the morphology as follows: Most of the membranes appear as packed sheets intermingled with layers or masses of fibrillar material. The membranes occur either as long sheets with free margins, as large vacuoles, or as paired sheets held together by recognizable junctional elements such as occluding zonules and desmosomes.

The fibrillar material mentioned above appears to be from two different sources: fragments of basement membrane layers which apparently firmly adhere to pieces of basal plasma membrane, or bundles of fibrils associated with desmosomes and identified because of this as fragments of the terminal web of the apical region of the exocrine cells.

These results provide an interesting dilemma. The fibrillar material, occluding zonules, and desmosomes attached to the plasma membrane fragments provide a morphological marker for the plasma membrane, yet these markers are not true components of plasma membranes but contaminants from the basement membrane. It is obvious that morphology cannot be used as sole evidence for plasma membrane isolation. Therefore, membrane workers must be used in unison with morphological evidence. Intrinsic membrane enzymes unique to certain membranes, i.e., cytochrome C oxidase in mitochondria, are often used and the relative specific activities are used to determine the degree of purity.

The majority of plasma membrane characterization has been done with rat liver cells. From these studies a battery of enzyme assays has been developed which have found use in plasma membrane isolations from other tissue.[49-59] A typical plasma membrane isolation may use: 5'nucleotidase, ATPases, alkaline phosphatase, and/or adenylcyclase. Again, problems arise when considering pancreas. Comparison of enzyme activities from guinea pig[57] shows that there is no one enzyme that is unique to or highly localized in the plasma membrane. The plasma membrane fracture has a relatively high Mg^+ ATPase activity, but the activity localized in the zymogen granule is ∿75% of the plasma membrane value. The activity of β leucyl naphthalaminidase in plasma membrane was approximately equal to that in smooth microsomes. NADH cytochrome C reductase (rotenone insensitive) activity in the plasma membrane was 25% of rough and smooth microsome values.

Before isolating a cell's plasma membrane, certain considerations should be decided on. First, it is advantageous to start with a homogeneous population of cells, instead of using whole tissue. In general, solid organs are composed of more than one cell type and the rat pancreas is no exception. The rat pancreas consists of 70-80% acinar (exocrine) cells, while the rest of the tissue is composed of endocrine (β) cells as well as duct cells. Connective tissue is also present in the pancreas, which results in further heterogeneity during tissue homogenization. This problem may be avoided since guinea pig exocrine cells can be dissociated and obtained in high yield. Properties of these cells will be considered later in this section.

Though plasma membranes have been isolated from a range of different tissues, as shown in Table 2,[47] the technique most often used involves utilizing basic properties of the membrane in question to separate it from other intracellular membranes of the homogenization. This technique involves the use of continuous and/or discontinuous sucrose gradients which rely on differences in density or sedimentation characteristics to achieve separation. An isolation procedure usually follows a procedure similar to the one outlined below:

> After homogenization of the tissue, or disruption of the cell suspension, the homogenate is centrifuged at low speeds in low density gradients for a short time, i.e., 500 x g .3 M sucrose for 10 minutes. This results in the separation of the cell particulate (cellular membranes, nuclei), which forms a pellet, from the cell supernatant (soluble cytoplasmic material), which cannot penetrate the gradient fully. The particulate pellet is resuspended and, depending on the particular case,

Table 2. Isolation of plasma membrane from different tissue

Organ	Animal	Reference
Liver	rat	48-53
	cow	54
	pig	55
	mice	56
	guinea pig	57-58
Solid hepatomas	rat	59
	mice	60
Small intestinal epithelium	rat	61
	hamster	62-65
	guinea pig	63-64
	rabbits	66
Kidney	rat	67
	guinea pig	68
	rhesus monkey	69-70
Bladder epithelium	rat	71
Skeletal muscle	rat	72
Pancreas	guinea pig	48
Mammary gland	bovine	73
Ascites tumor cells	mouse	74-75
Platelets	human	76

is placed on a continuous or discontinuous gradient of higher density (i.e., 0.5-2.3 M sucrose) and centrifuged at higher speeds for longer periods of time (i.e., 90,000 x g 2 hr). The result is that intracellular membranes travel through the gradient and separate into discrete bands at various densities according to their individual properties (density, shape).

A point of caution should be mentioned. Exocrine pancreas plasma membrane is a specialized membrane that is functionally divided. The basal side is involved with intercellular communication (i.e., hormone binding), whereas the apical membrane is involved with exocytosis, while the lateral membrane is involved with intracellular adhesion. It is reasonable to assume that these sections of the plasma membrane may be structurally different and this could result in different migration patterns in the gradients. This possible problem could be monitored with the use of ^{125}I as a marker for the plasma membrane. (Iodination techniques are reviewed later in this section.) After homogenization and gradient centrifugation, membrane bands would be monitored for membrane-bound -^{125}I. Lewis et al.[77] have used this approach to isolate basolateral plasma membrane of intestinal epithelial cells. Two gradient peaks of membrane-bound ^{125}I were detected. One coincided with brush border enzymes such as alkaline phosphatase and β leucyl naphthylaminidase, whereas the other was coincident with the major peak of (Na^+, K^+) ATPase which has been thought to be concentrated in the basolateral plasma membrane.

Chang, VannBennett, and Cuatrecasas[78] have used membrane receptors as general markers for plasma membrane isolation. ^{125}I labeled insulin, cholera toxin, wheat germ agglutinin, and concanavalin A have been used. These markers can be used at very low concentrations, 10^{-9} to 10^{-11} M, are of high affinity, and are reversible. Wheat germ agglutinin and concanavalin A seem to be the compounds of choice. Cholera toxin reacts readily with nuclear membrane as well as plasma membrane, and insulin is restricted to use with hormone sensitive tissue, and the uneven or selective labeling which occurs in cells whose membranes are functionally divided (pancreas).

Using a discontinuous sucrose gradient Meldolisi[48] obtained the plasma membrane from homogenized guinea pig pancreas. The preparation was characterized as free of subcellular components with the exception of occasional lysosomes or lysosomal residues, also, contamination contained basement membrane mentioned previously.

Brunette and Till[79] have isolated plasma membranes from L cells using an aqueous 2-phase dextran-polyethylene glycol system developed by Albertson.[80] This system separates particles on the basis of differences in their surface properties, primarily surface charge. This procedure is rapid (2 hours after homogenization) and results in high yields with good purity.

Warren[81] has isolated plasma membrane from L cells in culture. The underlying principle of the methods is simple: surface membranes are stabilized by heavy metal ions (Zn^{+2}) by blocking -SH groups with fluorescein mercuric acetate (FMA) or Tris base. Stabilization takes place in a hypotonic medium so the cells swell and the surface membrane rises off the underlying cytoplasm. The cells are broken by homogenization and membranes are isolated by differential centrifugation on sucrose gradients.

Properties of Isolated Exocrine Pancreas Cells

Amsterdam and Jamieson[82] have reported a procedure for the dissociation of adult guinea pig pancreas into exocrine cells using chromatographically pure collagenase and chymotrypsin and chelation of divalent cations by EDTA in the Kreb's Ringer bicarbonate buffer. They report also the properties of the isolated exocrine cells as follows: in the isolated exocrine cell desmosomes and hemedesmosomes were rarely seen, zonulae adherent when present were ill defined, collagen and basement membrane had completely disappeared. Although the isolated exocrine cells were rounded up, they retained their *in situ* polarity with regard to surface specialization of the plasma membrane and distribution of the cytoplasmic organelles. The former apical plasma membranes were identified by the presence of associated microvilli which overlapped a region of cytoplasm which contained the zymogen granule population. The remainder of the plasma membrane consisted of a unit membrane which was free of basement membrane. Invaginations similar to pinocytic pits were located along the former basal plasma membrane. Pinocytic pits in this region are characteristic features of exocrine cells *in situ*. In addition to the placement of zymogen granules other organelles characteristic of exocrine cells also retain their *in situ* polarity in the isolated cells. The Golgi complex is centrally located between the nucleus and the former basal pole, which also contains the majority of the rough endoplasmic reticulum (RER). Autophagic vacuoles containing ribosomes, RER, and unidentified membranes are also common. After 4 hours of incubation in KRB-1% BPA, isolated cells retained their polarity and were well preserved. A morphometric analysis showed that although there were fluctuations in volume of the various organelles, the increase in volume of the Golgi zone, condensing vacuole, and autophagic vacuoles appeared to result in the simultaneous and equivalent decrease in the volume of the RER and cytoplasmic matrix, and nuclear to cytoplasmic volume ratios of isolated exocrine cells were similar to those of exocrine cells *in situ*. Bolander reported steriological data on normal guinea pig pancreas which gave similar results for organelle volume.

In a subsequent paper Amsterdam and Jamieson[83] described the functional characteristics of the isolated exocrine cells with respect to the protein synthesizing capacity and the ability to discharge exportable proteins in response to secretogogues. The protein synthesizing capacity of the isolated cells was monitored by measuring the kinetics of incorporation of the (^{14}C) leucine into proteins of total cell population. (^{14}C) leucine was incorporated into acid precipitable protein at a linear rate over 4 to 5 hours with approximately 3% of total (^{14}C) leucine incorporated into protein.

Quantitative electron microscopy autoradiography showed approximately 70% of cells fell within a narrow grain density, 0.3-0.6 gm/cm^2, indicating that the entire exocrine cell population of the gland is reasonably synchronized and uniform in its ability to synthesize protein. Pulse-chase experiments with the chase ranging from 10-240 minutes showed a migration of label as follows: RER → Golgi peripheral region → condensing vacuoles → zymogen granules at the cell apex.

Response of isolated exocrine cells to secretogogue gave similar results to previous studies of Jamieson and Palade[1-4] using tissue slices in which a lag of 20-30 minutes is seen before labeled proteins begin to appear in the medium. The lag is attributed to the time required for nascent labeled protein to be transported from the RER to the zymogen granules. One major difference found with isolated exocrine cells was release of secretory proteins in response to secretogogues, occurring at a rate of 2 to 3 times less that of pancreatic slices. Hormone concentrations needed for secretory response are approximately 10 times greater *in situ*. A probable explanation is that hormone receptor sites were altered by the protease treatment used during dissociation either by decrease in the number of sites and/or decrease in their binding capacity for hormones.

Using Amsterdam and Jamieson's procedure, exocrine cells have been isolated from rat pancreas. Isolated exocrine rat cells incorporate (^{3}H) leucine into protein and release the pulse labeled secretory product into the media upon addition of secretogogue. Kronquist et al.[84] obtained similar results for $^{35}SO_4$ labeled products.

Labeling Techniques

The plasma membrane is the cell barrier to the external environment. Thus, it is possible to place extrinsic labels specifically on the cell surface. These labels can be used as markers for membrane isolation and characterization. These labeling techniques are also used to monitor changes in the cell surface.[85] For a labeling technique to be successful in specifically labeling the cell surface, it is of critical importance that the technique satisfy the following criteria: the cell surface must be impermeable to the labeling substance, and the label should not modify the cell surface dramatically. The specificities of different labeling techniques range from being very specific, reacting with very few moieties to very general labels which react with carbohydrate, protein and lipid. The labeling techniques summarized below satisfy these criteria and also possess different specificities.

Iodination. Morrison[86] has reported a procedure for the enzymatic incorporation of iodide^{125} or iodide^{131} into proteins utilizing lactoperoxidase and hydrogen peroxidose generated by a glucose oxidase-glucose system. Fraker and Speck[87] have reported a procedure for iodinating proteins utilizing glycoluril as the catalyst for iodination. In both systems once the reactive species is generated it readily reacts with the aromatic ring of tyrosines to give either mono- or di-iodotyrosine. The ^{125}I is found exclusively in the ortho position. ^{125}I also reacts with histidines, but the product is not as stable as iodotyrosine.

Galactose oxidase followed by reduction with sodium borotritide. Terminal galactose and galactosamine residues of membrane glycoproteins and glycolipids can be specifically labeled following the procedure of Ghanberg and Hakomori,[88] in which galactose oxidase oxidizes the galactosyl or W-acetyl galactosaminyl residues to the corresponding D-galacto-hexodialdose. This oxidation product is then reduced with sodium borotritide, which incorporates ^{3}H at the 6 carbon position of the galactose or galactosamine residue. Galactose oxidase shows a strict specificity for galactose and W-acetyl galactosamine. If it is found that most of the glycoproteins and glycolipids are terminated by the disaccharide sialylgalactose, the sialic acid moiety can be removed by treatment with neuraminidase, prior to labeling by the galactose oxidase-sodium borotritide system.

Taurine derivative. Storvo and Ricardo[89] have described a procedure for labeling the cell surface utilizing a hydrophilic nitrene precursor reagent N-(4-azido-2 nitro phenyl)-2-amino ethyl sulfonate (NAP taurine) which, once photo activated, is capable of reacting with proteins, carbohydrates, and lipids. The predominant stable products are most likely secondary amines produced by insertion of the nitrene into carbon hydrogen bonds.[110] With NAP taurine it is possible to obtain an overall view of the cell surface.

Statement of the Problem

Evidence for insertion of zymogen granule membrane into the plasma membrane during the process of secretion is indirect and equivocal. Although it is accepted theory and electron micrographs support the theory, there is no biochemical evidence for membrane fusion during exocytosis.

The aim of this study is to establish biochemically evidence for the fusion of the zymogen granule membrane to the plasma membrane in isolated rat acinar cells during secretogogue stimulated secretion.

There is a biochemical marker for zymogen granule membrane, GP-2.[15,44] This study attempts to develop a technique to monitor the appearance of GP-2 on the cell surface during secretion. There are 2 main problems that have to be considered. The first involves a comparison of the zymogen granule to the plasma membrane. How distinct is the plasma membrane from the zymogen granule membrane? Isolation and characterization of the plasma membrane is necessary to make this analysis. The first part of this study involves attempts to isolate the plasma membrane and to compare it with the intracellular membranes.

If GP-2 is unique to the zymogen granule membrane, then biochemical techniques must be devised to detect whether the appearance of GP-2 on the cell surface is correlated with stimulus coupled secretion. The second part of this study deals with this problem.

MATERIALS AND METHODS

Preparation of Homogenates of Adult Rat Pancreas

When whole tissue was used, the tissue was homogenized by the procedure of Ronzio.[13]

Homogenates of cell suspensions were prepared as follows. Isolated cells from 4-6 pancreas (0.5-1.0 ml packed cells) were suspended and incubated in 10 ml of hypotonic phosphate buffered saline (155 mOsm) for 30 minutes at 4°C. This caused the cells to swell but not lyse. After 30 minutes the cells were collected by centrifugation at 7 x g for 5 minutes and suspended in 4 ml of hypotonic phosphate buffered saline. The cells were lysed by homogenization in a Thomas Ten Broeck Tissue Grinder, 15 ml size. Homogenization was monitored by phase contrast microscopy. When the ratio of nuclei to whole cells was about 2/1 (8-10 strokes), the homogenization procedure was stopped. The homogenization was not carried out until all cells were lysed to avoid possible shearing and breaking of long strands of plasma membrane. The cell homogenate was centrifuged shortly (7 x g, 2 min) to remove most whole cells. The resulting supernatant was designated the cell homogenate.

Preparation of Nuclear Membrane

Nuclear membranes were by a modification of the procedure described by Kasper.[90] Modifications were made to allow for the preparation of nuclear membrane and plasma membrane from the same cell suspension. The procedure was as follows. The cells were lysed as previously described and the homogenate was centrifuged twice at 700 x g for 10 minutes in 0.3 M sucrose containing 0.1 mg/ml SBTI at 4°C. The pellet, containing nuclei, plasma membrane, and heavy cell debris, was made 1.58 M by adding 3.2 volumes of 2 M sucrose containing 0.1% Dextran 500 (Pharmacia). The 1.58 M suspension (8 ml) was layered over 2 ml of 2.3 M sucrose. The gradient was centrifuged 192,000 x g for 1 hour. The nuclei formed a pellet, while the cell debris did not penetrate the 2.3 M sucrose. No contaminating particles in the nuclear preparation were seen when viewed under phase contrast microscopy. The nuclear pellets were collected and lysed by sonication in 0.2 M $NaHCO_3$ containing 0.1 mg/ml SBTI and 0.1 mg/ml DNase (Worthington). The suspension was pulse sonicated with a Biosonick Sonic Oscillator (Bronwill Scientific) at a setting of 50 for 20 seconds, at 4°C. The suspension was centrifuged at 192,000 x g for 1 hour. The supernatant was discarded and the nuclear membrane pellets were stored at -80°C.

Preparation of Rough and Smooth Microsomal Membranes

Rough and smooth microsomes were prepared following the procedure of Ronzio.[13] Membranes from these microsomes were prepared as follows. Microsomal pellets were resuspended in 2 ml of 0.15 M NaCl and lysed by dropwise addition of 0.2 M $NaHCO_3$ containing 0.1 mg/ml SBTI and incubated at 4°C for 1/2 hour. The suspensions were centrifuged at 192,000 x g for 2 hours at 4°C. Rough and smooth microsomal membrane pellets were resuspended in 0.25 M NaBr containing 0.1 mg/ml SBTI by sonication at a setting of 50 for 20 seconds, at 4°C. The suspensions were centrifuged at 192,000 x g for 1 hour. This washing procedure was repeated once and the washed membrane pellets were stored at -80°C.

Preparation of Mitochondrial Membrane

The filtered homogenate from at least 6 gm of tissue was centrifuged at 1600 rpm for 10 minutes. The supernatant was collected and centrifuged at 2800 rpm for 30 minutes. The mitochondria settled on top of the zymogen granule pellet and could be easily removed by pipet. The mitochondria were lysed by sonication in 0.25 M NaBr, centrifuged 100,000 x g for 2 hours, and the pellets stored at -80°C.

Preparation of Zymogen Granule Membrane

The procedure used for preparing zymogen granule membranes was that of Ronzio and MacDonald[15] as modified by Kronquist, Elmahdy, and Ronzio.[84]

Preparation of a 700 x g Particulate Fraction

Preparation of a 700 x g particulate fraction was by a modification of a procedure of Meldolisi.[48] The filtered homogenate from at least 8 gm of tissue or from a cell suspension from at least 5 pancreases was centrifuged at 700 x g for 10 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 0.3 M sucrose and recentrifuged at 700 x g for 10 minutes at 4°C. The second centrifugation removed most of the contamination due to microsomes and cell debris that was not removed in the first centrifugation, as judged by the clarity of the supernatant. The pellet was resuspended in 14.3 ml of 0.3 M sucrose at 4°C, and 45.7 ml of 2 M sucrose containing 0.1% Dextran 500 (Pharmacia) and 0.1 mg/ml SBTI were added dropwise with constant stirring at 4°C to bring the molarity to 1.58 M sucrose. Discontinuous gradients consisting of 9 ml of the 1.58 M suspension overlaid with 3 ml of 0.3 M sucrose were centrifuged at 80,000 x g for 1 hour. The 700 x g membrane fraction rose to the interface while the cell debris and nuclei formed the pellet. The interface was collected and an equal volume of H_2O was added, and the suspension was centrifuged at 192,000 x g for 1 hour. The pellet was resuspended in 0.25 M NaBr containing 0.1 mg/ml SBTI by sonicating for 20 seconds at a setting of 50, at 4°C. The suspension was centrifuged at 192,000 x g for 1 hour. This washing procedure was repeated once and the pellet was stored at -80°C. The 700 x g particulate fraction contained smooth membrane fragments as judged by electron microscopy.

Preparation of Isolated Exocrine Cells

Pancreatic acinar cells were isolated by using a modification of a procedure of Amsterdam and Jamieson.[85] The procedure was as follows. All dissociation and incubation media consisted of Kreb's Ringer bicarbonate solution (KRB) equilibrated with 95% O_2 and 5% CO_2, and contained a complete amino acid supplement and 14 mM glucose.

After collection of the gland the procedure was initiated by injecting 5 ml of enzyme dissociation media (KRB containing 0.8 mg/ml collagenase II [Worthington], 0.36 mg/10 ml α-chymotrypsin [Worthington]) into the interstitium of the gland. This caused the gland to become distended and rapidly exposed the majority of the acini to the enzymes. The distended gland and the residual enzyme solution were transferred to a 25 ml Erlenmeyer flask and incubated at 37°C for 12 minutes.

The gland was incubated in the enzyme solution for 12 minutes at 37°C. The gland was then incubated for the following times at 37°C: 10 minutes in 10 ml KRB containing 2 mM EDTA (to remove divalent cations), then 5 minutes in 10 ml KRB containing 0.1 mM $CaCl_2$. The gland was then transferred to 5 ml KRB enzyme dissociation media (50.2 units/ml collagenase II, 26 units/ml α-chymotrypsin). The length of this incubation varied but usually was approximately 32 minutes. The extent of dissociation was monitored by the use of phase contrast microscopy. When cells spontaneously came off the periphery of the gland and cells were flowing through the intraluminal spaces, the dissociation was stopped by addition of 5 ml KRB containing 5% BPA, 0.1 mg/ml SBTI. The suspension was gently mouth pipetted approximately 20 times to complete the dissociation. From this point on, unless otherwise mentioned, the cells were kept at 4°C. The cell suspension was filtered through 26 μm and then 20 μm mesh nylon gauze to remove any undissociated tissue, or cell clumps, as well as any connective tissue.

To separate the intact cells from enzymes and cell debris, 4 ml of the suspension was overlaid on 8 ml of KRB containing 5% BPA, 0.1 mg/ml SBTI and centrifuged at 7 x g for 10 minutes in a Sorvall G-L2 centrifuge. The supernatant, which contained contaminants and cell debris, was discarded. The cells formed a tan pellet. These cells were rewashed by resuspending the pellet in KRB containing 5% BPA, 0.1 mg/ml SBTI and centrifuging at 7 x g for 5 minutes. The pellet obtained was a homogeneous suspension of single cell acinar cells, free of debris. Their viability was greater than 95% as measured by methyl-green exclusion.

In vitro Radioactive Labeling with ^{125}I

Proteins on the cell surface were iodinated following the procedure of Speck and Fraker,[87] which utilizes 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (glycoluril) as the catalyst for iodination. This method was mechanically easier while being as efficient as the lactoperoxidase-glucose oxidase catalyzed iodination. Glycoluril is insoluble in aqueous solution and therefore was dissolved in chloroform. A film of the catalyst formed on the walls of a 12 ml conical centrifuge tube when aliquots (50 μg glycoluril/250 μl chloroform) were evaporated under N_2. For all iodination experiments, unless otherwise noted, the reaction volume did not exceed 0.5 ml; cell concentration did not exceed 0.25 ml packed cells (approximately 20 x 10^6 cells). Two hundred microcuries ^{125}I was used for iodination. The reaction was carried out at 4°C for 30 seconds and was stopped by removing the reaction mixture from the glycoluril coated tube.

After iodination the cells were washed 3 X with KRB containing 0.25 M NaI and 0.1% SBTI to remove excess unbound ^{125}I.

A cell particulate fraction was obtained by lysing the cells by sonication at the maximum setting for 20 seconds, at 4°C. The lysate was centrifuged at 192,000 x g for 1 hour.

A fraction of the supernatant was collected and used to monitor the degree of cytoplasmic labeling. The specific activity of the supernatant was usually 5-10% that of the cell particulate. The rest of the supernatant was discarded.

The pellet which contained the cell particulate was resuspended in 0.25 M NaI containing 0.1 mg/ml SBTI by sonicating as mentioned above. The suspension was centrifuged at 192,000 x g for 1 hour and the pellet was collected and stored at -80°C.

The 0.25 M NaI extraction removed any unbound ^{125}I remaining and any cytoplasmic or secretory proteins which had adhered to the membranes. This was monitored by a reduction in the amylase band in the Coomassie blue profile of cell particulate fractionated on polyacrylamide gels.

Radioactive Labeling of Protein A with ^{125}I

Protein A (Pharmacia) was also iodinated using glycoluril as the catalyst using the following conditions: glycoluril (50 μg), Protein A (200 μg/0.5 ml H_2O), 2 mCi ^{125}I, with a reaction time of 30 seconds at 4°C. To remove any unbound ^{125}I, Protein A was dialyzed for 3 days at 4°C with at least 6 changes of dialysis buffer (H_2O). Protein A was then lyophilized to dryness and stored at 4°C.

Fluorography of ^{125}I in Polyacrylamide Gels

The procedure followed was a modification of the procedure of Bonner and Lasky.[91] Directly after electrophoresis or after staining, the gel which contained 30,000 CPM/slot was soaked in 20 times its volume in DMSO for 30 minutes. This was followed by a second incubation in 20 volumes of DMSO. The gel was then placed in 4 volumes of a solution containing 20% (w/v) PPO in DMSO (22.2% w/v) for 3 hours with constant stirring. The gel was then placed in 20 volumes of DDH_2O for 30 minutes with constant stirring. This step was repeated for another 30 minutes, for it was essential to remove all the DMSO from the gel as it exposes the x-ray film. The gel was then placed on filter paper and dried by a Bio-Rad Model 224 Gel Slab Dryer *in vacuo* at 90°C for 2 hours. The dried gel on filter paper was pressed against the x-ray film (Kodak RPO) and put between 2 glass plates which were wrapped with aluminum foil. The package was stored in the dark for 8-10 weeks.

Autoradiography of ^{125}I in Polyacrylamide Gels

The procedure for autoradiography of ^{125}I labeled protein in polyacrylamide gels was that of Gamberg, Itaya, and Hokomori.[92] The gel, which contained 30,000 CPM/slot, was dried, wrapped, and stored as described in a previous section.

Analytical Polyacrylamide Slab Gel Electrophoresis

Preparation of slab gel.

The procedure used was a modification of the procedure of Lammli.[93] The acrylamide bis-acrylamide ratio was changed from 37:1 to 20:1, the separating gel was adjusted from 8% acrylamide to 10% acrylamide, and the apparatus used was a Bio-Rad Model 220 Dual Vertical Slab Gel Electrophoresis Cell.

The standard gel thickness was 1.5 mm and was prepared in the following way:

Stock acrylamide:	30 gm acrylamide and 1.5 gm bis-acrylamide/100 ml H_2O
Separating buffer:	18.2 gm Tris acetate, 0.4 gm SDS/200 ml H_2O, pH 8.8
Stacking buffer:	3 gm Tris acetate, 0.2 gm SDS/100 ml H_2O, pH 6.8

The separating gel was prepared by mixing 9 ml of stock acrylamide, 15 ml stock separating buffer, and 5 ml of H_2O. The mixture was deaerated *in vacuo* by aspiration at 4°C for 15 minutes and then mixed with 50 µl TEMED (Bio-Rad) and 250 µl of freshly prepared 0.1% Ammonium Persulfate. The mixture was immediately applied between the glass plates (precoated with 2% Kodak Photo Flo 200) with a syringe to a level of 9.5 cm x 14 cm.

The stacking gel was prepared by mixing 5 ml stock stacking buffer, 1.5 ml stock acrylamide solution, and 4 ml H_2O. The mixture was deaerated *in vacuo* at 4°C for 15 minutes and then mixed with 25 µl TEMED and 100 µl 0.1% Ammonium Persulfate. This mixture was immediately overlaid on the solidified separating gel.

The template comb was placed in the stacking gel to provide the sample slots for when the gel polymerized.

The gel was allowed to polymerize overnight to assure complete and uniform polymerization.

Preparation of sample and electrophoresis. Membrane pellets were solubilized by sonication in the sample buffer solution (Tris Acetate, 1.5 gm; SDS, 4 gm; glycerol, 20 gm; 2-mercaptoethanol, 10 gm; bromphenol blue, 2 mg dissolved in H_2O to 100 ml with pH adjusted to 6.8) to give a protein concentration of 150 g/100 µl buffer. Prior to electrophoresis the samples were heated at 100°C for 5 minutes.

Protein samples (80-120 µl) were applied to each sample slot and electrophoresis was carried out at a constant current of 25 mA/gel. Electrophoresis was terminated when the dye front migrated 8.5 cm down the separating gel. This usually took 5-6 hours.

Staining of slab gels. After electrophoresis was completed, the gel was immediately placed in 500 ml of Coomassie brilliant blue staining solution (30% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue) and allowed to stain overnight with constant stirring. Destain was performed by transferring the gel into destaining solution (30% methanol, 10% acetic acid). With frequent changes of destaining solution (once every 6-8 hours), sufficient destaining could be accomplished within 36-48 hours.

Analytical Polyacrylamide Gel Electrophoresis - Cylindrical Gels

The procedure used for electrophoresis of cylindrical polyacrylamide gels (0.04 Tris Acetate containing 1% SDS) was that of Fairbanks et al.[94] Samples were dissolved by sonication in protein solvent (0.01 M Tris HCl, pH 8.0, containing 1% SDS and 5 mM EDTA). Prior to electrophoresis the sample solution was made 2% in 2-mercaptoethanol and the solution was heated at 100°C for 5 minutes. After heating the sample was mixed with 1/3 volume of sample solution containing 20% sucrose and 40 µg/ml pyronin B (9% gels with an acrylamide/bisacrylamide ratio of 37/1). Gels were polymerized in 0.5 x 11 cm glass tubes, precleaned with chromic acid and coated with dimethyldichlorosilane. Gels were allowed to set overnight to insure complete and uniform polymerization. When possible, samples were applied in a volume of less than 30 µl to promote sharp protein bands. For volumes greater than 30 µl, electrophoresis was initiated at a low current (2 mA/gel), allowing the sample to become more compact at the top of the gel. After the sample had migrated into the top of the gel, the current was raised to 6 mA/gel for the remainder of electrophoresis. Electrophoresis was run at 13°C to diminish curvature of polypeptide bands. A constant length of each electropherogram was obtained by allowing the tracking dye, pyronin B, to migrate 8.9 cm from the origin. This took an average of 4-5 hours.

Staining of cylindrical gels

Protein staining. The technique used for staining protein bands within polyacrylamide gels utilized Coomassie blue and required removal of SDS for optimal results. SDS was removed by placing gels in 30 ml screw-cap culture tubes containing 10% trichloroacetic acid immediately after electrophoresis. Gels were kept in trichloroacetic acid for at least 2 days with a minimum of 4 changes of trichloroacetic acid. Trichloroacetic acid extracted gels were stained overnight in 0.4% Coomassie blue in 10% trichloroacetic acid-33% methanol. Gels were destained by placing in 10% trichloroacetic acid-33% methanol for 12 hours and then in 10% trichloroacetic acid at 37°C until background stain was sufficiently removed. Gels were scanned at 550 nm on a Gilford linear transport.

Carbohydrate staining by the periodic acid-Schiff procedure (PAS). The procedure employed for detection of carbohydrate within polyacrylamide gels was a modification of the procedure of Fairbanks et al.[98] Removal of SDS by soaking in 10% trichloroacetic acid for 3-4 days, with numerous changes in trichloroacetic acid, was necessary to avoid artifactual staining of noncarbohydrate species. Acid extracted gels were stained as follows: 0.5% periodic acid for 2 hours, 0.5% sodium arsenite-5% acetic acid for 1 hour, 0.1% sodium arsenite-5% acetic acid for 30 minutes, this step was repeated, 5% acetic acid for 20 minutes. The genls were then placed in Schiff reagent overnight in the dark. Gels were destained by soaking in 0.1% sodium metabisulfite, 0.01% HCl with several changes until destaining solution failed to turn pink upon addition of formaldehyde. Gels were scanned at 560 nm with a Gilford linear transport.

RESULTS

Radioactive Labeling with ^{125}I

As discussed in the introduction, when using a labeling technique as a plasma membrane marker, it is of critical importance to verify the requirements for the reaction.

Iodination of proteins was chosen as the method for labeling rat pancreatic acinar cells for the following reasons: ^{125}I is incorporated into proteins via the reaction of ^{125}I with the aromatic ring of tyrosines of the imidazole ring of histidine.[86] Because of this, all proteins exposed to the catalyst should be labeled, although the degree of labeling may vary depending on the positioning of the tyrosines and histidines in the protein molecule. It is generally accepted that when lactoperoxidase catalyzes iodination is used with respect to whole cells or organelles, proteins on the outer surface are the labeling substrate. Studies on the reaction of lactoperoxidase have suggested that lactoperoxidase forms an enzyme-substrate complex with the protein which is to be iodinated.[86] Lactoperoxidase is a high molecular weight protein (77,500 m.w.) and it does not readily dissociate into subunits;[86] this should prevent its penetration of an intact membrane. Glycoluril should not penetrate intact membranes either, because of its relative insolubility in aqueous solutions.[87] With respect to rat pancreatic acinar cells, iodination via lactoperoxidase or glycoluril should be limited to the proteins on the cell surface.

The requirements for iodination of membrane proteins by lactoperoxidase and glucose oxidase are summarized in Table 3. The cell particulate fraction as described in Methods should contain all the membranes of the cell. Iodination of membrane proteins from acinar cells required both lactoperoxidase and glucose oxidase. Glucose oxidase was required as it provided hydrogen peroxide required by lactoperoxidase. Removal of either enzyme drastically limited incorporation. Lactoperoxidase alone was far less effective than the combination with glucose oxidase. These results confirm results from other laboratories.[95]

The requirements for iodination via glycoluril are summarized in Table 4. Glycoluril is necessary for iodination; virtually no labeling occurs in its absence. The specificity of labeling of both systems was investigated and the results are summarized in Table 5. With both labeling systems, greater than 90% of the acid preciptable radioactivity was found in the cell particulate fraction, indicating that the ^{125}I was preferentially bound to membrane. To further demonstrate that the ^{125}I was firmly bound to the membrane protein, the cell particulate fraction was prepared from labeled cells. Free ^{125}I was separated from membrane protein by column chromatography using a Bio Gel P-6 column. Bio Gel P-6 was utilized because of its low exclusion limit (4,600 m.w.). Free ^{125}I would elute at the void volume, while membrane protein would elute at the exclusion volume. The elution of ^{125}I and membrane protein is illustrated in Figure 1. Ninety-three percent of the radioactivity applied to the column was associated with the protein peak. The membrane was undegraded, by the fact that there was one major absorbance peak at the exclusion

Table 3. Requirements for lactoperoxidase-glucose oxidase catalyzed iodination

Cells were isolated as described in Methods. Complete reaction mixtures contained 10 x 10^6 cells, lactoperoxidase (1,000 units/ml), glucose oxidase (600 units/ml), ^{125}I (1.6 μCi), in a total volume of 12 ml.

Modification	Acid precipitable radioactivity (cpm)	Specific activity (cpm/mg protein)
None	201	265
(-)lactoperoxidase	33.4	15.1
(-)glucose oxidase	48.9	39.2
(-)lactoperoxidase and glucose oxidase	23.0	0.1

Table 4. Requirements for 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril catalyzed iodination

Cells were isolated as described in Methods. Complete reaction mixtures contained 10 x 10^6 cells, glycoluril (50 μg), ^{125}I (150 μCi), in a total volume of 100 μl.

Modification	Radioactivity acid precipitable (cpm)	Protein (μg)	Specific activity (cpm/μg protein)
None	2,151	60	35.8
None	3,674	150	24.5
(-)glycoluril	31	60	0.5
(-)glycoluril	30	150	0.2

Table 5. Relative incorporation of ^{125}I into cell particulate and cell supernatant fractions

Cells were isolated and particulate and supernatant fractions were prepared as described in Methods. Reaction mixtures for lactoperoxidase glucose oxidase catalyzed iodination were as follows: 10 x 10^6 cells, lactoperoxidase (615 units), glucose oxidase (615 units), ^{125}I (33 µCi), in a total volume of 330 µl. Reaction conditions for glycoluril (50 µg), ^{125}I (150 µCi), in a total volume of 100 µl.

Rxn	Catalyst[a]	Fraction	Radioactivity acid precipitable (cpm)	cpm part./ cpm super.
1	LPO/GO	particulate	1,824	9.7
		supernatant	187.9	
2	LPO/GO	particulate	1,927	13.4
		supernatant	143	
3	LPO/GO	particulate	1,444	8.1
		supernatant	178	
4	Glycoluril	particulate	2,151	10.8
		supernatant	200	
5	Glycoluril	particulate	3,674	26.2
		supernatant	140	

[a] LPO/GO, lactoperoxidase-glucose oxidase.

Figure 1. Analysis of membrane-bound radioactivity by column chromatography, Bio Gel P-6.

Cells were isolated, iodinated, and the particulate fraction prepared as described in Methods. The particulate fraction was acid precipitated and acid insoluble material was redissolved in protein solvent (10 mM Tris HCl, pH 8.6; 5 mM EDTA; 1% SDS) and applied to a Bio Gel P-6 column (40 cm x 1 cm) equilibrated in 0.1 SDS. Samples (1.25 ml) were collected and monitored for protein by measuring absorbance at 280 nm. Radioactivity was monitored by liquid scintillation. Absorbance units (458), containing 14,250 cpm, were applied to the column. Four hundred thirty-two absorbance units and 13.670 cpm were recovered, which represents 94.2% and 95.2% recovery, respectively. Solid circles represent distribution of radioactivity; open circles represent distribution of protein. Elution of molecular weight markers is signified by bars.

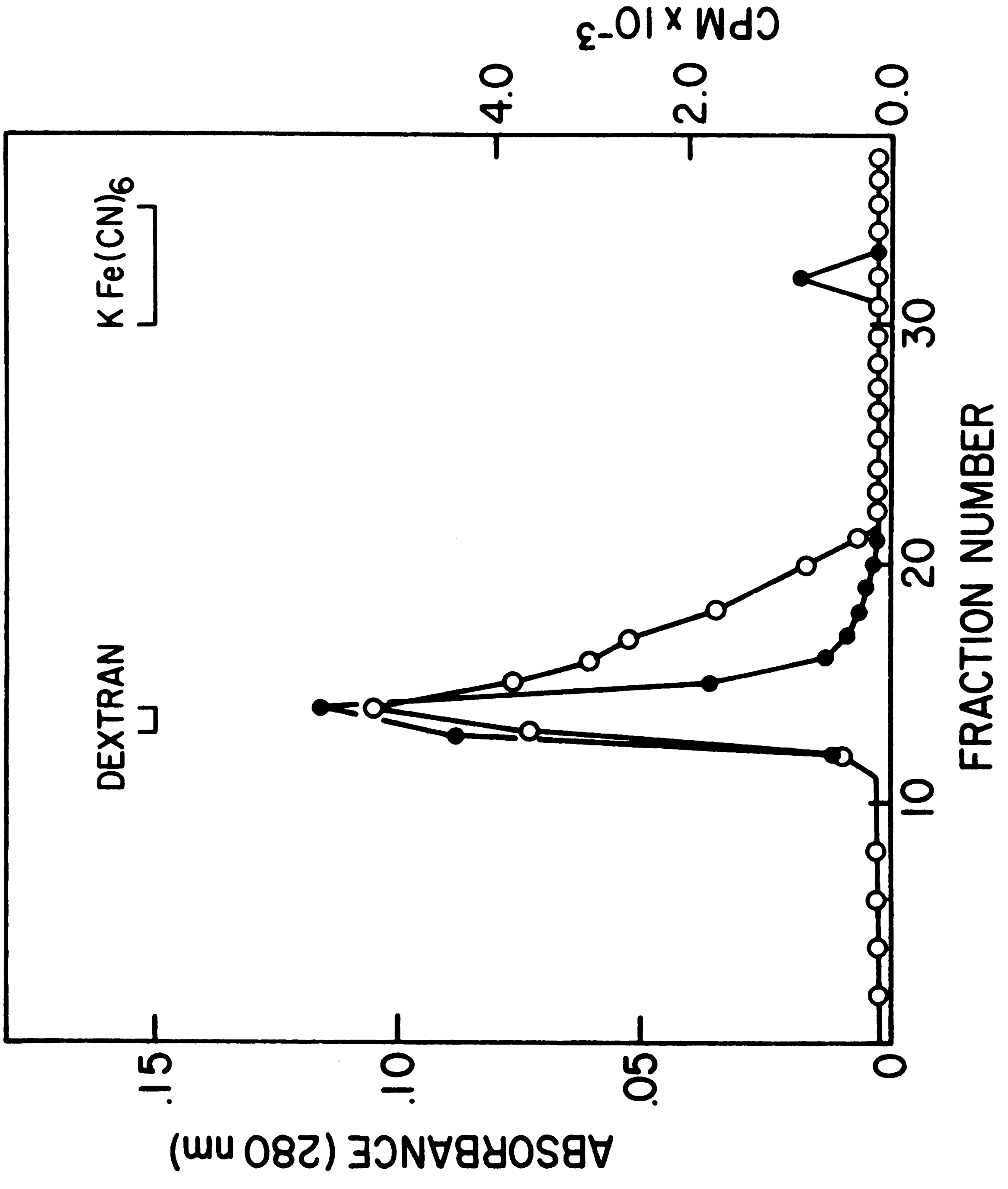
DEXTRAN
K Fe(CN)6
FRACTION NUMBER
10
20
30
ABSORBANCE (280 nm)
0
.05
.10
.15
CPM x 10-3
0.0
2.0
4.0

Figure 1

volume detected, and analysis by SDS polyacrylamide electrophoresis showed no degradation.

Whelton and Aust[96] previously reported that incorporation of ^{125}I into membrane protein of rat liver microsomes increased when butylated hydroxytoluene was added to the reaction mixture. Lipid peroxidation can occur during the iodination reaction if the hydrogen peroxide concentration exceeds 8 μM.[86] Peroxidation of membrane lipid has been shown to cause increased permeability of membranes or to cause lysis of cellular organelles such as mitochondria.[97] The effect of BHT on the iodination of acinar cells was investigated and the results are summarized in Table 6. Addition of 0.001% BHT to the reaction mixture decreased incorporation of label into lipid by 29%. Although results suggested that the ^{125}I was incorporated into membrane protein, when the labeled membrane protein was analyzed by SDS polyacrylamide electrophoresis, radioactivity could not be recovered. As shown in Figure 2, loss of membrane-bound ^{125}I was not due to membrane degradation. Membrane degradation can be detected in the polypeptide profile by a reduction of intensity of a high molecular weight species concomitant with an increase in a heterogeneous population of lower molecular weight species.[98] As seen in Figure 2, the Coomassie blue profiles from both cell particulate fractions are nearly identical and do not contain the broad low molecular weight band associated with membrane degradation. Loss of radioactivity was not a problem with soluble proteins, as bovine plasma albumin, acid precipitated and electrophoresed, retained 85% of its initial radioactivity.

The effect of washing procedures followed by SDS polyacrylamide electrophoresis was next investigated. Membrane preparations that

Table 6. Effect of butylated hydroxytoluene on the incorporation of ^{125}I into lipid

Cells were isolated as described in Methods. Reaction conditions were as follows: 10 x 10^6 cells, lactoperoxidase (4 units), glucose oxidase (0.03 units), ^{125}I (33 μCi), in a total volume of 500 μl. After iodination, particulate fraction was prepared and acid precipitated. Lipid extraction was performed following the procedure of Folch et al.

	Radioactivity		
Modification	total (cpm)	lipid extractable (cpm)	% in lipid phase
None	13,083	9,511	72.6
plus 0.001% BHT[a]	17,934	7,878	43.6

[a] BHT, butylated hydroxytoluene.

Figure 2. Effects of different washing procedures on recovery of protein-bound ^{125}I after SDS polyacrylamide gel electrophoresis.

Bovine plasma albumin was iodinated under the following conditions: bovine plasma albumin (2 mg), glycoluril (2 μCi), in a total volume of 250 μl. Cells were isolated, iodinated, and particulate fraction prepared as described in Methods. Bovine plasma albumin and half of the cell particulate fraction were acid precipitated and then solubilized in SDS and neutralized by addition of 5 μl of concentrated NH_4OH. The samples were applied on SDS polyacrylamide gels and electrophoresed as stated in Methods. The other half of the cell particulate fraction was dialyzed overnight at 4°C against 1% SDS containing 0.1 mg/ml SBTI, with at least 3 changes of dialysis buffer. Directly after dialysis, the sample was solubilized, applied on SDS polyacrylamide gels, and electrophoresed as described in Methods. Two gels were electrophoresed; one gel was stained for protein with Coomassie blue and the other gel was manually fractionated and radioactivity monitored by liquid scintillation.

A) Bovine plasma albumin (50 μg), 49.314 cpm applied with 41,917 cpm (85%) recovered after electrophoresis.

B) Acid precipitated particulate fraction (120 μg), 15,500 cpm applied with 5,136 cpm (33%) recovered after electrophoresis.

C) Dialysis washed particulate fraction (120 μg), 29,500 cpm applied with 15,228 cpm (51.6%) recovered after electrophoresis.

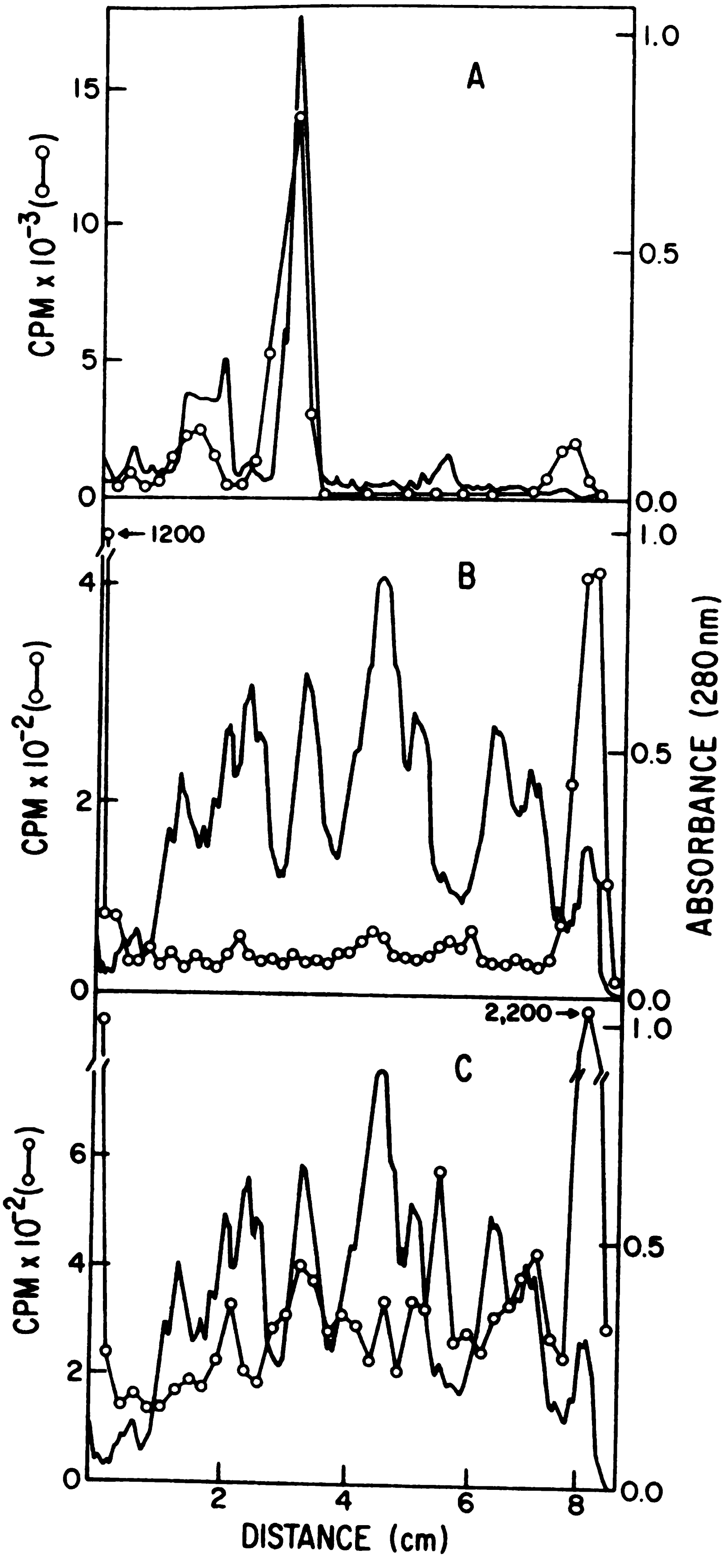
A
B
C
CPM x 10⁻³ (o—o)
CPM x 10⁻² (o—o)
CPM x 10⁻² (o—o)
ABSORBANCE (280nm)
DISTANCE (cm)
0
5
10
15
0
2
4
0
2
4
6
0.0
0.5
1.0
0.0
0.5
1.0
0.0
0.5
1.0
2
4
6
8
1200
2,200

Figure 2

were not acid precipitated before electrophoresis retained 18% more radioactivity than membrane preparations that were acid precipitated before electrophoresis (Figure 2). Recovery of membrane radioactivity was still substantially lower than the recovery of radioactivity from the soluble protein, bovine plasma albumin.

The effect of O_2 on the stability of the membrane-bound ^{125}I was investigated to see if O_2 present in the air caused oxidation of the protein-^{125}I bond. Oxygen did not contribute to the lability of the membrane protein-^{125}I bond (Figure 3). Membrane fractions that were exposed to the air retained radioactivity to the same extent as did the membrane fractions kept under N_2. In both cases the combination of acid precipitation and SDS polyacrylamide electrophoresis reduced the recovery of the radioactivity applied by 15-25%. Compare the ^{125}I from acid and nonacid precipitated membranes (Figures 2 and 3). The profiles are similar with the exception that peaks from acid precipitated profiles are greatly reduced. This implies that radioactivity was not lost from a few proteins in the membranes but that the combination of the acid precipitation and SDS polyacrylamide electrophoresis of the membrane casued destabilization of the membrane protein-^{125}I bonds in most, if not all, of the membrane proteins labeled. In subsequent studies, labeled membranes were directly solubilized for electrophoresis after labeling.

Glycoluril was chosen over lactoperoxidase as the catalyst for iodination because of its being mechanically easier than the lactoperoxidase-glucose oxidase system, while being as effective a catalyst for iodination.

Figure 3. Effect of O_2 on the iodination and recovery of radioactivity after SDS polyacrylamide gel electrophoresis.

Cells were prepared as described in Methods. Half of the cells were kept under N_2, while the other half were left exposed to air (O_2). From the point of isolation until electrophoresis, the N_2 fraction was kept under N_2. The cells were then iodinated as described in Methods. Washing procedures were the following for both fractions. The cells were divided into 2 portions, one group of cells being washed by acid precipitation before polyacrylamide electrophoresis whereas the other group of cells were directly solubilized for electrophoresis. Electrophoresis was performed as stated in Methods, and after electrophoresis the cylindrical gels were fractionated manually and radioactivity monitored by liquid scintillation.

A) Exposure to air. Open circles represent samples solubilized directly for electrophoresis, 12,280 cpm recovered, representing 58.4% of the radioactivity applied. Closed circles represent fractions acid washed before electrophoresis, 4,171 cpm recovered, representing 43.2% of the radioactivity applied.

B) N_2 samples. Open circles represent samples solubilized directly for electrophoresis, 9,649 cpm recovered, representing 53.7% of the radioactivity applied. Closed circles represent fractions acid washed before electrophoresis, 5,042 cpm recovered, representing 28% of the radioactivity applied.

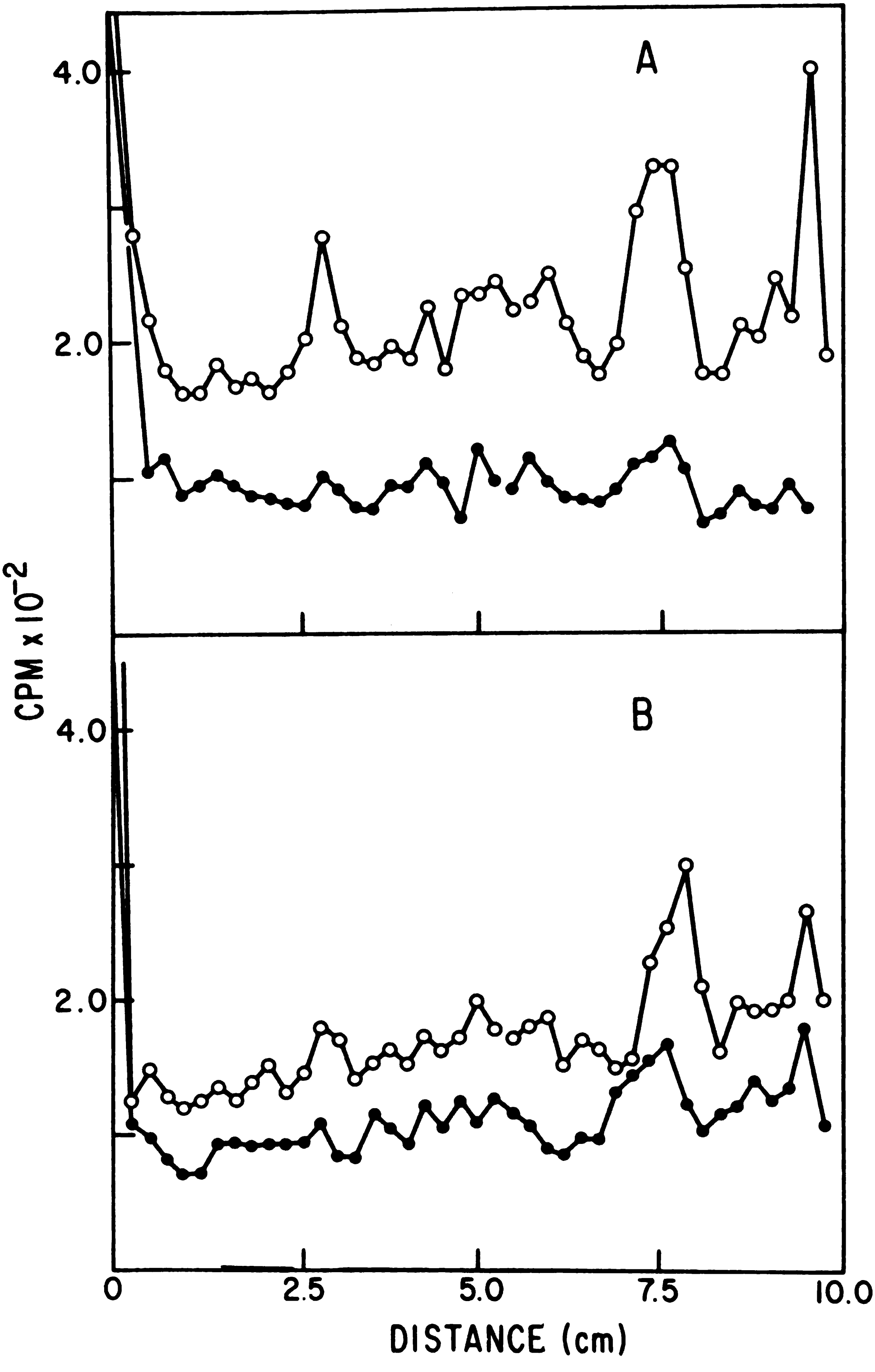

Figure 3

Table 7. Analysis of radioactivity present in the cell supernatant fraction after iodination by glycoluril

Cells were isolated, iodinated, and the cell particulate and cell supernatant fractions prepared as described in Methods. Samples of supernatant fractions were used to monitor total radioactivity present and the percent of protein bound in the supernatant fraction.

	Radioactivity		
Fraction	total present (cpm)	acid precipitated (cpm)	% protein bound
Particulate	26,533	11,956	45.1
Supernatant	16,903	554	3.2

Plasma Membrane Isolation and Characterization

Concanavalin A (Con A) binds to the terminal nonreducing sugars in glycoproteins and glycolipids.[78] Because of this Con A has been used as a tool for characterization of normal and neoplastic cell surfaces. Con A covalently bound to Sepharose beads was used in an attempt to isolate plasma membrane from rat exocrine cells. The objective of this procedure was to bind to membrane fragments to the beads. Cell homogenates had to be used instead of whole cell suspensions because homogenization of whole cells bound to the beads also resulted in the destruction of the beads. The high relative density of the Con A Sepharose beads provided a quick and easy way to separate beads from the cell homogenate. This was accomplished by low speed centrifugation (7 x g, 5 min). After absorbed membrane fragments adsorbed to the beads were separated from the cell homogenate, the fragments would be eluted from the beads.

Other membranes containing lectin receptors (e.g., rough or smooth microsomes or zymogen granules) might bind to the beads. Therefore, gradient centrifugation might be necessary to further purify the plasma membrane fragments.

Because elution of the membrane fragments from the beads should be done in a way that causes the least alteration in membrane structure (e.g., detergents such as triton X 100 should not be used), α-methyl-mannoside was used as the sugar of choice for competitive binding between monosaccharides and membrane lectin receptors.[78] Elution of membrane fragments from Con A Sepharose beads was attempted by incubation in 10% α-methyl-mannoside.

Results of incubation of Con A Sepharose beads are illustrated in Figure 4. Approximately 50% of the iodinated membrane fragments

Figure 4. Adsorption of iodinated membrane fragments to Concanavalin A-Sepharose beads.

^{125}I bound to membrane was used as a marker. Cells were isolated, labeled with ^{125}I, and then lysed as described in Methods. After homogenization, the cell homogenate was incubated with an equal number of Concanavalin A-Sepharose beads (number of beads equal to the number of cells before homogenization). Total volume, 1 ml; cells, 16 x 10^6. Incubation was at 4°C for 60 minutes. Samples (10 μl) of the supernatant (after centrifugation of the beads) were taken at the time points indicated to monitor binding of membrane fragments to the beads. Four samples at each time point were taken. After 60 minutes of incubation the beads were washed 3 times in KRB 1.0 mM $CaCl_2$, containing 5% BPA, 0.25 M NaI, 0.1 mg/ml SBTI, to remove any unbound cell material. To remove cell material bound to the beads, the beads were incubated with 10% α-methyl-mannoside for 90 minutes with 2 mM EDTA added at 45 and 75 minutes. Samples (10 μl) of the supernatant (after centrifugation of the beads) were taken at the times indicated. Four samples at each time point were taken. All samples collected were monitored for radioactivity as described in Methods.

A) Adsorption of iodinated fragments to Concanavalin A-Sepharose beads.

B) Release of iodinated fragments from Concanavalin A-Sepharose beads upon incubation with α-methyl-mannoside.

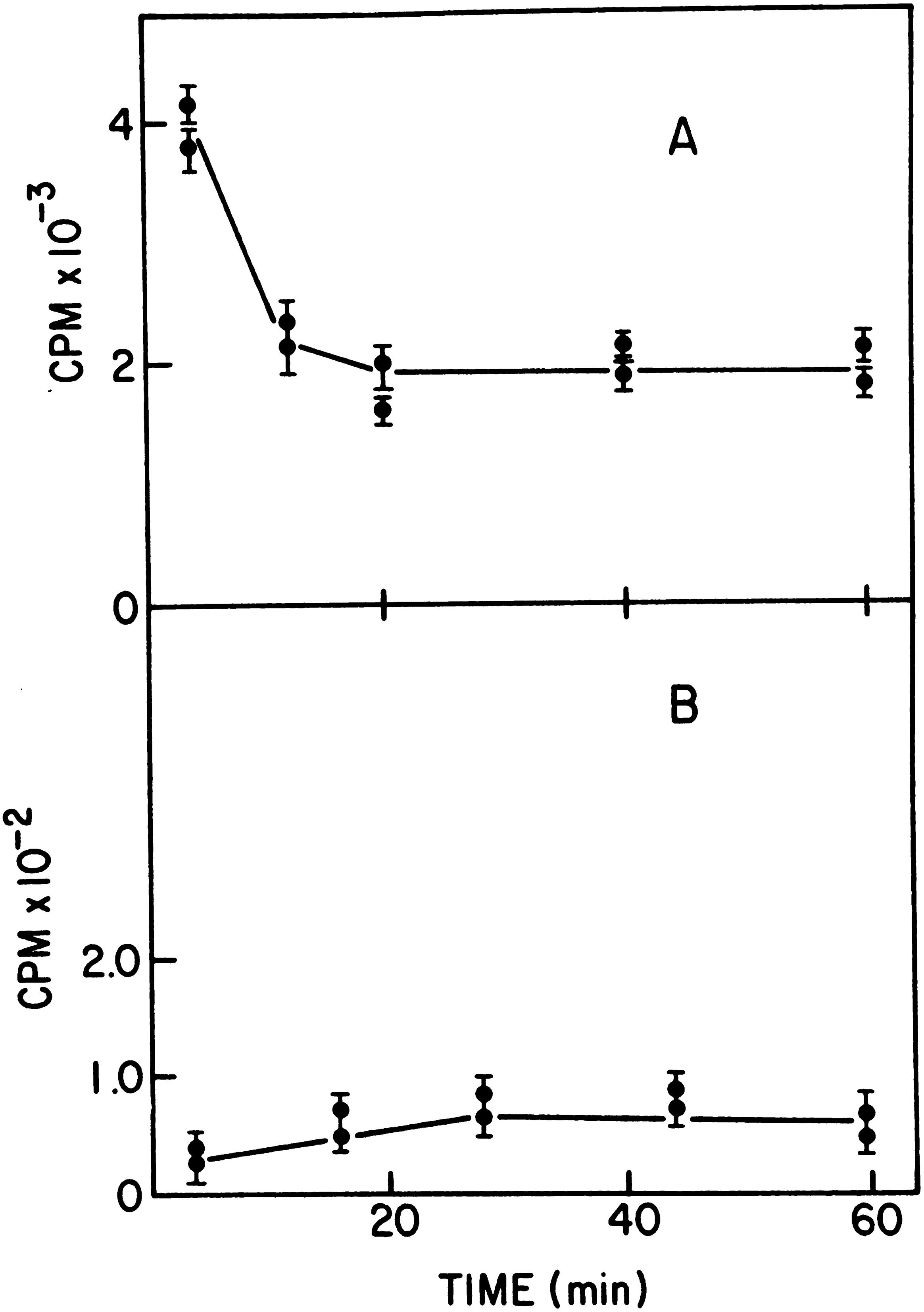

Figure 4

were bound to the beads. When viewed under phase contrast microscopy, membrane fragments were seen adhered to the beads. Incubation with 10% α-methyl-mannoside did not remove the membrane fragments from the beads, as is shown in Figure 4B. Ethylenediaminetetraacetate (EDTA) acts to destabilize membrane structure by chelation of divalent cations,[67] as well as causing breakdown of Con A.[78] Extraction with 2 mM EDTA at the indicated time points was not effective. When viewed under phase contrast microscopy, it could not be determined if any membrane fragments were being released by the Con A Sepharose beads. Free membrane fragments could not be detected. Less than 1% of the bound radioactivity was released.

The possibility of isolating plasma membrane from cells utilizing the aqueous two phase polymer system of Brunette and Till[79] was investigated. The results are summarized in Table 8. The majority of the labeled membrane remained in the pellet, which reduced the yield drastically. The yield was calculated as the percentage of the labeled membrane recovered at the interface of the two phase of the two polymer phases. Labeled membrane fragments that were not trapped in the pellet were evenly distributed throughout the gradient. Brunette and Till reported that the two phases separated in 10 minutes after mixing and centrifugation. In my experiments the phases usually took 2 hours to separate. Increases in duration or intensity of centrifugation did not shorten the time for separation of the two phases or a change in the distribution of the plasma membrane fragments. When working with pancreatic cell homogenates, long incubation times should be avoided, since activation of digestive zymogens may occur, resulting in degradation of membrane preparations.

Table 8. Distribution of membrane-bound ^{125}I in aqueous two phase polymer system

The 2 phases were prepared by mixing in a separatory funnel: 200 gm of a 20% (w/w) solution of Dextran 500 (Pharmacia), 103 gm of a 30% (w/v) solution of polyethylene glycol (Carbowax 6,000, Union Carbide), 99 ml of doubly distilled H_2O, 333 ml of 0.22 M phosphate buffer, pH 6.5, and 80 ml of 10^{-2} M $ZnCl_2$. The mixture was shaken and allowed to settle overnight at 4°C. After the 2 phases had formed, they were separated and stored at 4°C. Cells were isolated, labeled, washed, and lysed as described in Methods. The cell homogenate (in hypotonic PBS, pH 6.5) was centrifuged at 210 x g for 10 minutes at 4°C. The supernatant, which contained cytoplasmic materials and microsomes, was discarded. The pellet, which contained nuclei, heavy cell debris, and membrane fragments, was resuspended in 40 volumes of cold 1 mM $ZnCl_2$ and centrifuged at 210 x g for 10 minutes at 4°C. The supernatant was discarded and the centrifuge tube, still containing the pellet, was inverted and wiped with adsorbant paper. The pellet was vortexed in a solution consisting of 6 ml of each polymer phase. The suspension was centrifuged at 5,500 x g for 10 minutes at 4°C. The nuclei and heavy cell debris sedimented to the bottom of the tube, forming the pellet, and less dense membrane fragments migrated to the interface of the 2 phases. After centrifugation, 3 samples (20 μl) of each section of the 2 phase polymer system (upper phase, interface, lower phase, and pellet) were taken to monitor the distribution of membrane-bound ^{125}I. Samples were acid precipitated and radioactivity monitored by liquid scintillation.

Polymer fraction	Radioactivity acid precipitable (cpm)	% distribution acid precipitable (cpm)
1) Total gradient	2,048	100.0
2) Upper phase	101	4.9
3) Interface	98	4.7
4) Lower phase	93	4.5
5) Pellet	1,756	85.7

The procedure of Meldolesi and Cova[48] for preparing plasma membrane from guinea pig pancreas was used in an attempt to isolate a plasma membrane fraction from rat pancreas. The procedure was used with both whole tissue and a single cell suspension of acinar cells. In the collagenase wash step, material obtained at the discontinuous gradient interface was incubated in phosphate buffered saline (PBS) containing .01 mg/ml collagenase for 10 minutes at room temperature. The arrows in Figure 5 indicate the polypeptides attributed to collagenase which adhered to the membrane fractions throughout the remainder of the isolation procedure. Therefore, the collagenase wash step was omitted.

Electron microscopy of thin sections can provide evidence for pure membrane preparations. Electron micrographs from 4 different membranes prepared by the Meldolesi and Cova procedure are shown in Figure 6. Membrane preparations from tissue were less contaminated from rough endoplasmic reticulum and other cellular material than preparations from single cell suspensions. Smooth membrane fragments free of contaminating debris were prepared (Figure 6C), but this could not be done routinely (e.g., once out of 5 preparations). The usual result of membrane fractions prepared from tissue is shown in Figure 6D. Material at the discontinuous gradient interface was enriched in smooth membrane fragments but was also contaminated to a large extent with cellular debris, either adhering to the membrane or trapped inside membrane vesicles. Membrane preparations from acinar cell suspensions proved less fruitful. As seen in Figure 6A, smooth membrane fragments from the discontinuous gradient interface were contaminated with cell debris and, in addition, rough microsomes. Because membrane preparations obtained from tissue were not

Figure 5. Electrophoretic analysis of the 700 x g pellet subjected to collagenase.

The 700 x g membrane fraction was isolated as described in Methods, with the following addition. After material from the interface of the discontinuous gradient was collected, it was incubated for 10 minutes at room temperature in phosphate buffered saline, pH 6.5, containing 0.01 mg/ml collagenase.

A) Collagenase profile (50 µg).

B) 700 x g membrane profile (100 µg).

Samples were electrophoresed and stained with Coomassie blue, as described in Methods.

Arrows represent contaminating polypeptides.

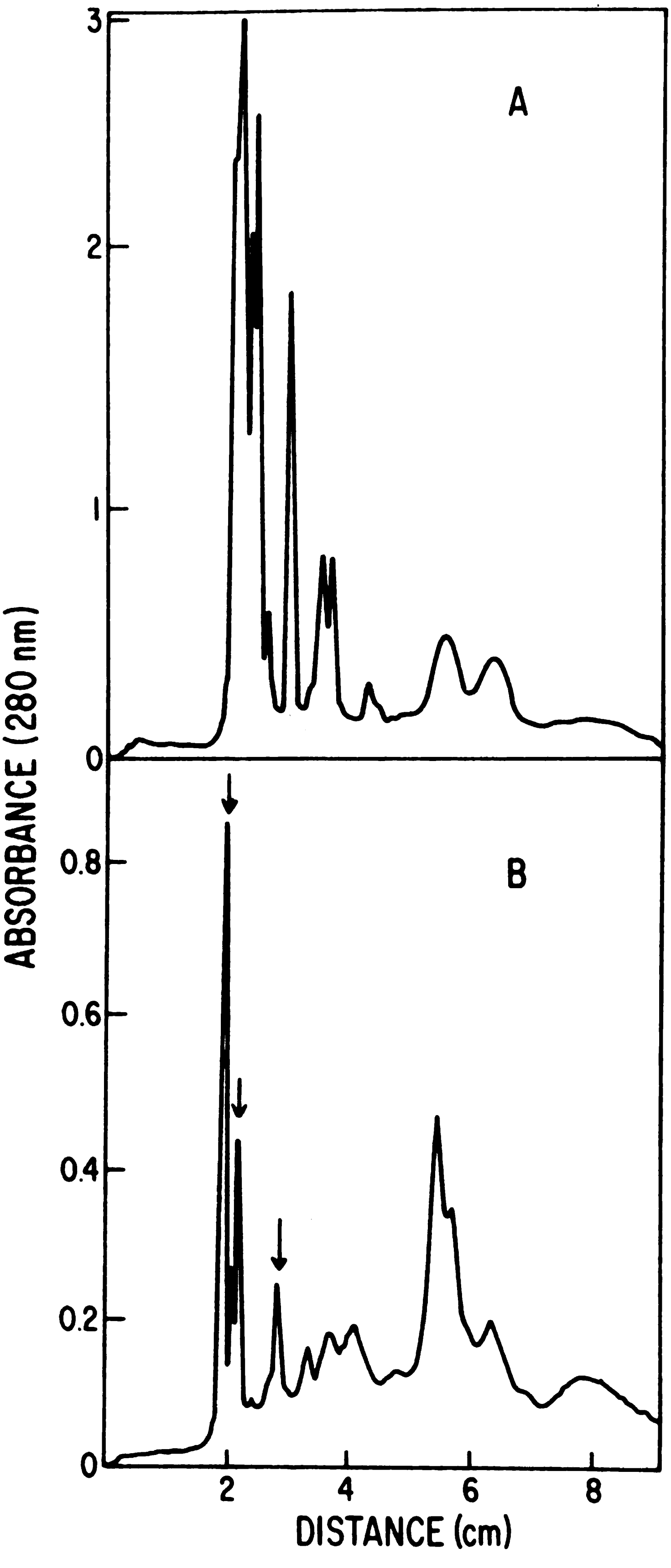

Figure 5

Figure 6. Electron microscopic analysis of 700 x g fractions.

Details of preparation of membrane fractions can be found in Methods. Fixation of fractions of membranes was by the procedure of Sanderson.[103] Membrane preparations were seen at a magnification of 40,000 x.

A) 700 x g fraction from cells.

B) 700 x g fraction from cells.

C) 700 x g fraction from tissue.

D) 700 x g fraction from tissue.

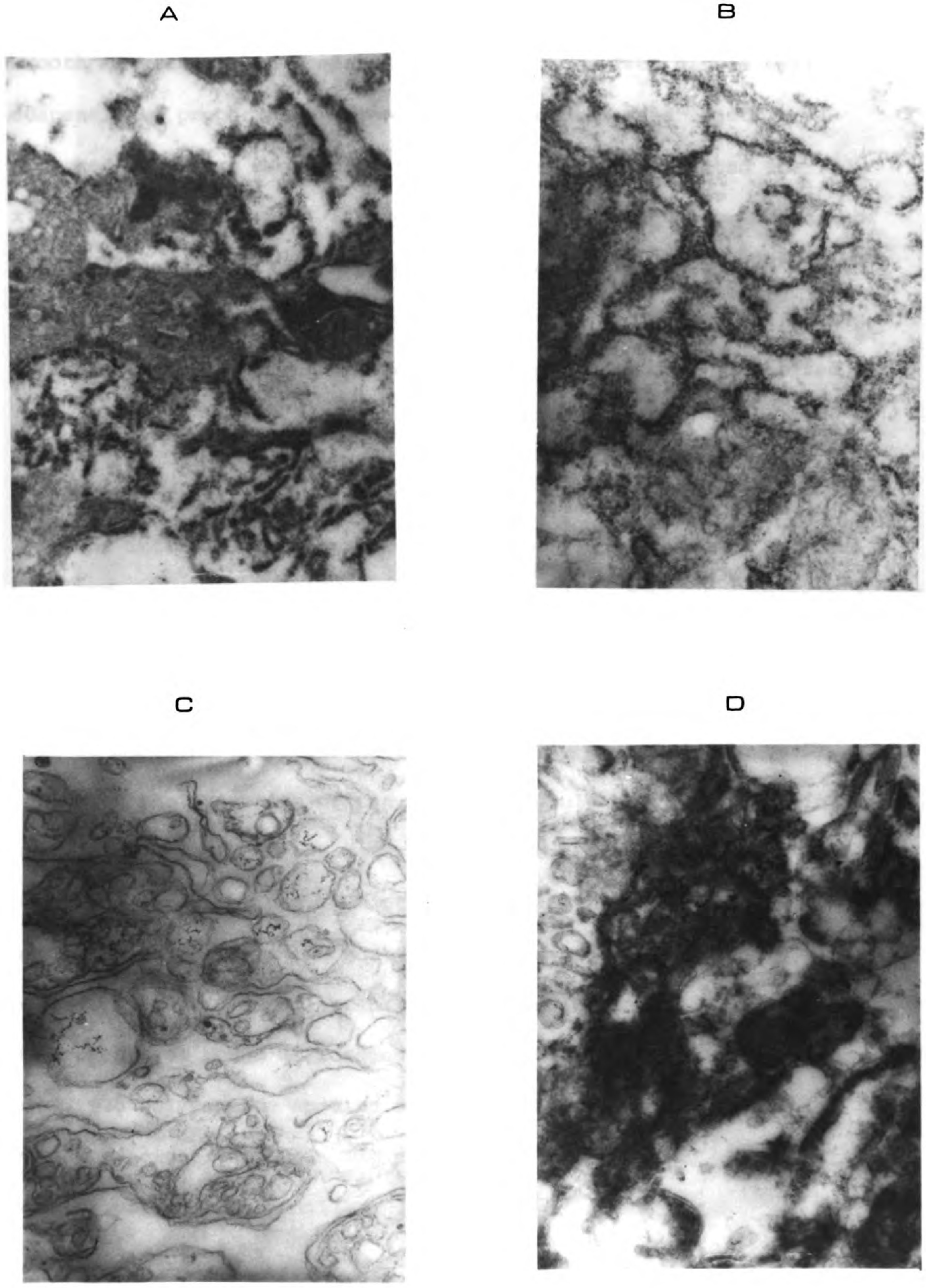

Figure 6

contaminated with rough microsomes and had a higher enrichment of smooth membranes when compared to preparations from single cell suspensions, preparations from tissue were used as a fraction enriched in plasma membranes. The fraction was designated the 700 x g fraction. Meldolesi and Cova[48] reported an in-depth analysis of a plasma membrane fraction prepared from guinea pig pancreas. This evidence came mainly from electron microscopy and enzyme activities present in the fraction. These observations have been summarized in the introduction.

Comparison of Intracellular Membranes

The intracellular membranes, zymogen granule content, and membranes from the 700 x g pellet were compared to determine whether the proteins of the zymogen granule membrane were unique to that membrane. Zymogen granule content was included to monitor contamination of membrane preparations from secretory proteins (Figure 7).

With the exception of the zymogen granule membranes, the intracellular membranes of the rat pancreas are characterized by a complex pattern of polypeptides. Smooth microsomal, mitochondrial, and nuclear membranes are the most complex, having greater than 40 polypeptide species with molecular weights ranging from 150,000 to 12,000. Membranes from rough microsomes and the 700 x g pellet are less complex, ∿25 polypeptide species and containing fewer high molecular weight species (80,000). The zymogen granule membrane has a much simpler polypeptide profile and has been well documented.[15,44] It is composed of 9 polypeptide species, with the second polypeptide from the top of the gel (GP-2) representing approximately 40% of the membrane protein. Smooth microsomes have a polypeptide which

Figure 7. Electrophoretic analysis of polypeptides of pancreatic membranes and zymogen granule content.

Membranes were prepared, solubilized, and electrophoresed on slab gels as described in Methods. The gels were stained with Coomassie blue. The following amounts of protein were analyzed: lane 1, 150 µg of nuclear membranes; lane 2, 150 µg of rough microsomal membranes; lane 3, 125 µg of smooth microsomal membranes; lane 4, 100 µg of zymogen granule membranes; lane 5, 100 µg of the zymogen granule content; lane 6, 165 µg membranes from the 700 x g pellet; lane 7, 150 µg of mitochondrial membranes. The positions of the molecular weight markers are indicated (a, thyroblobulin; b, bovine plasma albumin; c, chymotrypsin). Tracking dye is denoted by td.

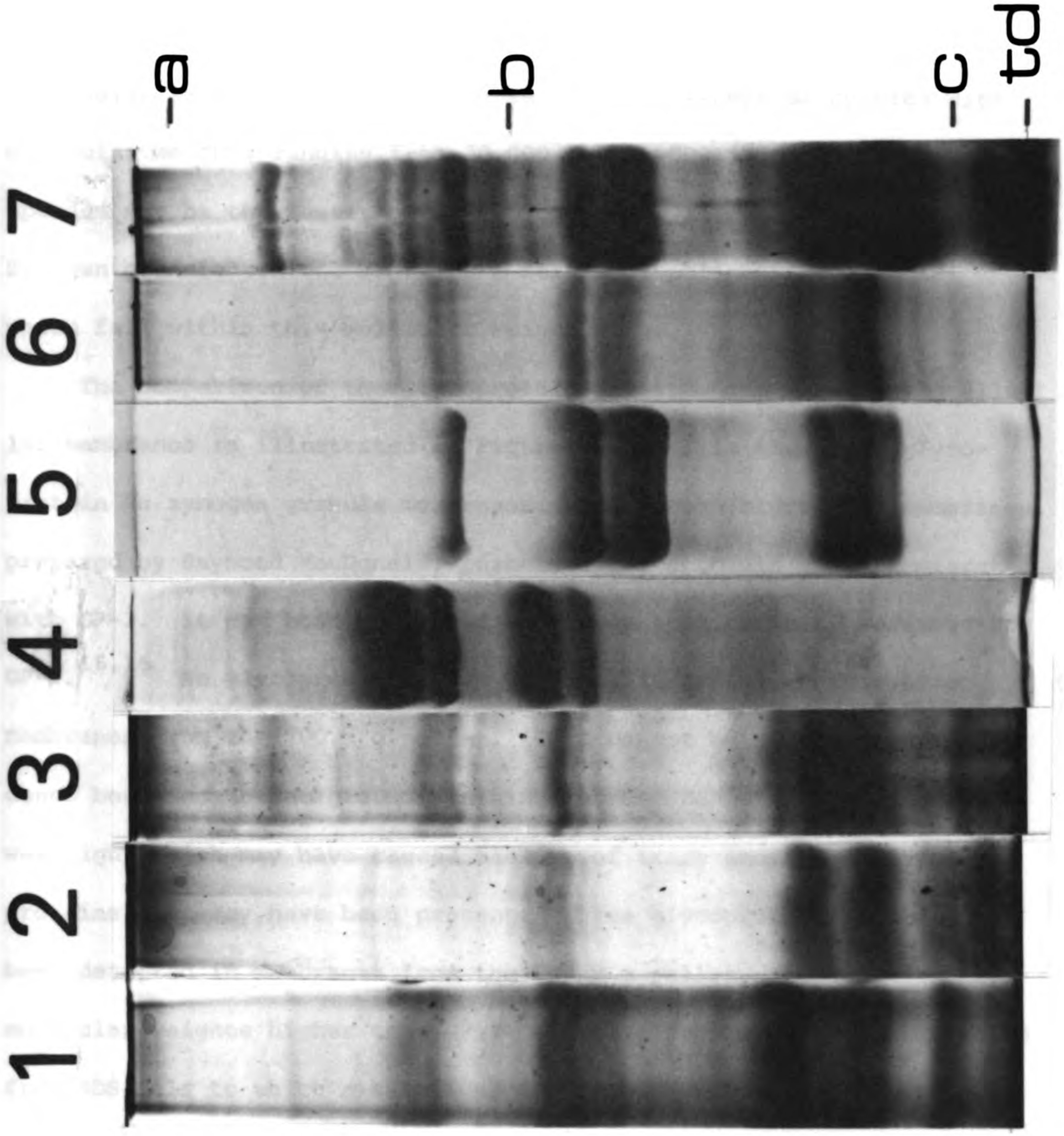

Figure 7

comigrates with GP-2, but this represents only 3% of the total membrane protein. Membranes from the 700 x g pellet contain a minor polypeptide that comigrates with GP-2. This may be due to contamination from smooth microsomes or be a characteristic of the plasma membrane. With the exception of the zymogen granule membrane, all intracellular membranes contain a series of polypeptide species with molecular weights ranging from 30,000 to 20,000. Some of these species may be the result of contamination by secretory protein. Zymogen granule content contains a series of polypeptide species which fall within this molecular weight range.

The comparison of the glycoprotein distribution among intracellular membranes is illustrated in Figure 8. GP-2 is the major glycoprotein in zymogen granule membranes. The smooth microsomal membranes, prepared by Raymond MacDonald, possess a glycoprotein which comigrates with GP-2. It has been suggested that this species is a precursor to GP-2.[15,16] No glycoproteins were detected in nuclear membranes or membranes from the 700 x g pellet. This cannot be stated with confidence because the background staining in the SDS polyacrylamide gels was high, which may have caused masking of trace amounts of glycoproteins that may have been present. Three glycoprotein species have been detected in membranes from the 700 x g pellet, all of which have molecular weights higher than GP-2.[46] The glycoproteins were detected from SDS gels to which a sample of 250 μg was applied. Samples analyzed contained only 175 μg. This was probably the main reason for failure to detect the glycoproteins present in membranes from the 700 x g pellet.

Figure 8. Electrophoretic analysis of glycopeptides of pancreatic membranes.

Membranes were prepared, solubilized, and electrophoresed on cylindrical gels as described in Methods. The gels were stained for carbohydrate as described in Methods. The following amounts of protein were analyzed: membranes from the 700 x g pellet, 175 µg; zymogen granule membrane, 50 µg; smooth microsomal membranes, 200 µg; rough microsomal membranes, 200 µg; and nuclear membrane, 200 µg. Electropherograms were scanned at 560 nm, and the results are expressed as relative absorbance. Tracking dye is denoted by td.

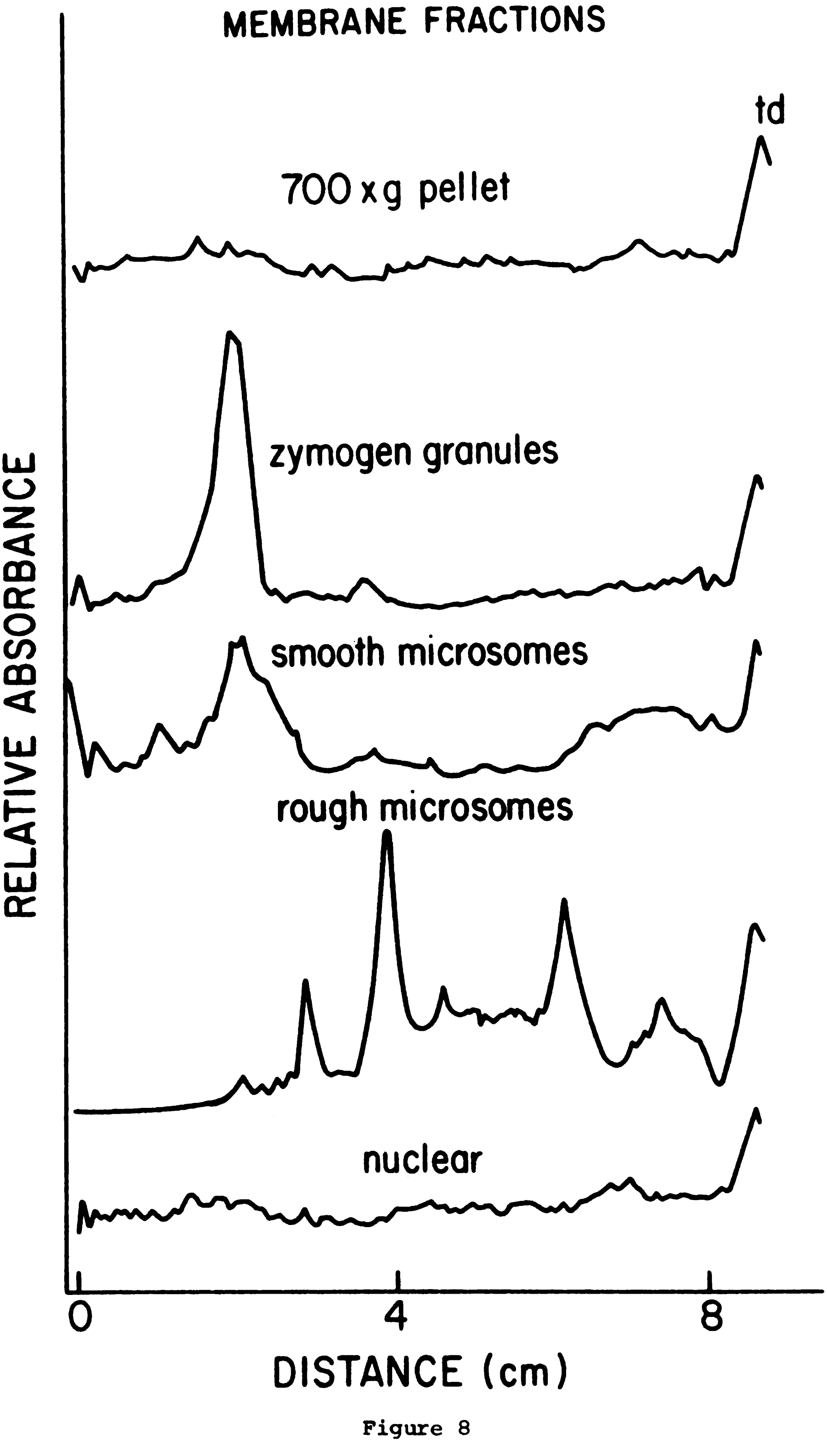
MEMBRANE FRACTIONS
td
700 x g pellet
zymogen granules
smooth microsomes
rough microsomes
nuclear
RELATIVE ABSORBANCE
0
4
8
DISTANCE (cm)

Figure 8

Comparison of Iodinated Membrane Proteins of the Cell Surface of Secreting and Non-Secreting Acinar Cells of Rat Pancreas

Studies[46] have shown GP-2 to be localized on the interior of the zymogen granule membrane. According to the accepted theory of exocytosis, after fusion of the zymogen granule membrane to the plasma membrane, GP-2 would be located on the outer surface of the plasma membrane. Fluorographic analysis of secreting cells labeled with ^{125}I was performed in an attempt to detect GP-2 on the cell surface. Isolated acinar cells were induced to secrete by addition of 10^{-5} M carbamylcholine chloride to the incubation media. Cell samples were taken at different time points after initiation of secretion and iodinated via glycoluril. Cell viability remained greater than 95% throughout the incubation when monitored by methyl green exclusion under phase contrast microscopy. After iodination the cell particulate fractions were prepared and analyzed by SDS polyacrylamide gel electrophoresis and fluorography. The results are shown in Figure 9. Comparison of the Coomassie blue profiles indicates no degradation of membranes occurred during the incubation and cell particulate preparation. The polypeptide profiles are identical and there is no decrease in intensity of high molecular weight bands with the concomitant increase in heterogeneous lower molecular weight bands. No detectable change in the GP-2 region could be detected. Although analysis of ^{125}I profiles by fractionation of individual gels provides quantitative data on proteins labeled, fluorographic analysis of labeled proteins has much greater resolution. Fluorography can distinguish individually labeled proteins to the same extent which proteins are separated by polyacrylamide electrophoresis. Gel fraction cannot provide this resolution.

Figure 9. Fluorographic analysis of polypeptides of the pancreatic cell surface of secreting and nonsecreting cells.

Cells were prepared as described in Methods, except for the following modification: from the pipetting step until the cells were induced to secrete, the cells were kept at 4°C to prevent uninduced secretion. After isolation, half the cells were placed in KRB 1.0 mM $CaCl_2$, containing 5% BPA, 0.1 mg/ml SBTI, and 10^{-5} M carbamylcholine chloride, and incubated at 37°C for 2 hours. The other half of the cells were incubated in the same media, minus the carbamylcholine chloride, at 4°C. At the following time points cell samples (1.5×10^6) were taken for iodination: 0, 5, 30, 60, and 105 minutes. The cells were washed once in KRB 1.0 mM $CaCl_2$ containing 0.1 mg/ml SBTI to remove any BPA adhered to the cells. The cells were iodinated, washed, and the cell particulate prepared as described in Methods. Polyacrylamide slab gel electrophoresis was performed with an equal amount of radioactivity (150,000 cpm) placed in each sample slot. Duplicate gels were electrophoresed. One was stained for protein with Coomassie blue and the other was prepared for fluorography.

S, zymogen granule content; a, 0 time - carbamylcholine;
b, 0 minutes + carbamylcholine; c, 5 minutes - carbamylcholine;
d, 5 minutes + carbamylcholine; e, 30 minutes - carbamylcholine;
f, 30 minutes + carbamylcholine; g, 60 minutes - carbamylcholine;
h, 60 minutes + carbamylcholine; 1, 0 time - carbamylcholine;
2, 60 minutes + carbamylcholine; 3, bovine plasma albumin.

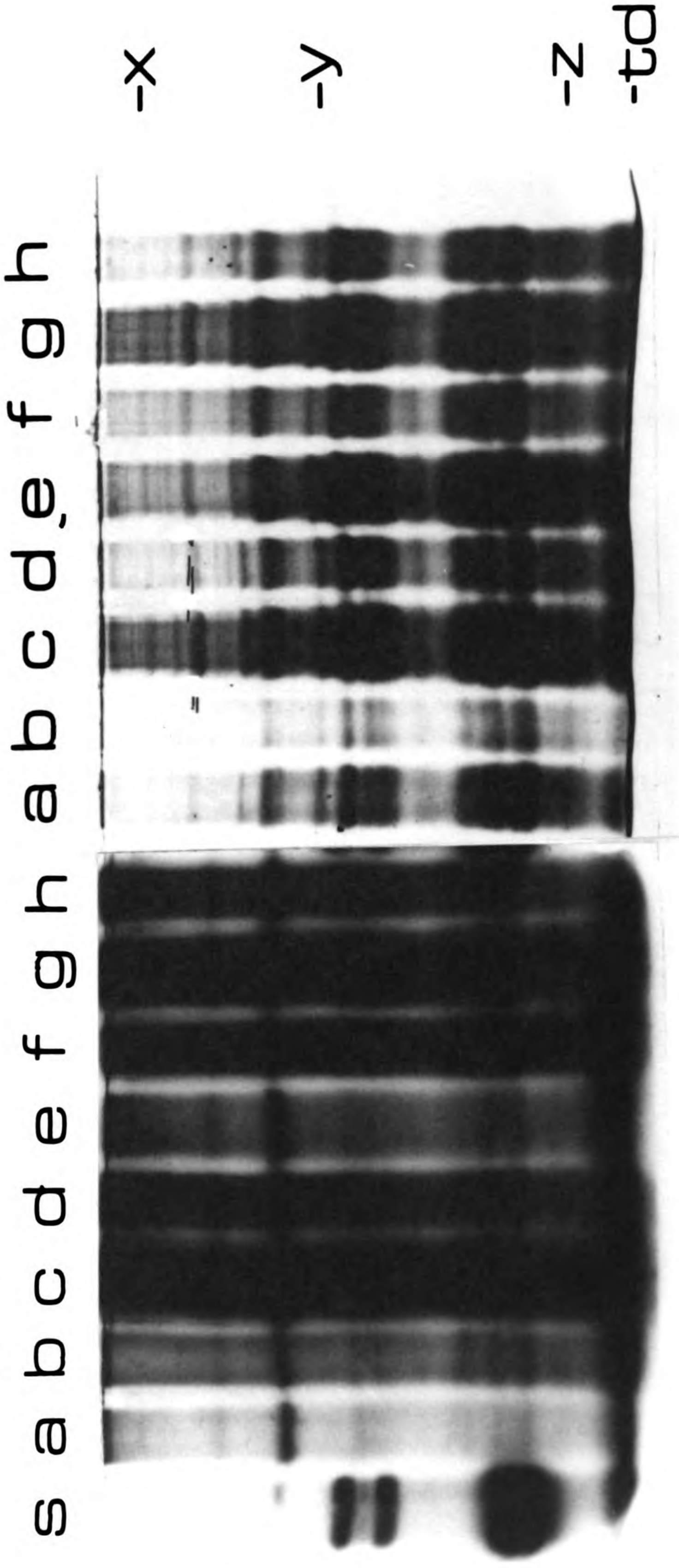

Figure 9

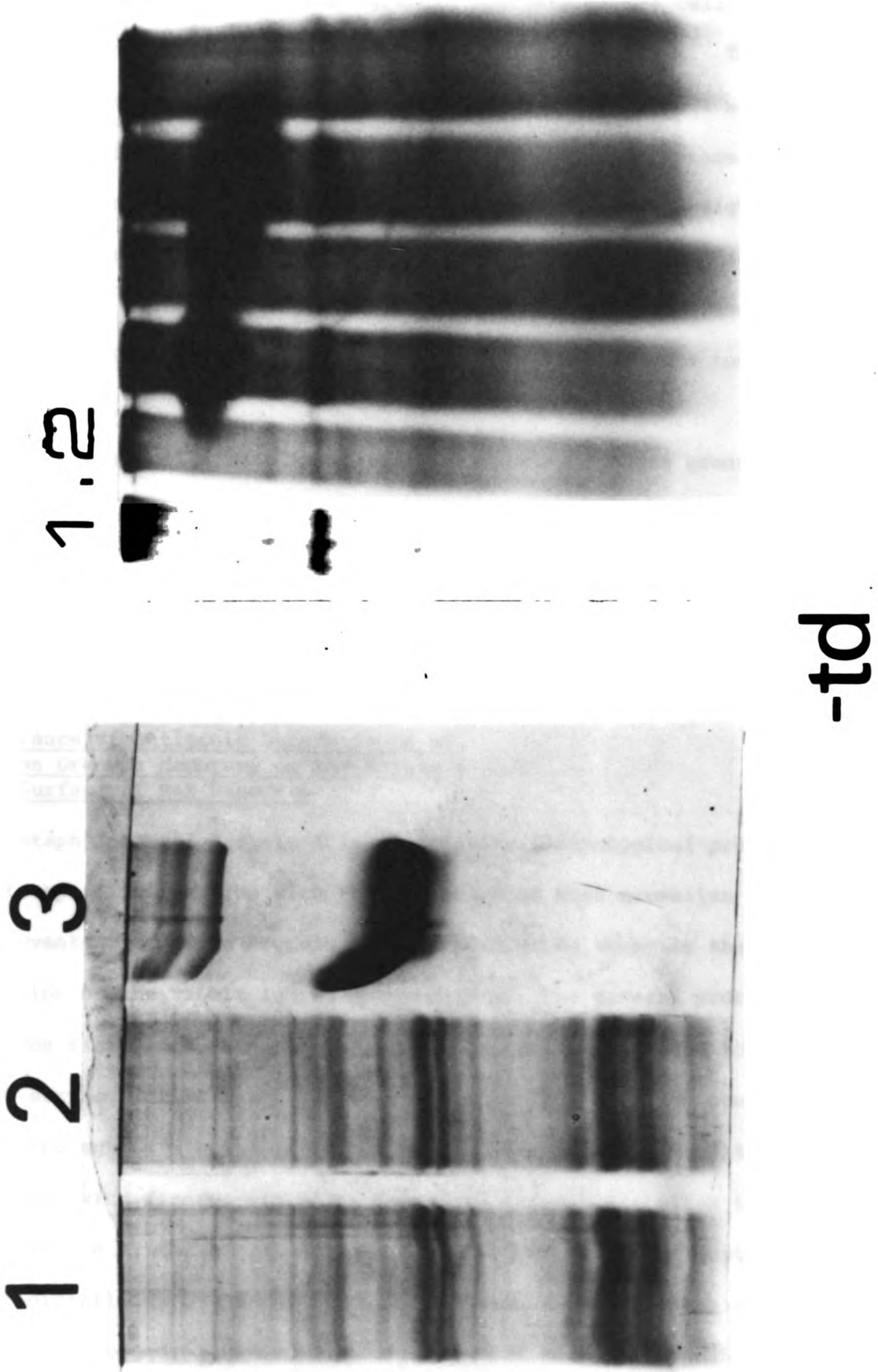

Figure 9A

As seen in Figure 9, fluorographic analysis of the cell surface did not detect the appearance of GP-2 on the cell surface. There were approximately 30 polypeptide species labeled with ^{125}I with molecular weights ranging from 150,000 to 12,000. A decrease in intensity during secretion of a polypeptide of molecular weight ∿60,000 was visible in Figure 9A. Comparison of fluorographic profiles of the cell particulate fractions with the zymogen granule content profile indicated no significant contamination due to secretory protein.

Unlabeled cells were incubated with labeled zymogen granule content to determine the extent of membrane contamination due to adsorbed secretory protein, and the results are summarized in Table 9. Four percent of the zymogen granule content remained with the cell particulate fraction throughout its preparation.

Appearance of Antigenic Determinants of Zymogen Granule Membrane on the Acinar Cell Surface of Rat Pancreas

Staphylococcal Protein A is a sensitive immunological probe due to its specific reaction with the Fc region of most mammalian IgG.[99] The advantage of using Protein A as a radioimmune assay is that the isolation of the rabbit IgG is not required. The general procedure involves first incubating the cells with antiersa, directed toward a given surface determinant. After incubation the cells are centrifuged through 5% albumin to remove unbound antibody. The cells are incubated with fluorescein or ^{125}I labeled Protein A. After incubation with Protein A, the excess Protein A is removed by centrifugation. The radioactivity bound to the cells represents the relative amount of antisera bound. Protein A is a protein of molecular weight

Table 9. Adsorption of secretory protein to the pancreas cell surface

Unlabeled cells (11.3 mg) were incubated in KRB 1.0 mM $CaCl_2$ containing zymogen granule content (0.73 mg, 4.67 x 10^5 cpm), labeled with ^{125}I as described in Methods. After incubation for 15 minutes at 25°C, the cell particulate fraction was prepared, and the amount of radioactivity was measured as described in Methods.

Fraction	Protein (μg)	Radioactivity (cpm)	Zymogen granule content bound (%)
Zymogen granule content	730	467,650	---
Cell particulate fraction	12,300	19,374	4.1

42,000 and contains 4 tyrosines/molecule, and is therefore easily iodinated. Ronzio and Mohrlok[45] have developed rabbit antisera directed against determinants of rat zymogen granule membranes. Protein A labeled with ^{125}I was used in concert with rabbit anti-zymogen granule membrane sera in an attempt to detect the appearance of zymogen granule membrane determinants on the cell surface during secretion. Figure 10 illustrates the specificity of the rabbit anti-zymogen granule sera to the isolated acinar cells. In all cases cells incubated with specific sera bound Protein A at least twice that of preimmune sera, Protein A alone, or specific sera incubated with ascites hepatoma cells. Figure 11 summarizes the results of the Protein A assay. There was a 1-hour lag phase during secretogogue-stimulated secretion before there was an increase in Protein A bound to the cell surface. During the second hour of secretion there was a tenfold increase in the amount of Protein A bound to the cells. The binding declined to initial levels during the third hour of incubation.

Figure 10. Analysis of specificity of rabbit anti-zymogen granule membrane to cell surface of isolated acinar cells.

Cells were isolated, Protein A iodinated as described in Methods. The Protein A assays were performed as described in Figure 11. Variation in the controls included incubation with Protein A only, Ascites Novikoff cells incubated with anti-zymogen sera, and incubation with preimmune sera at different time points in secretion. Variations in assays of specific sera included addition of hormone, different amounts of Protein A. Open circles represent rabbit anti-zymogen granule membrane sera. Closed circles represent control assays.

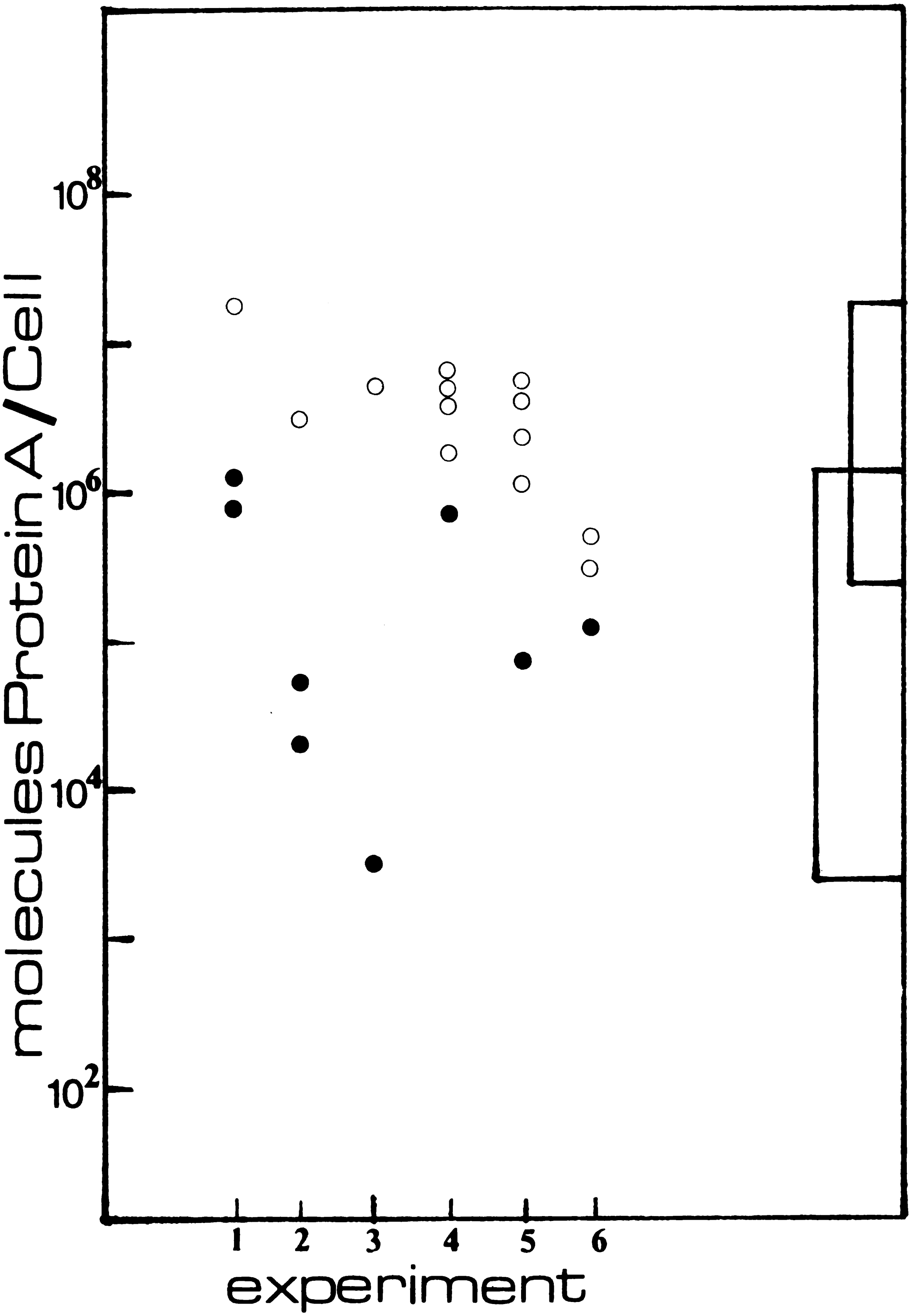

Figure 10

Figure 11. Determination of antigenic determinants of zymogen granule membranes on the cell surface during secretion.

Cells were prepared as described in Methods. A cell suspension containing 10^6 cells/200 l in KRB containing 5% BPA, 0.1 mg/ml SBTI were induced to secrete by making the suspension 10^{-5} M carbamylcholine chloride. The media were changed every 20 minutes to avoid damage to cells by secreted enzymes. Cell viability was monitored every 15 minutes. Cell samples (0.5×10^6) were collected at the time points indicated during the incubation and were incubated as follows at 4°C: to each sample 10 l of anti-zymogen granule membrane preadsorbed with zymogen granule lysate was added and incubated for 45 minutes. After incubation the cells were washed 2 X in KRB containing 5% BPA, and 0.1 mg/ml SBTI to remove any unbound antibody. After the second wash the cells were transferred to new reaction vials to avoid contamination due to antibodies adhering to the walls of the vials. The cells were then washed 2 more times to insure absence of unbound antibodies and were then incubated with 1 g of iodinated Protein A (10^6 cpm/ g Protein A) for 30 minutes. After incubation the cells were washed in the same manner as after the incubation with antisera for the same reasons. After the washings were completed the cells were solubilized in 1% SDS, 0.1 M NaOH overnight, neutralized, and radioactivity monitored by liquid scintillation.

Solid line represents incubation with specific sera; dashed line represents incubation with preimmune sera.

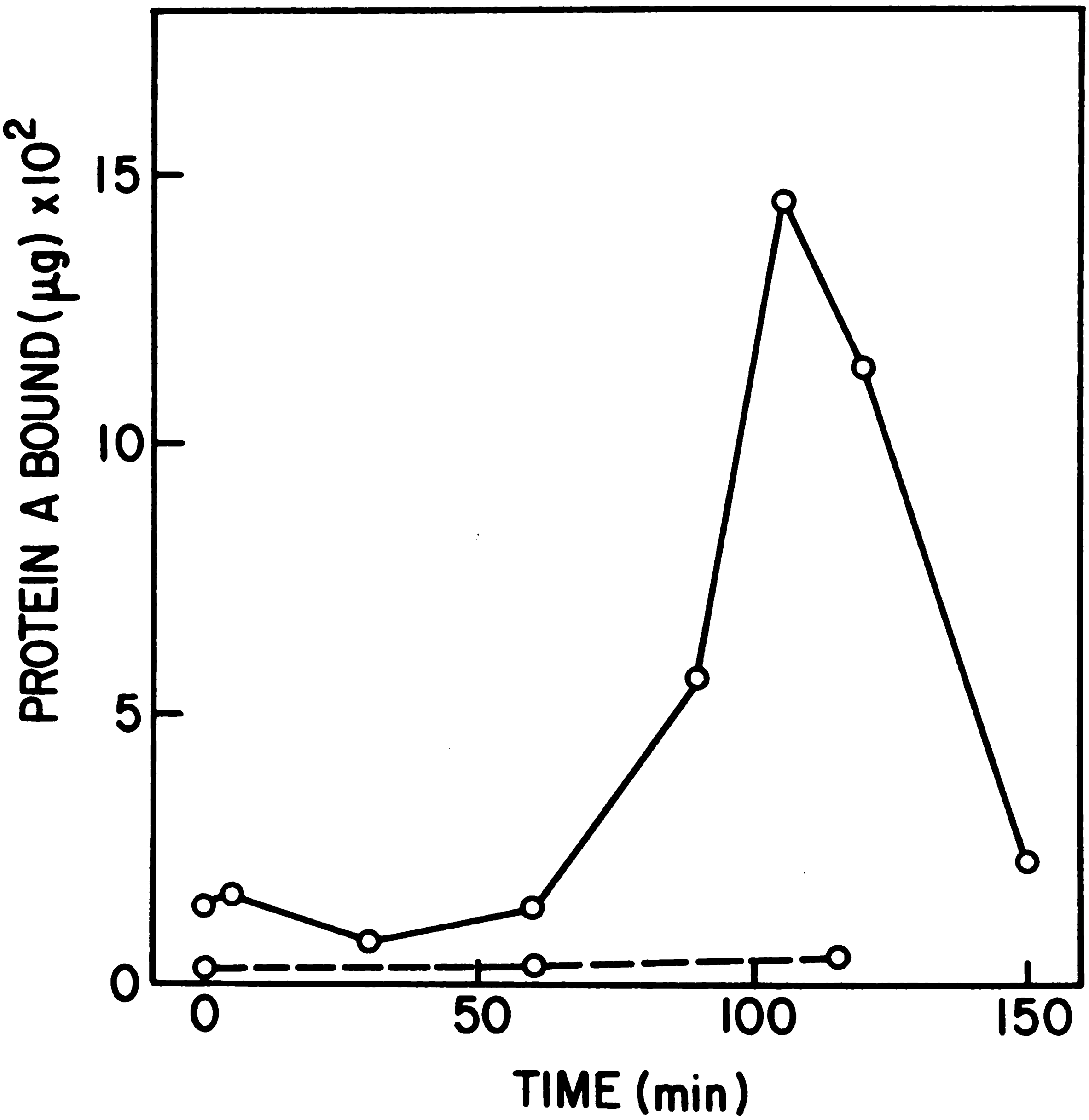

Figure 11

DISCUSSION

^{125}I Labeling of Isolated Acinar Cells of Rat Pancreas

For a labeling technique to be used as a cell surface marker, certain prerequisites must be met. The cell surface must be impermeable to the catalyst, as well as the reactive species. In addition, labeling should be irreversible and result in minimal perturbation of the cell surface.

Both lactoperoxidase glucose oxidase-catalyzed and glycoluril-catalyzed iodinations of proteins were investigated. Iodination catalyzed by glycoluril was chosen as the method for labeling acinar cells, because glycoluril was mechanically easier while its labeling efficiency was similar to iodination of proteins catalyzed by lactoperoxidase and glucose oxidase.

The cell surface is impermeable to glycoluril because of glycoluril's limited solubility in aqueous solution. From the data presented it cannot be stated with certainty that the reactive species did not penetrate the cell surface. Significant amounts of radioactivity were found to be pumped into the cell during the labeling procedure; however, greater than 95% of the radioactivity found in the cell supernatant fraction was free ^{125}I. Similar observations have been reported with human erythrocytes labeled by lactoperoxidase-glucose oxidase catalysis.[95] Ninety to ninety-five percent of the protein-associated ^{125}I was located in the cell particulate fraction.

The labeling technique did not cause significant perturbation of the cell surface as judged by the high viability (95%) retained by the cells throughout the incubation procedure.

It is possible that all of the radioactivity associated with the cell particulate fraction is located on the plasma membrane present in that fraction. The second possibility with respect to rat acinar cells is that ^{125}I is preferentially incorporated into membrane protein. If this were the case, then ^{125}I penetrating the cell surface would label all intracellular membranes as well as the plasma membrane. In order to resolve this problem, it is necessary to isolate the intracellular membranes from labeled cells and determine the relative incorporation of ^{125}I. The techniques for isolation of intracellular membranes from isolated acinar cell suspensions have not been developed, with the exception of the nuclear membrane. Isolated cells cannot be obtained in sufficient quantity to allow isolation of organelles. Because of this it cannot be concluded which is the more likely explanation.

A major complication involves the loss of protein-bound ^{125}I. The results indicate that the combination of acid wash followed by SDS polyacrylamide gel electrophoresis drastically decreased recovery of radioactivity. This occurred only with membrane protein; recovery from soluble proteins was not affected by this procedure. Comparisons of profiles from acid-precipitated particulate fractions and particulate fractions not precipitated suggest that radioactivity was not lost from a few proteins in the membrane. The combination of acid precipitation and SDS polyacrylamide gel electrophoresis of the membrane caused destabilization of the membrane protein-^{125}I bonds in most, if not all, membrane proteins labeled.

Plasma Membrane Isolation and Characterization

As previously stated, evidence for purity must be morphological as well as biochemical. The isolation and characterization of plasma membranes were attempted in order to compare and contrast them with zymogen granule membranes. Attempts to isolate plasma membranes were unsuccessful for various reasons. Low recovery was a major complication in the procedure involving Con A Sepharose beads. Labeled membrane fragments bound to the beads, but subsequent elution of membrane fragments from the beads was unsuccessful.

The aqueous two phase system of Brunette and Till was unsuccessful. Eighty-four percent of the membrane-bound ^{125}I remained in the pellet, whereas only 4.5% was recovered at the interface; 6.5% recovery was reported by Brunette and Till.[79] A high recovery of membrane is important when dealing with plasma membranes which are functionally divided, as is the case with pancreatic acinar cells. Because these cells possess basal, lateral, and apical surfaces, it can be assumed that the structure of membrane may not be homogeneous. Fragments of these different surfaces could equilibrate at different densities throughout the system being utilized. Wisher and Evans[100] have reported the fractionation of rat hepatocyte plasma membrane into 6 subfractions, by the combination of differential and gradient centrifugation. The 6 subfractions possessed different density, morphological, enzymic, and chemical properties. Thus, a membrane band in a gradient may be mistaken as representing the entire plasma membrane when it is in fact a segment of the plasma membrane.

A fraction enriched in membranes was prepared from rat tissue following a modified procedure of Meldolesi and Cova[48] for the isolation of plasma membrane from guinea pig pancreas. This enriched fraction was designated membranes from the 700 x g pellet. Electron microscopy revealed an enrichment of smooth membranes contaminated with cytoplasmic adherents. Addition of 0.1% Dextran 500 (m.w. 500,000) to compete with contaminants in binding to the membranes had limited success in the reduction of cytoplasmic contamination. The 700 x g pellet was used in subsequent studies involving comparison of the intracellular membranes to determine the uniqueness of the zymogen granule membranes.

Developing a technique for isolation of plasma membrane is a complicated task. The techniques involved in most preparations rely on the density of sedimentation characteristics of the membrane in question. A major complication in isolating plasma membranes from the pancreas is the absence of a clear-cut morphological or biochemical marker for the plasma membrane.[48] Conventional biochemical markers involve enzyme activities which are known to be localized on the plasma membrane. In the pancreas there is no one enzyme localized on the plasma membrane that is not also found on other intracellular membranes. Meldolesi and Cova[48] presented an in-depth analysis of the plasma membrane fraction prepared from guinea pig. 5'-Nucleotidase is present in plasma membranes, but it is also localized in the smooth microsomes, with an activity which is 60% of that found in plasma membrane. High activities of β-leucyl-naphthylaminidase were localized in the plasma membrane, but the level found in smooth microsomes prevents its use as a membrane marker. Mg^{++} ATPase levels in plasma membranes are slightly higher than the levels found in zymogen granule membranes. In addition, cytoplasmic proteins contain digestive enzymes which results in degradation of membrane preparations if inhibitors are not present throughout the preparation (e.g., SBTI).

It may be possible to isolate plasma membranes by affinity chromatography, in which a pancreatic hormone would be attached to a solid support, and a cell homogenate or previously enriched membrane fraction would be passed over the column. Hormone receptors are segregated on the basal side of the acinar cells, and therefore techniques involving hormone receptors are limited to that region of the plasma membrane.

The absence of enzyme activities associated with other intracellular membranes, e.g., cytochrome c oxidase in mitochondrial membrane, has been taken as evidence for plasma membrane purity. Lack of enzymic activities from other intracellular membranes leaves the plasma membrane as the alternative. Evidence of this type is questionable, as disruption of organelles may cause sufficient alteration in the membrane structure so as to result in the loss of enzymic activity. It is possible that a plasma membrane preparation may be contaminated by membrane fragments which have low activities of the enzyme marker and therefore will not be identified as contaminants.

Comparison of Intracellular Membranes

One objective of this investigation was to determine the uniqueness of the zymogen granule membranes. Comparison of Coomassie blue profiles showed the zymogen granule membranes to be much less complex than the other intracellular membranes. A minor polypeptide in smooth microsomal membranes and membranes from the 700 x g pellet comigrated with the major polypeptide of the zymogen granule membrane, GP-2. Because of the uncertainty of the composition of the 700 x g pellet, it cannot be stated with confidence that the comigrating species is a true plasma membrane species. It could be a result of contamination from smooth microsomal membranes, or from zymogen granule membranes.

The comparison of the glycopeptide distribution in the intracellular membranes was performed using the periodic acid-Schiff procedure developed by Fairbanks.[94] Exhaustive extraction in acid was required to remove SDS, which resulted in high background staining if not sufficiently removed. GP-2 was shown to be the major glycopeptide in the zymogen granule membrane, in agreement with results of previous studies. The smooth microsomal membrane profile, prepared by Raymond MacDonald, revealed a glycopeptide which comigrated with GP-2. This result, along with other studies, led to the proposal that this glycopeptide is a precursor to GP-2.[15] Rough microsomal membranes contained 3 major glycoproteins, all having molecular weights lower than GP-2. No glycoproteins were detected in nuclear membranes or membranes from the 700 x g pellet.

In more recent studies Douglas Lewis[46] detected 3 high molecular weight glycoproteins present in the membranes from the 700 x g pellet. One of these glycopeptides comigrates with GP-2. Again, a definitive conclusion as to the significance of this finding is prevented by the uncertainty of the composition of the 700 x g pellet. The glycopeptides may be components of the plasma membrane or the result of contamination present in the pellet.

Failure to detect these glycopeptides in my analysis is due to a combination of two factors. First, only 175 μg of protein from the 700 x g pellet was analyzed in my study. Second, high background staining prevented high resolution necessary for detection.

From the polypeptide and glycopeptide analyses of the intracellular membrane; and from previously published observations of Ronzio and MacDonald,[12-15,44] it was concluded that the polypeptide profile of zymogen granule membranes is unique with respect to other intracellular membranes of the rat exocrine pancreas. Zymogen granule membranes are characterized by GP-2. Significant amounts of glycoprotein or protein which comigrates with GP-2 were not detected in other intracellular membranes or in soluble protein fractions.

Fluorographic Analysis of Membrane Proteins from Secreting and Nonsecreting Acinar Cells of the Rat Pancreas

Fluorography provided the most sensitive analysis of labeled membrane proteins. Fluorographic analysis resolves labeled protein to the same degree which polyacrylamide gel electrophoresis separates proteins.

Isolated acinar cells were induced to secrete by the addition of carbamylcholine chloride (10^{-5} M). Cell samples were iodinated at various time points up to 2 hours after initiation of secretion. No change in the iodination profile of cell particulate fractions from the time points taken (0-2 hours) were detected by fluorography.

Changes in iodination patterns of intact pancreatic acinar cell surfaces during secretion could result from 2 phenomena. Reports[48] suggest that GP-2 should appear on the outer surface of the plasma membrane during fusion of zymogen granules with the plasma membrane during exocytosis. If GP-2 appeared on the cell surface, it could have been labeled with ^{125}I and a band with increasing intensity in the region of the gel where GP-2 migrates would have been present in the fluorographs. A major change in the surface topology during secretion could have been detected if the change had also resulted in the susceptibility of membrane proteins to iodination. This could be visualized either as a decrease or an increase in band intensity.

However, negative results for GP-2 are not conclusive. It is possible that GP-2 is endocytosed and reutilized as rapidly as it appears on the surface during exocytosis. GP-2 tyrosines may have been inaccessible to labeling because of GP-2's tertiary structure or by blockage from adjacent molecules. Isolated zymogen granule membranes should be labeled with ^{125}I to determine the degree to which GP-2 can be labeled.

These results suggest no major change in the cell surface topology during exocytosis. These findings cannot be stated conclusively until it is demonstrated that labeling is specific for cell surface.

Appearance of Antigenic Determinants of Zymogen Granule Membranes on the Cell Surface of Rat Pancreas Acinar Cells During Secretion

Protein A used in concert with rabbit antisera directed against determinants of zymogen granule membranes was used to detect the appearance of zymogen granule membrane on the cell surface during secretion. The specificity of the antisera provided the means for a sensitive test for membrane fusion. Protein A bound to acinar cells increased tenfold after 2 hours of hormone secretion and returned to the original value during the third hour of secretion. Although an increase in binding occurred during the first 2 hours of secretion, the binding kinetics of Protein A do not agree with the kinetics of amylase release. Douglas Lewis observed that 80% of secretion occurs within the first hour of incubation with carbamylchcline, with 90% of secretion completed after 2 hours. The increase in Protein A binding occurred exclusively in the second hour of incubation with secretogogue; no increase was observed during the first hour.

Protein A is taken up by damaged or dead cells.[101] This did not contribute to the increase in binding, as the cell viability remained above 95% during all incubations. Extensive nonspecific binding of Protein A did not occur when cells were first treated with preimmune sera.

The decrease in binding ability of Protein A during the third hour of incubation is in agreement with electron microscopic studies investigating endocytosis, and the reutilization of membrane protein. Gueze and Port,[43] using tissue slices, have reported that during the first 2 hours of secretion in the rat acinar pancreas there is an increase in the cell surface area of 300%. During the third hour of secretion the cell surface area is reduced to its normal size. This is attributed to the formation of multivesicular bodies from endocytosis of excess membrane.

Comparison of control values with rabbit anti-zymogen granule membrane sera revealed an interesting pattern. The binding of Protein A with control sera was always lower than the binding with specific sera, yet the variation among controls was much greater than the variance among values using the membrane-specific serum. These variations may have resulted from the different preparations of labeled Protein A. Protein A has 4 tyrosines, all of which are involved in the binding of Protein A to the Fc region of the IgG.[101] It has been reported that extensive labeling of Protein A with ^{125}I can result in the alteration of the binding capacity of Protein A with IgGs.[102] Different concentrations of Protein A were also used. Another cause might have resulted from use of different cell preparations. The cell might have been altered to different degrees during the cell isolation procedures. In all cases Protein A binding was significantly higher with specific sera than the binding of each respective control.

These results present preliminary biochemical evidence for membrane fusion during exocytosis in the rat acinar pancreas. In order to conclusively demonstrate membrane fusion during exocytosis, amylase release should be monitored for each time point, and single cell preparations should be used for each time course. The results demonstrate the success of the assay, but further quantitation is necessary.

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